A QUANTITATIVE AND QUALITATIVE HISTOLOGICAL ASSESSMENT OF SELECTED ORGANS OF OREOCHROMIS MOSSAMBICUS AFTER ACUTE EXPOSURE TO CADMIUM, CHROMIUM AND NICKEL

By

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SEDERT MY EERSTE LEWENSLIG
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HET JUL MY KOMPAS HELP RIG
OP DIT WAT GOED EN EERBAAR IS
SOOS NET TWEE GOEIE OUERS KON

MY TWEE BETROUBARE BAKENS
JUL BYSTAND EN LIEFDE VIR MY
HET DIE PAD NA MY IDEALE GELYK GEMAAK,
MY WESE TOT VOLLE POTENSIAAL LAAT GEDY
SOOS NET TWEE GOEIE OUERS KON

MY TWEE UITMUNTENDE VOORBEELDE
WANT AL WAS DIE PAD TOT HIER SOMS ROF
EN JUL SIELE SE SOME SOMS EERG GETOETS IS
HET JUL MOED EN YWER NOOIT VERDOF

EN HET JUL MY GELEER VAN DEURDRUK
NA OORWINNING, NA SELFVERWENSLIKING
SOOS NET TWEE GOEIE OUERS KON

VANDAG, BY HIERDIE MYLPAAL, IS EK DANKBAAR
EN TROTS OM JUL STEEDS TE Hê AAN MY SY -
DIE BESTE TWEE OUERS WAT EK Ooit kon Kry

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**Figure 6.8:** Median, quartiles, and extreme values of the calculated total spleno-somatic index (SSI) of *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (× - not significantly different; √ - significantly different).

**Figure 6.9:** Median, quartiles, and extreme values of the calculated gonadal-somatic index (GSI) of female *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (× - not significantly different; √ - significantly different).
Figure 6.10: Median, quartiles, and extreme values of the calculated gonadal-somatic index (GSI) of male _O. mossambicus_ after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (× - not significantly different; ✓ - significantly different).

Figure 6.11: Median, quartiles, and extreme values of the calculated fat-tissue index (FTI) of _O. mossambicus_ after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (× - not significantly different; ✓ - significantly different).

Figure 6.12: Quantitative histopathological assessment of the liver of _O. mossambicus_ after short-term exposure to various metals. Mean values of the reaction index (I<sub>org rp</sub>): CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I-inflammation, T-tumour. Sum of the 5 reaction index indicates the organ index (I<sub>org</sub>). Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (× - not significantly different; ✓ - significantly different).

Figure 6.13: Quantitative histopathological assessment of the gill of _O. mossambicus_ after short-term exposure to various metals. Mean values of the reaction index (I<sub>org rp</sub>): CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I-inflammation, T-tumour. Sum of the 5 reaction index indicates the organ index (I<sub>org</sub>). Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (× - not significantly different; ✓ - significantly different).
**Figure 6.14:** Quantitative histopathological assessment of the ovary of *O. mossambicus* after short-term exposure to various metals. Mean values of the reaction index (I \(_{\text{org rp}}\)): CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I-inflammation, T-tumour. Sum of the 5 reaction index indicates the organ index (I \(_{\text{org}}\)). Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (\(\times\) - not significantly different; \(\checkmark\) - significantly different).

**Figure 6.15:** Quantitative histopathological assessment of the testis of *O. mossambicus* after short-term exposure to various metals. Mean values of the reaction index (I \(_{\text{org rp}}\)): CD-circulatory disturbance, RC-regressive changes, PC-progressive changes, I-inflammation, T-tumour. Sum of the 5 reaction index indicates the organ index (I \(_{\text{org}}\)). Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests (\(\times\) - not significantly different; \(\checkmark\) - significantly different).

**Figure 6.16:** Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 10% LC\(_{50}\) Ni exposure.

- Congestion (C) of sinusoids (S) of blood vessels (BV) and the central vein (CV).

  **Intercellular deposits (I)** are scattered throughout the section, but are concentrated around the central vein.

  Fatty degradation identified by fatty vacuoles (F) are scattered throughout the section.

  (Stained with H & E; 40 X magnification.)

**Figure 6.17:** Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 10% LC\(_{50}\) Ni exposure.

- The general disruption of cord structure caused by severe fat accumulation (*) with in hepatocytes throughout the
Nuclear activity, such as pyknosis (P, encircled in black) and karyorrhexis (K, encircled in white), can also be identified on this section.

(Stained with H & E; 100 X magnification.)

**Figure 6.18:** Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 20% LC$_{50}$ Ni exposure.

Degeneration is the state in which the physiological substances present in tissues increase or appear in other places. Both fatty degeneration (6.13 A) and granular degradation (6.13 B) are clearly identifiable in this section.

(Stained with H & E; 40X magnification)

**A) Fatty degeneration**

Fat is stored in the cytoplasm in the form of triglyceride-rich droplets (*) are stored in the hepatocytes which appear as big vacuoles throughout this section. The hepatocytes lost their hexagonal shape and the general structure of liver tissue is disrupted. Fatty degeneration is accompanied by pyknosis (encircled in black on insert).

(Insert: Stained with H & E; 100 X magnification.)

**B) Granular degeneration**

Proteinous granules are visible in the cytoplasm of the hepatocytes throughout this section. Cytoplasm appears to clumped. Hepatocytes appear swollen and lost their hexagonal shape. Pyknosis of the nucleus is observed (encircled in white).

(Insert: Stained with H & E; 100 X magnification.)

**Figure 6.19:** Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 20% LC$_{50}$ Ni exposure.

- Intercellular deposits (I) were found around the central vein (CV).
- Mono macrophage centres (MMC) are located between the intercellular deposits.
- Infiltration of **mono nuclear leucocytes (MNL)** was also found around the central vein. (Stained with H & E; 40X magnification).

**Figure 6.20:** Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 10% LC$_{50}$ Cr exposure.
- Hepatocytes show varied degrees of **hypertrophy (Ht)** throughout the section.
- **Granular degradation (G)** of the cytoplasm of the hepatocytes can be seen in most hepatocytes on this section.
- Fragmentation of the nucleus, **karhorrhexis (K)**, was found throughout the section. (Stained with H & E; 40X magnification).

**Figure 6.21:** Representative histopathological characteristics observed in the gill of *O. mossambicus* after acute exposure to 10% LC$_{50}$ chromium exposure.
- The epithelial cells on the primary lamellae are **vacuolated (V).**
- **Deposits (De)** are found within the epithelial cells. (Stained with H & E; 100X magnification).

**Figure 6.22:** Representative histopathological characteristics observed in the gill of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel exposure.
- **Hyperplasia (Hp)** of the primary gill epithelium is noted.
- **Deposits (De)** are found within the epithelial cells. (Stained with H & E; 100X magnification)

**Figure 6.23:** Representative histopathological characteristics observed in the gill of *O. mossambicus* after acute exposure to 10% LC$_{50}$ cadmium exposure.
- **Deposits (De)** are found within the epithelial cells throughout the section.
- **Hyperplasia (Hp)** of both the secondary and the primary gill epithelia are demonstrated in this section.
- Epithelia lifting (EL).
- A slight increase in mucus cells (MC) was noted. (Stained with H & E; 40X magnification).

**Figure 6.24:** Representative histopathological characteristics observed in the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel exposure.
- The presence of ovum (O) in the testis (intersex).
- All developmental stages were identified in this section.
- All developmental stages were vacuolated (V)
- Primary and secondary spermatocytes stained hyperchromatic (Hc). (Stained with H & E; 100X magnification)

**Figure 6.25:** Section thought the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel.
- General lobular organization is lost.
- Primary spermatocytes are vacuolated (V) and stain hyperchromatic (Hc). (Stained with H & E; 40X magnification)

**Figure 6.26:** Section through the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel.
- General lobular organization is retained.
- Not all developmental stages identified in this section (only primary and secondary spermatocytes identified)
- Primary spermatocytes are vacuolated (V).
- Spermatocytes stain hyperchromatic (Hc). (Stained with H & E; 100X magnification)

**Figure 6.27:** Section through the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel.
A) General lobular structure is retained, and all developmental stages are represented. (Stained with H & E; 400 X magnification.)
B) Spermatocytes stained hyperchromatic (Hc) and are vacuolated (V). (Stained with H & E; 100 X magnification.)

**Figure 6.28:** Section through the testis of *O. mossambicus* after
acute exposure to 10% LC$_{50}$ cadmium.

- General lobular structure is lost.
- No spermatids or spermatozoa identified.
- Primary and secondary spermatocytes appear vacuolated (V) and stain hyperchromatic (Hc).
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>CD</td>
<td>Circulatory disturbances</td>
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<tr>
<td>CF</td>
<td>Condition factor</td>
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<td>DO</td>
<td>Dissolved oxygen</td>
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<td>FTI</td>
<td>Fat-tissue index</td>
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<td>I</td>
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<td>I&lt;sub&gt;org&lt;/sub&gt;</td>
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<td>L</td>
<td>Leucocrit</td>
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<td>NFHAI</td>
<td>Necropsy based fish health assessment index</td>
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<tr>
<td>PC</td>
<td>Progressive changes</td>
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<td>RC</td>
<td>Regressive changes</td>
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<td>SSI</td>
<td>Spleno-somatic index</td>
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<td>T</td>
<td>Tumours</td>
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<td>TDS</td>
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<td>[TP]</td>
<td>Total plasma protein concentration</td>
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<td>Tot-I</td>
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<td>σ</td>
<td>Conductivity</td>
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South Africa is renowned for its exploitable mineral resources and continues to be a major player in the world’s mineral markets. The country is well known for containing the world’s largest gold and platinum repositories and electroplating industries, which is the major cause for delivering by-products such as cadmium (Cd), chromium (Cr) and nickel (Ni).

Environmental pollution caused by active mining and seepage from closed mines, continuously threatens South African water resources. Such pollution can cause a shift in water chemistry and increase the availability of certain metals to the living organisms of such a system. Even at low concentrations metals are amongst the most toxic environmental pollutants. As a result of their persistence and capacity to accumulate in the environment, metals have a lasting detrimental effect on the ecosystem. Although there is progress in the treatment of metallic wastes, the discharge thereof by industries is still a serious water pollution problem.

In the past, chemical analysis of water has proven to be of great use for the detection of pollutants within the environment. The value of chemical analysis alone has become limiting, as chemical analysis supplies information on the levels of chemicals at a certain time. Furthermore, the monitoring of water quality variables often does not reflect long-term events that may play a critical role in determining the ecosystem health. It is now generally understood that measurements of only the physical and chemical attributes of water cannot be used as surrogates for assessing the health of an aquatic ecosystem. The new trend is to incorporate biological monitoring into
existing monitoring strategies. Fish are entirely dependent on the aquatic environment for their survival, rendering them a good monitor of water pollution.

Macroscopic changes in organs are preceded by changes at the tissue, cellular or molecular level. These changes are the net result of adverse biochemical and physiological changes within an organism. Histological analysis is therefore a very sensitive parameter and a valuable technique in determining cellular changes in target organs as a result of exposure to stressors. Fish histology can thus be used as an indicator of exposure to contaminants and assess the degree of pollution.

Because of the subjective nature of morphological studies correlations with other quantitative studies are difficult. However, incorporation of quantitative methods is essential to the continued development of histopathology as a biomarker of pollution exposure, and to the interpretation of histological responses. The aim of this study is to qualitatively and quantitatively describe the toxic induced histological changes in the selected organs of *Oreochromis mossambicus* after acute exposure to Cd, Cr and Ni. Fish were exposed to 10% (n=20) and 20% (n=20) of the LC$_{50}$ concentration of Cd, Cr and Ni respectively under controlled conditions (23 ± 1°C) for 96 hours in an environmental room with a control group (n=5) for each exposure.

Assessment methods chosen to determine the toxic effects of Cd, Cr and Ni include a necropsy based fish health assessment index (NFHAI), organo-somatic indices (liver, spleen, gonads) to access the general health of the fish. Histological changes to the liver, gills and gonads were described (qualitative) and graded (quantitative) according to a standardized histological tool.
After 96 hours of exposure to 10% and 20% of the LC<sub>50</sub> of Cd, Cr and Ni respectively, the average calculated condition factors of all the groups fell within normal the range of ± 1. No significant difference in the condition factor (CF), heamatocrit (Hct) values and the leucocrit (L) values was noted between the exposure groups and the control group. The total plasma protein concentration ([TP]) of the 20% LC<sub>50</sub> Cd exposure group was significantly lower than that of the control group.

The average NFHAI value as calculated for the 20% LC<sub>50</sub> Cd exposure group were significantly higher than the value calculated for the control group. From these results it was concluded that the general health of the specimens from the 20% LC<sub>50</sub> Cd exposure group were compromised but not adversely affected by the exposure. The increase in the NFHAI is attributed to discoloured liver, low total plasma protein concentration, increase in liver discolouration and the low haematocrit of this exposure group. Except for 10% LC<sub>50</sub> Cd exposure group, all the hepato-somatic indices (HSI) determined for all the exposure groups ranged between the normal 1-2%. The HSI determined for both the 10% and 20% LC<sub>50</sub> Cd exposure groups are significantly lower than the average HSI determined for the control group.

Similar histological changes, similar in degree, were identified in all the exposure groups including the control group for the liver, testis and ovaries. It was accepted that these histological changes were not as a result of the exposure, but rather as a result of previous exposure to toxic substances. Similar histological, changes variant in degree, were identified in the gills from the cadmium exposure group and the chromium exposure group. This indicated that these histological changes are not metal specific.

From the results obtained from this study it was concluded that after 96 hours of exposure to relatively low concentrations of Cr and Ni the general health of the fish were not adversely affected. Although exposure to relatively low concentrations of Cd resulted in a decrease in the general
health of the exposed fish, these fish were able to adapt and cope with the insult and the liver retained the ability to detoxify. The application of the quantitative histological assessment tool allows for a comparison of the exposure groups to the control group which then elucidates toxic histological changes as a result of the metal exposures. Histological analysis of the gill is a sensitive biomarker for low concentrations of metal exposure.
Opsomming

Suid-Afrika is bekend vir sy minerale rykdom en is 'n kragtige speler in die wêreld se minerale mark. Suid-Afrika besit die wêreld se ryskste goud en platinum erts asook die wêreld se grootste elektroplateringswerke wat lei tot die vorming van by-produkte soos kadmium (Cd), chroom (Cr) en nikkel (Ni).

Omgewings besoedeling veroorsaak deur mynbou aktiwiteite bedreig Suid-Afrika se kosbare varswater reserwes. Mynboubesoedeling veroorsaak 'n verandering in die water se chemiese samestelling wat lei tot 'n toename in die beskikbaarheid van sekere metale aan die akwatiese organismes van die geaffekteerde stelsel. As gevolg van metale se volharding en kapasiteit om te bio-akkumuleer het dit 'n voortdurende skadelike effek op die ekosisteem. Alhoewel daar vordering is in die behandeling van metaal afvalstowwe, is die vrystelling daarvan deur nywerhede steeds 'n ernstige waterbesoedelings probleem.

In die verlede was chemiese analyse van water van groot belang vir die identifisering van besoedeling in die omgewing. Die waarde van chemiese analyse alleenlik is nie voldoende nie, aangesien chemiese analyse inligting verskaf van chemiese vlakke op 'n spesifieke oomblik in tyd. Bowendien weerspieël die monitering van waterkwaliteit nie lang-termyn gebeure wat 'n kritiese rol kan speel in die gesondheid van die ekosisteem nie. Dit is nou algemeen bekend dat metings van alleenlik die fisiese en chemiese eienskappe van water nie gebruik kan word as 'n plaasvervanger vir die assessering van die gesondheid van 'n akwatiese ekosisteem nie. Die nuwe
 Opsomming

Neiging is om biologiese monitering te inkorporeer in bestaande moniterings programmere. Vis is afhanklik van die akwatiese omgewing vir oorlewing, wat hulle 'n goeie moniteringsmiddel van waterbesoedeling maak.

Makroskopiese veranderings in organe word voorafgegaan deur veranderings in die weefsel, sellulêre of molekülêre vlak. Hierdie veranderings is die netto resultaat van verskeie biochemiese en fysiologies veranderings binne 'n organisme. Histologiese analise is daarom 'n sensitiewe veranderlike en 'n waardevolle tegniek om sellulêre veranderings in teken organe te bepaal as gevolg van blootstelling aan besoedeling. Vis histologie is dus 'n aanwyser van blootstelling aan besoedeling en kan gebruik word om die graad van besoedeling te bepaal.

Weens die subjektiewe aard van morfologiese studies is korrelasies met ander kwantitatiewe studies moeilik. Inkorporasie van kwantitatiewe metodes is essensieel vir die voortgesette ontwikkeling van histopatologie as 'n merker van die effek van blootstelling aan besoedeling, en tot die interpretasie van histologiese resultate. Die mikpunt van hierdie studie is om kwalitatief en kwantitatief die histologiese veranderings weens toksisiteit in teken organe van *Oreochromis mossambicus* na 'n 96 uur blootstelling aan Cd, Cr en Ni te beskryf. *O. mossambicus* is blootgestel aan 10% (n=20) en 20% (n=20) van die LC50 konsentrasie van Cd, Cr en Ni onderskeidelik, onder gekontroleerde toestande (23 ± 1°c) vir 96 uur in omgewings kamer met 'n kontrole group (n=5) vir elke blootstelling.

Assesserings metodes gekies om die toksiese effek van die gekose metale op die algemene gesondheid van die vis te bepaal bestaan uit 'n nekropsy gebasseerde gesondheid assesserings indeks (NFHAI) en organo-somatiese indekse (OSI). Histologiese veranderings in die lewer, kieue en gonades is volgens 'n gestandardiseerde histologiese hulpmiddel kwalitatief en kwantitatief beskryf.
Opsomming

Na 96 uur blootstelling aan 10% en 20% van die LC$_{50}$ van Cd, Cr en Ni onderskeidelik, val die gemiddelde berekende kondisie factor steeds binne die grense wat as normaal beskou word (± 1). Geen beduidende verskil in die kondisie factor (CF), hematokrit (Hct) en die leukokrit (L) waardes is gevind wanneer die blootstellings groepe met die kontrole group vergelyk word nie. Die totale plasma proteine konsentrasie ([TP]) bereken vir die 20% LC$_{50}$ Cd blootstellings groep was beduidend laer as die berekende totale plasmaprotein konsentrasie van die kontrole groep.

Die gemiddelde NFHAI waarde bereken vir die 20% LC$_{50}$ Cd blootstellings groep was beduidend hoër as die waarde bereken vir die kontrole groep. Aan die hand van hierdie resultate is die gevolgtrekking gemaak dat die algemene gesondheid van die 20% LC$_{50}$ Cd blootstelling groep negatief geaffekteer is deur die blootstelling aan Cd. Die verhooging in die NFHAI is as gevolg van ‘n toename in verkleurde lewers, lae totale plasma protein konsentrasies, en die lae hematokrit waarde van hierdie blootstelling groep.

Die hepato-somatiese indeks (HSI) bepaal vir beide die Cr en die Ni blootstellings groeppe lê tussen die normaal van 1-2%. Die HSI bepaal vir beide die 10% en 20% LC$_{50}$ Cd blootstellings groeppe is beduidend laer as die gemiddeld bepaal vir die kontrole groep.

Soortgelyke histologiese veranderings is geïdentifiseer in al die blootstelling groepe, insluitende die kontrole groep, vir die lewer, testis en ovaria. Hieruit is afgelei dat dié histologiese verandering nie is as gevolg van die metaal blootstelling nie, maar eerder ‘n gevolg van vroëer blootstelling aan skadelike stowwe. Soortgelyke histologiese veranderings, verskillend in graad, is geïdentifiseer in die kieue van beide die Cd blootstellings groepe asook beide die Cr blootstellings groepe, wat daarop dui dat hierdie histologiese veranderings nie metaal spesifiek is nie.
Die resultate verkry gedurende die studie dui daarop dat na 96 uur van blootstelling aan lae konsentrasies van Cr en Ni die algemene gesondheid van die vis is nie ongunstig geaffekteer word nie. Alhoewel blootstelling aan relatiewe lae konsentrasies van Cd wel die algemene gesondheid van die vis nie merkwaardeg affekteer word nie, en die lewer behou die vermoë om die skadelike stowwe te detoksifiseer. Die kwantitatiewe histologiese metode maak dit moontlik om die blootstellings groepe met die kontrole groep vergelyk. Histologiese veranderings wat deur besoedelstowwe veroorsaak is, word tydens blootstelling experimente kwalifiseerdaar. Histologiese analisering van die kieue is 'n sensitiewe biomarker vir lae konsentrasies van metaal blootstelling.
Chapter 1

Introduction

1.1 General introduction

Water is the most abundant of all compounds on earth, and is essential for life on this planet. It covers approximately 71% of the planet and is present in all living matter. Of all available water on earth, only 0.25% can be classified as surface water (Davis and Day, 1998).

South Africa is classified as a semi-arid to hyper-arid developing country, with a below average rainfall. Wise utilisation of this precious resource in a sustainable manner is therefore, essential for the future of the country. The aquatic environments are constantly being exposed to pollution from industrial processes, mining operations, as well as agricultural and domestic run-off (Förstner and Wittmann, 1981).

In the past, water quality management in South Africa, mainly focused on measuring physical and chemical water parameters. Chemical analysis is however costly and because water samples collected for chemical analysis are obtained at a specific point in time, they do not reflect average exposure conditions. Physical and chemical variables alone cannot provide and accurate account of the general health of an aquatic ecosystem. The use of biological systems, to determine the general health of a water body, is continuously incorporated into standard water analysis procedures (Roux et al., 1993).
Biomonitoring allows for the analysis of the effect of pollution on biological systems, providing an indication of both the past and the present water quality. Biomonitoring at the lower levels of biological organisation allows for an “early warning system”. Stressors can thus be detected at an early stage, and dealt with before they exert their effects at higher levels of biological organization (Newman and Jagoe, 1996).

Fish are routinely used in biomonitoring programs because they are in direct contact with their surrounding environment. Increased levels of contaminants might disrupt the physiology of fish by changing biochemical and metabolic pathways, reducing survival, growth and reproductive potential (Heath, 1995). Fish are able to demonstrate synergistic, antagonistic and other combined effects which cannot be detected by physical-chemical analysis (Luus-Powell, 1997).

Macroscopic alterations in fish are preceded by changes at the tissue, cellular or molecular level. These alterations are the net result of adverse biochemical and physiological changes within an organism. Fish histology can thus be used as an indicator of exposure to contaminants and assess the degree of pollution (Hinton and Laurén, 1990).

### 1.2 Motivation and problem statement

South Africa's available freshwater resources are already almost fully-utilised. Most of the country's major rivers have been dammed; in some areas over 50% of the wetlands have been converted for other land-use purposes; industrial and domestic effluents are polluting the ground- and surface waters (DWAF, 1999).

At the projected population growth and economic development rates, it is unlikely that the projected demand on water resources in South Africa will be sustainable. Water will increasingly become the limiting resource in South
Africa, and supply will become a major restriction to the future socio-
economic development of the country (DWAF, 1999). The aquatic
environments are constantly being exposed to pollution from industrial
processes, mining operations, as well as agricultural and domestic run-off
(Förstner and Wittmann, 1981).

South Africa is one of the world’s major mining and mineral-processing
nations. In 2003 South Africa produced more than 59 different mineral
commodities from about 920 mines and quarries. South African mineral
resources include gold, manganese, tin, vanadium, copper, platinum,
uranium, chromium and nickel. The contribution of the mining industry to
the gross domestic product (GDP) in 2003 was estimated to be 11%.
Primary mineral accounted for 34% of all merchandise trade in 2003.
Beneficiated mineral exports and primary mineral exports accounted for
about 42% of total merchandise exports (Chamber of Mines, 2003).

The release of metals into the environment, as a result of the many mining
activities, has serious implications on the biota. Even at low concentrations,
metals are amongst the most toxic environmental pollutants. As a result of
their persistence and capacity to bioaccumulate, metals have a lasting
detrimental effect on the ecosystem (Mance, 1987).

Fish have been shown to accumulate contaminants such as metals in their
tissues (Barnhoorn, 2001; Coetzee, 1996; Giesy and Wiener, 1977; Luus-
consumption of fish containing elevated levels of metals can cause health
problems. Chronic cadmium exposure has been linked to renal damage,
hypertension, and cardiovascular effects in humans (ODEQ, 2003).

The ability to identify the effects of metals at the lower levels of biological
organization will provide valuable tools in the assessment of possible insults
on environments (Grant, 2004). Histopathological investigations have been
used to relate the health status of fish from polluted sites to those from
rather unpolluted locations (Overstreet, 1988; Schmidt et al., 1999). However, little research has been done on the histology and histopathological responses of endemic fish species of South Africa (Van Dyk et al., 2007).

For the purpose of this study, three target organs were selected: liver, gills and gonads. The liver is a detoxification organ and essential for both the metabolism and excretion of toxic substances. The liver has the ability to degrade toxic compounds, but its regulating mechanisms can be overwhelmed by elevated concentrations of these compounds, resulting in structural damage (Van Dyk et al., 2007). Exposure to toxicants, such as metals, may cause histological changes in the liver, which in turn could be used as a biomarker to indicate prior exposure (Hinton and Laurén, 1990).

The fish gill is involved in gaseous exchange, acid-base balance, ionic transport and nitrogenous waste excretion. Gill epithelium provides an extensive surface of contact with the environment to facilitate ion transport and gaseous exchange. Because of the highly vascular structure of the epithelium, it is also a primary target for water borne toxicants (Wong and Wong, 2000).

Tolerance to stress is likely to be lower in the reproductive tract than in any other organ in fish (Gerking, 1980). A decrease in population, stemming from reproductive impairment, is potentially the most serious biological impact of a toxicant-compromised environment (Pieterse, 2004).

The subjective and descriptive nature of morphological and histological studies in the past made correlation with other quantitative approaches difficult (Hinton & Laurén, 1990). To assess degree of damage it’s important to use a method which will lead to comparative results (Bernet et al., 1999).

It is important to examine and explore the details of individual biomarker responses to individual pollutants without external factors influencing the results. This is only possible under laboratory conditions, where external
conditions (e.g. temperature and pH) can be controlled and fish can be experimentally exposed to one or more metals known to be present in natural waters (Grant, 2004). The results of such laboratory studies will be useful in predicting the toxic effects of metal exposure in aquatic systems and to eventually determine species and metal specific histological lesions and changes (Van Dyk et al., 2007).

The motivation for this study can be summarised as follow:

- Aquatic ecosystems must be protected, monitored and managed to ensure the sustainability of South African water resources.
- Metal pollution is of great environmental concern.
- Fish histology can be used as an indicator of exposure to contaminants, such as metals, and assess the degree of pollution.
- Studies under controlled laboratory conditions are necessary to determine metal specific lesions.
- Limited histological data exists on endemic fresh-water fish of South Africa.

1.3 **Aim of study**

**The aim of this study is:**
To qualitatively and quantitatively describe the toxic induced histological changes in selected organs of *Oreochromis mossambicus* after acute exposure to 10% and 20% of the LC$_{50}$ concentration of cadmium, nickel and chromium, respectively.

In order to accomplish this, a laboratory study was conducted under controlled conditions in an environmental room, at the research aquarium, University of Johannesburg.
The specific objectives of this study are to:

**Objective 1:** To determine the general health of fish exposed to cadmium, chromium, and nickel by calculating the necropsy based fish health assessment index (NFHAI).

**Objective 2:** Determine the effect of metal exposure on specific organo-somatic indices.

**Objective 3:** Qualitatively describe histological changes as a result of metal exposure.

**Objective 4:** Quantify histological changes as a result of metal exposure in the target organs.

The hypothesis tested during the current study includes:

After acute exposure to 10% and 20% of the LC$_{50}$ concentration of cadmium, chromium and nickel;

- the general health of the exposed specimens will decline, which would be reflected as an increase in the calculated necropsy based fish health assessment index (NFHAI).
- the organ weight (liver, spleen and gonads) to body weight ratio (organo-somatic indices) of exposed specimens will change, which indicate a change in organ function;
- metal specific histological changes will be identified (qualitative assessment);
- statistically significant histological changes will be identified within the target organs exposure (quantitative assessment);
- histological changes in the 20% LC$_{50}$ exposure group would be more pronounced than the changes found in the 10% LC$_{50}$ exposure group.

### 1.4 Research frame work

Table 1.1 provides an outline of the research framework as followed in this study.
Table 1.1: Research framework followed for this study.

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<td>Chapter 3</td>
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<td></td>
<td>• Necropsy-based health assessment index.</td>
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<td>Chapter 4</td>
<td>Literature review: Target organs</td>
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<td></td>
<td>• Liver.</td>
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<td>Chapter 5</td>
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<td>• Test organism selection.</td>
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<td></td>
<td>• Exposure (period of exposure and method).</td>
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<td></td>
<td>• Water quality monitoring (physical and chemical variables).</td>
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<tr>
<td></td>
<td>• Necropsy (necropsy based health assessment index and somatic indices).</td>
</tr>
<tr>
<td></td>
<td>• Processing (tissue and data).</td>
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<td></td>
<td>• Histological analysis (qualitative and quantitative).</td>
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<tr>
<td>Chapter 6</td>
<td>Results:</td>
</tr>
<tr>
<td></td>
<td>• Water quality.</td>
</tr>
<tr>
<td></td>
<td>• Necropsy (necropsy based health assessment index and somatic indices).</td>
</tr>
<tr>
<td></td>
<td>• Histological assessment (qualitative and quantitative).</td>
</tr>
<tr>
<td>Chapter 7</td>
<td>Discussion:</td>
</tr>
<tr>
<td></td>
<td>• Final conclusions.</td>
</tr>
</tbody>
</table>
1.5 References


2.1 Introduction

South African freshwater ecosystems exhibit natural variability in physical and chemical properties due to differences in geology, geomorphology, climate and soils (Davis and Day, 1998). Freshwater ecosystems are therefore more susceptible to anthropogenic influences than the more consistent and stable marine environments (Rainbow and Dallinger, 1993).

The cause of pollution in major river systems can be attributed to complex industrial processes, mining operations, agricultural and domestic effluents and atmospheric precipitation (Förstner and Wittmann, 1979). Metals belong to a group of elements whose hydrological cycles have been greatly influenced by man (Landis and Yu, 1995), and are one of the major types of pollutants commonly present in surface waters (Bourg, 1988; Mason, 1991; Sanders, 1997). Water serves as the medium in which metal undergoes biological and chemical reactions (Bourg, 1988).

Virtually all metals are toxic to aquatic organisms when present in excessive levels, and are therefore considered as one of the most toxic forms of aquatic pollution (Laws, 2000). Increased body concentrations of metals in fish tissue disrupts physiological homeostasis, which leads to increased susceptibility to disease, reduced performance and ultimately results in death (Friberg et al., 1974). Metals are considered to be environmentally important pollutants because of their persistence and ability to accumulate
in organisms; they are readily water-soluble, chemically stable, and show poor biodegradability (Hellawell, 1986; Sanders, 1997).

2.2 Metals and the environment

Metals exist as elements and compounds throughout the biosphere as a result of natural and anthropogenic processes. Most of the naturally occurring forms of metals in the environment originate from weathering of the mineral rich lithosphere. Runoff of these mineral rich soils during rainfall leads to the entrance of metals into the aquatic environment. These metals are present as a component of the suspended particulate matter, suspended solids, and in material that settles from the water column to form the bedded aquatic sediments in surface waters. Dissolution of a portion of these bedded aquatic sediments will result in metals being present in the dissolved form as well (Di Toro et al., 2001).

Metals enter the aquatic environment, from anthropogenic sources in both the particulate and dissolved form. These include point source discharges from industrial plants, mining activities and other diffuse sources (i.e. runoff from highways, residential and urban areas, and leaching from construction materials and anti-foulant coatings on boat hulls (Di Toro et al., 2001).

Some metals are essential elements and are required for the normal metabolism of organisms, while others are non-essential and play no significant biological role (Förstner and Wittmann, 1979). Excessive levels of essential metals can however be toxic to the organism by damaging the life functions of the organism. Essential metals include copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and zinc (Zn). Non-essential metals are cadmium (Cd), chromium (Cr), mercury (Hg), lead (Pb), arsenic (As), and antimony (Sb) (Kennish, 1992).
Aquatic ecosystems are sensitive to pollution influences because of the structure of their food chains. The small biomass in aquatic environments occurs in a variety of different trophic levels, which may lead to accumulation of xenobiotic substances at higher trophic levels (Förstner and Wittmann, 1979).

A complex set of reactions occurs upon the release of a metal or a metal compound into a water body, resulting in the metal being present in a variety of chemical forms (Morel, 1983; Stumm and Morgan, 1996). The chemical forms in which the metals occur determine the toxicity of the metal ions to aquatic organism (Brenzonik et al., 1990; Di Toro et al., 2001; Förstner and Wittmann, 1979). Table 2.1 provides a summary of the factors that influence metal toxicity.

### 2.3 Fish used as indicator to metal pollution

When selecting an organism as an indicator for ecological studies it’s important to consider a test organism that’s sensitive to environmental pollution. Fish are the ideal models for addressing questions regarding pollution in the aquatic environment because they are totally dependant on their surrounding environment for survival. When fish are subjected to stressors such as metals, they have immediate neuro-endocrine changes (primary responses) that produce biochemical and physiological changes (secondary responses). These changes ultimately lead to changes at the individual, community or population level (Pickering and Pottinger, 1995).

Fish are found on all rungs of the trophic ladder and are therefore able to integrate the effects of abiotic and biotic variables acting in the ecosystem (Figure 2.1: Di Toro et al., 2001). Fish are able to demonstrate synergistic, antagonistic and other combined effects which cannot be detected by
**Table 2.1:** Factors influencing the toxicity of metals in the aquatic environment (adapted from Bryan, 1976).

<table>
<thead>
<tr>
<th>Form of metal in water</th>
<th>inorganic</th>
<th>organic</th>
<th>soluble</th>
<th>particulate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ion</td>
<td>complex ion</td>
<td>chelate ion</td>
<td>molecule</td>
</tr>
<tr>
<td></td>
<td>colloidal</td>
<td>precipitated</td>
<td>absorbed</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Presence of other metals/poisons</th>
<th>joint action</th>
<th>no action</th>
<th>antagonism</th>
<th>more-than-additive</th>
<th>additive</th>
<th>less-than-additive</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Factors influencing physiology of test organism and chemical form of metal</th>
<th>temperature</th>
<th>pH</th>
<th>dissolved oxygen</th>
<th>light</th>
<th>salinity</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Condition of organisms</th>
<th>stage in life history (egg, larva, adult)</th>
<th>changes in life cycle (e.g. reproduction)</th>
<th>age</th>
<th>size</th>
<th>sex</th>
<th>starvation</th>
<th>activity</th>
<th>adaptation to metals</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Behavioural response</th>
<th>altered behaviour</th>
</tr>
</thead>
</table>

physical-chemical analysis (Luus-Powell, 1997). Studies have shown that fish have the ability to accumulate metals in their tissues (Barnhoorn, 2001; Coetzee, 1996; Giesy and Wiener, 1977; Nussey, 1998; ODEQ, 2003; Robinson, 1996).

Fish are found on all rungs of the trophic ladder and are therefore able to integrate the effects of abiotic and biotic variables acting in the ecosystem (Figure 2.1: Di Toro et al., 2001). Fish are able to demonstrate synergistic, antagonistic and other combined effects which cannot be detected by physical-chemical analysis (Luus-Powell, 1997). Studies have shown that fish have the ability to accumulate metals in their tissues (Barnhoorn, 2001; Coetzee, 1996; Giesy and Wiener, 1977; Nussey, 1998; ODEQ, 2003; Robinson, 1996).

When conducting ecotoxicological studies there are a number of advantages to using fish as a test organism. These include (Powers, 1989):

- fish are easily bred and raised under laboratory conditions;
- fish are inexpensive to purchase;
- several fish species are economically important.

Mining in South Africa is of great economical importance, which results in the production of cadmium, chromium and nickel as by-products. In the following section these metals will be discussed focusing on the economical importance, source of pollution, occurrence in the aquatic environment, the lethal and sub-lethal effects, as well as their bioaccumulation in the environment.

2.4 Cadmium

Cadmium is natural element found in the earth's crust, combined with other elements such as oxygen, chlorine, or sulphur. Cadmium is mostly present
in nature as complex oxides, sulphides, and carbonates in zinc, lead, and copper ores (ATSDR, 1999).

Cadmium is a non-essential trace element with no biological function and is implicated in numerous human deaths and delirious effects on fish and wildlife. In sufficient amounts cadmium is toxic to all forms of live (Eisler, 1985)

2.4.1 Economical importance and uses of cadmium

Cadmium ore does not exist since cadmium is commercially obtained as a by-product of zinc, lead and copper production. Cadmium is considered a contaminant in mining because it is not economically viable to mine for it solely. Rather than disposing of it as a waste, engineers have been able to utilise its unique properties for many important industrial applications (ATSDR, 1999).

A primary use for cadmium metal is as an anticorrosive because cadmium provides outstanding corrosion protection to metallic structures together with low friction coefficients and low electrical receptivity. The combination of these properties along with others such as good brazability and solderability, good plateability and galvanic compatibility make it the preferred coating when critical or safety related applications are considered such as fasteners or equipment in the aerospace, electrical, defence, mining, nuclear and off-shore industries. Cadmium serves as an electrode component in alkaline batteries and is used in alloys, silver solders, and welding. Cadmium is also used for pigment and plastics manufacturing. Cadmium pigments produce intense colourings such as yellow, orange and red, and are well known pigments in artists' colours, plastics, glasses, ceramics and enamels (ATSDR, 1999).
2.4.2 Source of cadmium pollution

It is estimated that about 25 000 to 30 000 tons of cadmium are released to the environment each year, about half from the weathering of rocks into river water and then to the oceans. Forest fires and volcanic emissions also release cadmium to the air (ATSDR, 1999; Bewers et al., 1987).

Release of cadmium from human activities is estimated at from 4 000 to 13 000 tons per year, with major contributions from mining activities, and burning of fossil fuels. Fertilizers often contain cadmium that will enter the soil and ground water when fertilizers are applied to crops (ATSDR, 1999).

2.4.3 Cadmium in the aquatic environment

The introduction of cadmium into natural waters has increased drastically since the turn of the century. Aquatic ecosystems are particularly sensitive to cadmium pollution mainly because of the very low levels of this metal in waters and biomass of prehistoric times, and the strong tendency of food webs to bioaccumulate this element (Nriagu and Sprague, 1987). Cadmium is widely regarded as a priority contaminant, and guidelines have been established for cadmium levels in public water supplies and other designated uses (ATSDR, 1999; Bewers et al., 1987).

The availability of cadmium to aquatic organisms from their immediate physical and chemical environments depends on numerous factors, including: absorption and desorption rates of cadmium from organic material; pH; chemical speciation of cadmium; and the redox potential of the water (USEPA, 1979; Eisler, 1985). Free (ionic) cadmium seems to be the toxic form and becomes much more prevalent at low salinity. Cadmium has a relatively long residence time in aquatic systems (Sprague, 1987).

Changes in pH affects cadmium sorption, with the proportion of available cadmium to sorbed cadmium changing from 90% at pH 5 to 5% at pH 9
Absorption and desorption processes are likely to be major factors in controlling the concentration of cadmium in natural waters. In most natural surface waters, the affinities of complexing ligands for cadmium generally follow the order of: humic acids > CO$_3^{2-}$ > OH$^-$. In unpolluted natural waters, most cadmium transported in the water column exist in the dissolved state as the hydrated ion Cd(H$_2$O)$_6^{2+}$. In polluted organic-rich waters, adsorption of cadmium by humic substances plays a dominant role in transport, partitioning, and remobilization of cadmium (USEPA, 1979).

2.4.4 Lethal and sub-lethal effects of cadmium on aquatic biota

The lethal effects of cadmium are thought to be caused by free cadmium ions that are not bound to metallothioneins or other proteins. These free cadmium ions may inactivate various metal dependent enzymes. Free cadmium ions also have the capacity to directly damage renal tubular membranes during uptake. The exact mechanism of acute cadmium poisoning is however unknown, but it depends in part on exposure period, concentration of cadmium in the medium, water temperature and salinity (Eisler, 1985).

The uptake of cadmium in fresh water fish occurs mainly thought the gills (Williams and Giesy, 1978). Cadmium presumably enters the gill epithelia via an apical Ca$^{2+}$-channel (Verbost et al., 1987) causing inhibition of the basolateral Ca$^{2+}$-transport (Verbost et al., 1988). Basolateral Ca$^{2+}$-transport depended on a high-affinity Ca$^{2+}$-ATPase. Ca$^{2+}$-ATPase is highly sensitive to cadmium (Flik et al., 1985; Verbost et al., 1988). The inhibition site is suggested to be on the Ca$^{2+}$-binding site of the Ca$^{2+}$-ATPase, mainly located in chloride cells (Whong and Chan, 1999). According to studies done by Pratap and Wendelaar Bonga (1993) ambient cadmium exposure accelerated the turn over rate of chloride cells and reduced Na$^+$/K$^+$-ATPase activity.
Substantial toxicological information on cadmium and freshwater biota demonstrates that ambient cadmium water concentrations exceeding 10 µg/l are associated with high morality, reduced growth, inhibited reproduction and other adverse effects. Teleosts exhibit significant mortality at cadmium concentrations of 0.8 to 9.9 µg/l during exposures of 4 to 33 days. Mortality generally increased as exposure time increased, water hardness decreased, and organisms age decreased (Eisler, 1985). Resistance to cadmium is higher in marine than in fresh water organisms and survival is usually higher at the lower temperature and higher salinities for any given level or cadmium in the medium (Sorensen, 1991).

Studies with three comparatively sensitive freshwater fish exposed to concentrations as low as > 1 and < 3 µgCd/l in water of low alkalinity, of 30 to 60 days, caused reduction in growth, survival and fecundity. Cadmium concentrations of 0.47 to 5.0 µg/l were associated with decreases in growth inhibition of reproduction, immobilization and population decrease in freshwater biota (Eisler, 1985).

Exposure of marine organisms to ambient cadmium concentrations between 0.5 and 10 µg/l resulted in decreases in growth, respiratory function disruption, molt inhibition, altered enzyme levels and abnormal muscular contractions. Effects were more pronounced at lower salinities and higher temperatures (Eisler, 1985).

At elevated concentrations (100 µg/l) cadmium inhibits the ATPase activity in different animal tissues including trout, and sometimes at concentrations as low as 16 µg/l in the case of gill basolateral membrane vesicles of European Eel (Anguilla anguilla). Inhibition of ATPase activities may lead to disturbances of osmoregulatory processes (Eisler, 2000). Table 2.2 provides a summary of sub-lethal effects on caused by cadmium exposure to fish.
**Table 2.2:** Sub-lethal effects of cadmium (adapted from Eisler, 2000; Meyers and Hendricks, 1982).

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Histological alterations</strong></td>
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<tr>
<td><strong>Gills</strong></td>
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<tr>
<td><strong>Gonads</strong></td>
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<tr>
<td><strong>Liver</strong></td>
<td></td>
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<tr>
<td><em>Halobarachus didactylus</em></td>
<td>CdCl₂</td>
<td>-</td>
<td>▪ Increase in connective tissue in liver.</td>
<td>Gutierrez et al., 1978.</td>
</tr>
</tbody>
</table>
Table 2.2: Sub-lethal effects of cadmium (adapted from Eisler, 2000; Meyers and Hendricks, 1982).

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
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<tr>
<td><strong>Other histological alterations</strong></td>
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</tr>
<tr>
<td><em>Cyprinus carpio</em></td>
<td>Cd (22 µg/l)</td>
<td>21 days</td>
<td>- Skin histopathology 24h – day 21, no deaths.</td>
<td>Igar et al., 1994.</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em></td>
<td>Cd (560 µg/l)</td>
<td>8 days</td>
<td>- Skin histopathology after 1h, all dead day 8.</td>
<td>Igar et al., 1994.</td>
</tr>
<tr>
<td><em>Fundulus heteroclitus</em></td>
<td>Cd (50 ppm)</td>
<td>48 hours</td>
<td>- Increase in intestinal mucous cell activity.</td>
<td>Gardner and Yевич, 1970.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Intestinal epithelial swelling.</td>
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<td></td>
<td></td>
<td></td>
<td>- Degeneration of proximal kidney tubes.</td>
<td></td>
</tr>
<tr>
<td><em>Salmo gairdneri</em></td>
<td>Cd (100 ppm)</td>
<td>4 weeks</td>
<td>- Hyaline droplet degeneration of kidney proximal tubes.</td>
<td>Forlin et al., 1986.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Dilated kidney lumina, reduced brush borders.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>- Proliferation of mitochondrial like bodies.</td>
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<td><strong>Physiological responses</strong></td>
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<td></td>
</tr>
<tr>
<td><em>Oreochromis mossambicus</em></td>
<td>Cd (22 µg/l)</td>
<td>96 hours</td>
<td>- LC(_50) for 3-day-old larvae, survivors had accumulation rate of 4.9 ng/larva daily.</td>
<td>Hwang et al., 1995.</td>
</tr>
<tr>
<td><em>Oreochromis mossambicus</em></td>
<td>Cd (83 µg/l)</td>
<td>96 hours</td>
<td>- LC(_50) for 1-day-old larvae, survivors had accumulation rate of 2.6 ng/larva daily.</td>
<td>Hwang et al., 1995.</td>
</tr>
</tbody>
</table>

Chapter 2 | Literature review: Metals
Table 2.2: Sub-lethal effects of cadmium (adapted from Eisler, 2000; Meyers and Hendricks, 1982).

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Physiological responses continue...</em></td>
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<td></td>
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<tr>
<td>Phoxinus phoxinus</td>
<td>Cd (7.5 ppb)</td>
<td>-</td>
<td>Spinal deformities.</td>
<td>Bengtson <em>et al.</em>, 1975.</td>
</tr>
<tr>
<td>Lepomis macrochirus</td>
<td>Cd (0.8 µg/l)</td>
<td>-</td>
<td>Inhibition of hepatic metal binding protein.</td>
<td>Cope <em>et al.</em>, 1994.</td>
</tr>
<tr>
<td>Puntius conchonius</td>
<td>Cd (0.63 ppm)</td>
<td>12 weeks</td>
<td>Erythrocyte abnormalities.</td>
<td>Gill and Plant, 1985.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Anaemia.</td>
<td></td>
</tr>
<tr>
<td>Plathichthys flesus</td>
<td>Cd (5-500 ppb)</td>
<td>4 - 9 weeks</td>
<td>Hypocalcemia, hypocalcemia.</td>
<td>Larsson <em>et al.</em>, 1981.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hypomagnesaemia, hyperphosphatemia.</td>
<td></td>
</tr>
<tr>
<td>Oncorhynchus mykiss</td>
<td>Cd (1-5 µg/l)</td>
<td>30 days</td>
<td>Reduction in glycogen content.</td>
<td>Richard <em>et al.</em>, 1998.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reduction in growth rate.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower plasma calcium concentration.</td>
<td></td>
</tr>
<tr>
<td>Salvelinus fontinalis</td>
<td>Cd (2 µg/l)</td>
<td>8 weeks</td>
<td>Disrupted lactic dehydrogenase activity and blood glucose levels.</td>
<td>Christiansen <em>et al.</em>, 1977.</td>
</tr>
<tr>
<td>Salmo salar</td>
<td>Cd (0.47 µg/l)</td>
<td>12 weeks</td>
<td>Alevin growth reduction.</td>
<td>Rombough and Garside, 1982.</td>
</tr>
<tr>
<td>Salmo salar</td>
<td>Cd (2 µg/l)</td>
<td>60 days</td>
<td>Cranial pathology, reduced growth, death.</td>
<td>Peterson <em>et al.</em>, 1983.</td>
</tr>
</tbody>
</table>
Table 2.2: Sub-lethal effects of cadmium (adapted from Eisler, 2000; Meyers and Hendricks, 1982).

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reproductive responses</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>Cd (1.8–3.4 µg/t)</td>
<td>-</td>
<td>• Eggs taken from adults exposed for 90 weeks failed to develop to fry stage.</td>
<td>Brown et al., 1994.</td>
</tr>
<tr>
<td><em>Salvelinus fontinalis</em></td>
<td>Cd (25 ppb) 24 hours</td>
<td></td>
<td>• Hormonal disturbances.</td>
<td>Sangalang and O'Halloran, 1972.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• 60 day embryo survival rate decrease.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Embryo weight decrease.</td>
<td></td>
</tr>
<tr>
<td><em>Lepomis macrochirus</em></td>
<td>Cd (0.08–2.14 ppm)</td>
<td>9 months</td>
<td>• Congenital deformities.</td>
<td>Eaton, 1974.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Pericardial and abdominal edema.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Shortened and deformed caudal fin and peduncle.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Delayed yolk sorption.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Microcephalia.</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2: Sub-lethal effects of cadmium (adapted from Eisler, 2000; Meyers and Hendricks, 1982).

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproductive responses  cont.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lepomis macrochirus</em></td>
<td>Cd (0.08 ppm)</td>
<td>-</td>
<td>▪ Larva from exposed adults move abnormally.</td>
<td>Eaton, 1974.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Tendency to low blood volume and blood clotting.</td>
<td></td>
</tr>
<tr>
<td><em>Lepomis macrochirus</em></td>
<td>Cd (2.14 ppm)</td>
<td>-</td>
<td>▪ Larva from exposed adults capable of only weak, quivering motions.</td>
<td>Eaton, 1974.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Tendency to low blood volume and blood clotting.</td>
<td></td>
</tr>
<tr>
<td><em>Oncorhynchus mykiss</em></td>
<td>Cd (1-5 µg/l)</td>
<td>30 days</td>
<td>▪ Endocrine disruption.</td>
<td>Richard <em>et al.</em>, 1998</td>
</tr>
</tbody>
</table>
2.4.5 Bioaccumulation and biomagnification of cadmium

Cadmium biomagnifies in terrestrial food chains and tends to accumulate in liver and kidneys of older organisms. This process was documented in the chain of soil to vegetation to invertebrates to upper trophic consumers (Eisler, 2000).

Fresh water and marine aquatic organisms accumulate cadmium from water containing cadmium concentrations not previously considered hazardous to public health or to most species of aquatic life (Eisler, 2000). Accumulation of cadmium in fish occurs principally from the water (Ferard et al., 1983; Hatakeyama and Yasuno, 1982; McFarlane and Franzin, 1980; Peterson et al., 1983; Rehwoldt and Karimian-Teherani, 1976). Uptake of cadmium from the medium by aquatic organisms usually increased with increasing water temperature in the range of 5–25ºC (Eisler, 2000).

Several studies have shown that cadmium accumulates in specific organs namely the kidney, liver, and gills of exposed specimens (Eaton, 1974; Kumada et al., 1980; Roberts et al., 1979; Sorensen, 1991). Cadmium was accumulated by fish hepatoma cells to a greater degree than were other metals (Eisler, 2000).

There is considerable variation in the ability of teleost tissues to accumulate cadmium from the ambient medium (Eisler, 2000). For example, rainbow trout exposed to 9 ppb cadmium for 2 weeks, bioconcentration factors (BCF) were 260 for gill, 17 for liver, 26 for kidney, and zero for spleen and heart tissues. At higher ambient cadmium levels of 10 ppb and exposure for 3 months, BCF values were substantially higher: 1,740 for gill, 4,900 for liver, 740 for kidney, 160 for spleen, and 100 for heart tissues (Roberts et al., 1979).

Bioaccumulation depends on exposure period, concentration of cadmium in the medium, water temperature and salinity. Under conditions of high
cadmium concentration and short exposure, the gill seems to be the primary site of damage and accumulation; under conditions of prolonged exposure and low cadmium levels, the intestine, kidney, and possibly other tissues were measurably affected. Retention of cadmium by teleosts depends on tissue biomagnification potential, length of post-exposure recovery period, and other factors. Evidence of cadmium transfer through various trophic levels suggests that only the lower trophic levels exhibit biomagnification (Eisler, 2000).

2.5 Chromium

Chromium is a natural component of the earth crust and is mined as chromite (FeCr$_2$O$_4$) ore. About 58% of the west’s chromium ores and 68.3% of the world’s known reserves of chromite, are located in South Africa's Bushveld Igneous Complex (Chamber of Mines, 2003).

The most common oxidation states of chromium are +2, +3, and +6, with +3 being the most stable. Chromium compounds of oxidation state 6 are powerful oxidants. Trivalent chromium (Cr$^{3+}$) is biologically essential and required in trace amounts for normal insulin and glucose metabolism in humans (Langard and Norseth, 1979), and for regulating carbohydrate metabolism in mammals (Gale, 1978; Onkelinx, 1977; Preston et al., 1976; USEPA., 1978). Trivalent chromium is considered to be a co-factor for insulin activity and part of an organic glucose tolerance factor in fish (Shiau and Chan, 1993) and have been shown to increases glucose utilization in tilapia and carp (Hertz et al., 1989). Hexavalent chromium (Cr$^{6+}$) is a non-essential element and considered to be toxic because of its powerful oxidative potential and it ability to cross cell membranes (Eisler, 2000).
2.5.1 Economical importance and uses of chromium

In 1999 South Africa produced 50.9% of world chrome ore output and chromite production reached 6 817 kilotons. The United States imports three quarters of its chromium and 60% of its ferrochrome from South Africa (Chamber of Mines, 2003).

More than 85% of all chromium produced is destined for the production of stainless steels and the closely related heat-resistant steels. Chromium, together with metals such as copper, nickel, titanium and vanadium, is added to iron to produce steels with greatly increased strength and rust- and corrosion-resistance. Ferrochrome is an essential ingredient of stainless steel. The chemical industry absorbs about 25% of chromium production. Chromium chemicals have a great variety of uses. Widely used as pigments, they also feature as tanning salts, oxidising agents and catalysts. Other applications include the chromium plating of metal surfaces, photography and pyrotechnics. Another main use for chromium and its compounds is in refractories, which account for 14% of production. Because of their high melting point, stability and chemical neutrality, chromite sands and refractory bricks containing chromite are used for casting moulds and furnace linings (ATSDR, 2000).

2.5.2 Sources of chromium pollution

Chromium is the seventh most abundant element on the earth with more than 2 108 million tons of chromium metal (most of it residing in the core and mantle of the earth). Although natural mobilization of chromium by weathering processes is estimated at 32 000 tons/year, the amounts of chromium added to the environment as a result of anthropogenic activities are far greater (Eisler, 2000).

Major atmospheric emissions of chromium were from chromium alloy and metal producing industries, and to a lesser amount from the coal
combustion, municipal incinerations, cement production, cooling towers, use of chromium containing phosphate fertilizers and landfill dumping of chromium contaminates sewage sludge and consumer products (ATSDR, 2000; Eisler, 2000;).

In aquatic environment the major source of chromium are the electroplating and metal finishing industries and publicly owned treatment plans, relatively minor sources other than localized contamination are ion and steel foundries, inorganic chemical plants, tanneries, textile manufacturing and runoff from urban and residential areas. Chromium in phosphates use as fertilizers may be an important source of chromium in soil and water (Eisler, 2000).

2.5.3 Chromium in the aquatic environment

Biological effect of chromium on aquatic organisms is modified by a variety of biotic and abiotic factors. These include the species, age, and developmental stage of the organism; the temperature, pH, salinity, and alkalinity of the medium; interaction effects of chromium with other contaminants; duration of exposure; and chemical form of chromium tested (Eisler, 2000).

In both freshwater and marine environments, hydrolysis and precipitation are the most important processes that determine the fate and effects of chromium. Adsorption and bioaccumulation play a relatively minor role in determining bioavailability of chromium to aquatic organisms (Ecological Analysts, 1981). Both Cr$^{3+}$ and Cr$^{6+}$ can exist in water with little organic matter, and Cr$^{6+}$ is usually the major species in seawater (USEPA, 1978). Under oxygenated conditions, Cr$^{6+}$ is the dominant dissolved stable chromium species in aquatic systems. The hexavalent (Cr$^{6+}$) form exists as a component of a complex anion that varies with pH and may take the form of chromate (CrO$_4^{2-}$), hydrochromate (HCrO$_4^{-}$), or dichromate (Cr$_2$O$_7^{2-}$). These ionic Cr$^{6+}$ forms are highly soluble in water and thus mobile in the
aquatic environment. All stable Cr\(^{6+}\) anionic compounds strongly oxidize organic matter on contact and yield oxidized organic matter and Cr\(^{3+}\) (Ecological Analysts, 1981). Trivalent chromium tends to form stable complexes with negatively charged inorganic or organic compounds, and thus is unlikely to be found un-complexed in aqueous solution if anionic or particulate compounds (such as decaying plant or animal tissues, or silt or clay particles) are present (Ecological Analysts, 1981; Pfeiffer et al., 1980; NRCC, 1976). Precipitated Cr\(^{3+}\) hydroxides remain in the sediments under aerobic conditions. At low pH and anoxic conditions Cr\(^{3+}\) hydroxides may solubilize and remain as ionic Cr\(^{3+}\) or oxidized to Cr\(^{6+}\) through mixing and aeration (Ecological Analysts, 1981).

### 2.5.4 Lethal and sub-lethal effects of chromium on aquatic biota

Inside the cell, the hexavelant (Cr\(^{6+}\)) form is reduced to the trivalent (Cr\(^{3+}\)) form which complexes with intracellular macromolecules, including genetic material, and is ultimately responsible for the toxic and mutagenic capacities of chromium (Eisler, 2000). All toxic effects of Cr\(^{6+}\) seem to be related to the strong oxidizing action of chromates, and all biological interactions of chromates seem to result in reduction to the Cr\(^{3+}\) form and subsequent coordination to organic molecules (Langard and Norseth, 1979).

There are at least five ionic species of hexavalent chromium. The hydrochromate ion and the chromate ion are the predominant species, and probably the agents that are toxic to freshwater life (Van der Putte et al., 1981a). Water pH dramatically affects the concentration of the ionic species present. A decrease in pH from 7.8 to 6.5 led to a increase in the hydrochromate ion by a factor of about 3, and a decrease in the chromate ion by a factor of about 6.8 (Van der Putte et al., 1981a). Studies had confirmed that Cr\(^{6+}\) is more toxic to freshwater daphnids and teleosts in water of comparatively low alkalinity, low pH, and low total hardness (Muller, 1980). In marine teleosts, the toxicity of Cr\(^{6+}\) increased at elevated temperatures (Eisler, 2000).
Aquatic plants and marine polychaete worms appear to be the most sensitive groups tested. Algae exposed to \( \text{Cr}^{6+} \) showed growth inhibition at 10 ppb and reproduction of polychaete worms was inhibited at 12.5 ppb. At higher concentrations, \( \text{Cr}^{6+} \) is associated with abnormal enzyme activities, altered blood chemistry, lowered resistance to pathogenic organisms, behavioural modifications, and disrupted feeding, histopathology, osmoregulatory upset, alterations in population structure and species diversity indices, and inhibition of photosynthesis (Eisler, 2000).

The effects and rate of uptake of chromium in teleosts were modified significantly by many biological and abiotic variables, including water temperature and pH, the presence of other contaminants or compounds, and sex and tissue specificity (Eisler, 1986). In rainbow trout, only males showed significant changes in liver enzyme activity during exposure to 0.2 ppm \( \text{Cr}^{6+} \) for 6 months; and the effects were intensified by the presence of nickel and cadmium salts in solution (Arillo et al., 1982). However, acute chromium poisoning caused macroscopic morphological changes in gills, kidney, and stomach tissues at pH 7.8, but only in the gills at pH 6.5 (Van der Putte et al., 1981a). Tests preformed on goldfish exposed to high \( \text{Cr}^{6+} \) concentrations indicated that toxic and sub-lethal effects were more pronounced at comparatively high water temperatures and reduced pH (Eisler, 2000). Table 2.3 provides a summary of the sub-lethal effects of chromium on fish.
**Table 2.3:** Sub-lethal effects of chromium (adapted from Eisler, 2000).

<table>
<thead>
<tr>
<th>Species</th>
<th>Cr\textsuperscript{6+} concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oncorhynchus tshawytscha</em></td>
<td>24 µg/l</td>
<td>105 days</td>
<td>• Nuclear DNA damage associated with impaired growth and reduced survival at higher concentration.</td>
<td>Farag <em>et al.</em>, 2006.</td>
</tr>
<tr>
<td></td>
<td>54 µg/l</td>
<td>105 days + 29 days</td>
<td>• Decrease weight.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increase: 24-120 µg/l</td>
<td></td>
<td>• Decrease survival.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>54-266 µg/l</td>
<td></td>
<td>• Increased lipid peroxidation products.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Reduced growth and survival.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Variations in DNA in the blood, associated with pathological changes in the kidney and spleen.</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.3 continues....

<table>
<thead>
<tr>
<th>Species</th>
<th><strong>Cr^{6+} concentration</strong></th>
<th>Exposure period</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dicentrarchus labrax</em></td>
<td>2m(M)</td>
<td>24 hours</td>
<td>▪ Total haemolysis of erythrocyte.</td>
<td>Roche and Bogé, 1993.</td>
</tr>
<tr>
<td></td>
<td>1-00 µ(M)</td>
<td>24 hours</td>
<td>▪ Increase glutathione peroxidase activity.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5-1 m(M)</td>
<td>24 hours</td>
<td>▪ Decrease glutathione peroxidase activity.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1m(M)</td>
<td>24 hours</td>
<td>▪ Increase lipid peroxidase activity.</td>
<td></td>
</tr>
<tr>
<td><em>Salmo gairdneri</em></td>
<td>16-21 ppb</td>
<td>14 - 16 weeks</td>
<td>▪ Reduced growth.</td>
<td>USEPA, 1980a.</td>
</tr>
<tr>
<td><em>Oncorhynchus tshawytscha</em></td>
<td>16-21 ppb</td>
<td>14 - 16 weeks</td>
<td>▪ Reduced growth.</td>
<td>USEPA, 1980a.</td>
</tr>
<tr>
<td><em>Salmo gairdneri</em></td>
<td>16-21 ppb</td>
<td>7 days</td>
<td>▪ Altered plasma cortisol levels.</td>
<td>USEPA, 1980a.</td>
</tr>
<tr>
<td><em>Channa punctatus</em></td>
<td>2.6 ppm</td>
<td>30 day</td>
<td>▪ Altered enzyme activities for variety of organs.</td>
<td>Sastry and Sunita, 1984.</td>
</tr>
</tbody>
</table>
**Table 2.3** continues...

<table>
<thead>
<tr>
<th>Species</th>
<th>Cr&lt;sup&gt;6+&lt;/sup&gt; concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physiological reactions cont.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Oncorhynchus kisutch</em></td>
<td>0.5 ppm</td>
<td>2 weeks</td>
<td>• Lower disease resistance.</td>
<td>Sugatt, 1980.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Lower serum agglutin production.</td>
<td></td>
</tr>
<tr>
<td><em>Oncorhynchus kisutch</em></td>
<td>0.23 ppm</td>
<td>4 weeks</td>
<td>• Lower salinity tolerance.</td>
<td>Sugatt, 1980.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Lower serum osmolarity.</td>
<td></td>
</tr>
<tr>
<td><em>Salmo gairdneri</em></td>
<td>0.2 ppm</td>
<td>6 months</td>
<td>• Altered liver enzyme activities.</td>
<td>Arillo <em>et al.</em>, 1982.</td>
</tr>
</tbody>
</table>

**Histological and morphological reactions**

<table>
<thead>
<tr>
<th><em>Oncorhynchus tshawytscha</em></th>
<th>54 µg/t</th>
<th>105 days</th>
<th>+ 29 days</th>
<th>Lipid droplets in liver.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase:</td>
<td></td>
<td></td>
<td></td>
<td>Increase gill epithelium.</td>
</tr>
<tr>
<td>24-120 µg/t</td>
<td></td>
<td></td>
<td></td>
<td>Apoptosis of chloride cells.</td>
</tr>
<tr>
<td>54-266 µg/t</td>
<td></td>
<td></td>
<td></td>
<td>Chloride cells cytoplasm hypereosinophilic, pyknosis, karyorrhexis.</td>
</tr>
</tbody>
</table>
2.5.5 Bioaccumulation and biomagnification of chromium

Accumulation of chromium in tissues and organism shows great variability. Accumulation depends on the chemical form in which chromium are present, the route of exposure, the amount administered and the effect of numerous biotic and physicochemical modifiers (Eisler, 1986). Chromium concentrations also vary among different species of fish collected from the same geographic area. For example, chromium concentration in muscle of a porgy (*Pachymetopan grande*) was 1430 times greater than in a goosefish (*Lophius piscatorius*) from the same area (Van As *et al*., 1973).

Accumulation of chromium under controlled conditions has been documented for speckled sanddab (Mearns and Young, 1977) and atlantic croaker (Baptist *et al*., 1970). Sanddabs held in seawater solutions containing 3 to 5 ppm Cr\(^{6+}\) contained up to 100 mgCr/kg intestine (dry weight), 10 in liver, and 3 in muscle (Mearns and Young, 1977). Sanddabs accumulated significant concentrations of chromium in various tissues during long-term exposure in seawater concentrations as low as 16 ppb Cr\(^{6+}\) (Mearns and Young, 1977).

At high concentrations of Cr\(^{6+}\) (i.e. 2.0 ppm in water) and at alkaline pH, chromium concentrations in rainbow trout tissues were greatest in gill, liver, kidney, and digestive tract. After transfer of these exposed fish to chromium-free media, residues tended to remain high in kidney and liver; concentration in gill tissues tended to be greater at pH 7.8 than at pH 6.5 (Van der Putte *et al*., 1981b).

Long-term exposure of rainbow trout to Cr\(^{6+}\) concentrations of 0.2 ppm resulted in elevated levels of chromium in kidney (3.5 mg/kg fresh weight), liver (2.0), and muscle (0.6). After 90 days in chromium-free media, chromium levels were 1.6, 1.3, and 0.5, respectively (Calamari *et al*., 1982). The rudd (*Scardinus erythrophthalmus*), exposed to Cr\(^{6+}\) for 24 hours, did not accumulate detectable levels of chromium in tissues during...
exposure to 16 ppm, but did during exposures to 20 ppm; the kidney contained residues up to 10.3 mgCr/kg fresh weight (Van Hoof and Van San, 1981).

High accumulations of chromium have been recorded among organisms from the lower trophic levels, but there is little evidence of biomagnification through food chains (Eisler, 2000).

2.6 Nickel

Nickel is a natural component of the earth crust and is mainly mined as (Wikipedia, 2006):

- laterites
  - principal ore minerals are nickeliferous limonite \((\text{Fe,Ni})_\text{O(OH)}\);
- garnierite
  - hydrous nickel silicate \((\text{Ni,Mg})_\text{3Si}_\text{2O}_\text{5(OH)}\);
- magmatic sulfide deposits
  - principal ore mineral is pentlandite \((\text{Ni,Fe})_\text{9S}_\text{8}\).

Approximately 8.5% of the world’s nickel reserves are located in South Africa’s Bushveld Igneous Complex. South Africa’s nickel is derived mainly as a byproduct from platinum mining (Chamber of Mines, 2003).

Nickel is essential for the normal growth of many species of microorganisms and plants and several species of vertebrates. Studies on chicks and rats suggest that nickel is essential for proper liver function. Various nickel salts, (including the sulphate, chloride, and bromide), were used in human medicine during the mid- to late 1800’s to treat headache, diarrhoea, and epilepsy and as an antiseptic. Therapeutic use of nickel compounds was abandoned in the early 1900’s after animal studies demonstrated acute and chronic toxicity of these salts (NAS, 1975; Nriagu, 1980).
2.6.1 Economical importance and uses of nickel

In 1999 South Africa produced an estimated 36,2 Kt of nickel (Chamber of Mines, 2003). Nickel is vital to the steel industry and especially the stainless steel industry, and it has played a key role in the development of the chemical, aerospace and armaments industries (Chamber of Mines, 2003).

The metal's greatest value lies in the alloys it can form with other elements, where it adds strength and corrosion resistance over a wide range of temperatures (Chamber of Mines, 2003). These alloys are used in ship building, jet turbines and heat elements, cryogenic installations, magnets, coins, welding rods, electrodes, kitchenware, electronics, and surgical implants; other nickel compounds are used in electroplating, battery production, inks, varnishes, pigments, catalysts, and ceramics (Eisler, 2000).

Nickel compounds are preferred for use in (ATSDR, 2005):

- nickel electroplating (nickel sulfate, nickel ammonium sulfate, nickel chloride, nickel fluoborate, nickel sulfamate),
- refining (nickel carbonyl),
- nickel-cadmium batteries (nickel hydroxide, nickel fluoride, nickel nitrate),
- the manufacture of stainless steel and alloy steels (nickel oxide),
- electronic components (nickel carbonate),
- mordant in textile industry (nickel acetate),
- catalysts and laboratory reagents (nickel acetate, nickel hydroxide, nickel nitrate, nickel carbonate, nickel mono-sulfide, nickelocene).

Some nickel salts have been incorporated into fungicides to combat plant pathogens, although their use has not been approved by regulatory agencies (Eisler, 2000).
2.6.2 Sources of nickel pollution

Nickel is ubiquitous in the biosphere. Nickel introduced into the environment from natural or human sources is circulated through the system by chemical and physical processes and through biological transport mechanisms of living organisms (NAS, 1975; Sevin, 1980; WHO, 1991).

Natural sources of airborne nickel include soil dust, sea salt, volcanoes, forest fires, and vegetation exudates and account for about 16% of the atmospheric nickel burden. Human sources of atmospheric nickel; which account for about 84% of all atmospheric nickel; include emissions from nickel ore mining, smelting, and refining activities; combustion of fossil fuels for heating, power, and motor vehicles; incineration of sewage sludges; nickel chemical manufacturing; electroplating; nickel-cadmium battery manufacturing; asbestos mining and milling; and cement manufacturing (Eisler, 2000).

Weathering and erosion of geological materials release nickel into soils (Chau and Kulikovsky-Cordeiro, 1995), and acid rain may leach nickel from plants into soils as well (WHO, 1991). The primary human sources of nickel to soils are emissions from smelting and refining operations and disposal of sewage sludge or application of sludge as a fertilizer. Secondary sources include automobile emissions and emissions from electric power utilities (USEPA, 1986).

Chemical and physical degradation of rocks and soils, atmospheric deposition of nickel-containing particulates, and discharges of industrial and municipal wastes release nickel into ambient waters (USEPA, 1986; WHO, 1991). Nickel enters natural waterways from waste water because it is poorly removed by treatment processes (Cain and Pafford, 1981). The main anthropogenic sources of nickel in water are primary nickel production, metallurgical processes, combustion and incineration of fossil fuels, and chemical and catalyst production (USEPA, 1986).
2.6.3 Nickel in the aquatic environment

Nickel normally occurs in the 0 and +2 oxidation states, although other oxidation states have been reported. In aquatic systems, nickel occurs as soluble salts adsorbed onto or associated with clay particles, organic matter, and other substances. The divalent ion is the dominant form in natural waters at pH values between 5 and 9, occurring as the octahedral, hexahydrate ion \((\text{Ni(H}_2\text{O})_6\text{)}^{2+}\). Nickel chloride hexahydrate and nickel sulfate hexahydrate are extremely soluble in water. Nickel forms strong, soluble complexes with \(\text{OH}^-\), \(\text{SO}_4^{2-}\), and \(\text{HCO}_3^-\). Less soluble nickel compounds in water include nickel nitrate, nickel hydroxide and nickel carbonate (Eisler, 2000).

The fate of nickel in fresh water and marine water is affected by the pH, pE, ionic strength, type and concentration of ligands, and the availability of solid surfaces for adsorption. Under anaerobic conditions, typical of deep groundwater, precipitation of nickel sulfide keeps nickel concentrations low (Eisler, 2000).

2.6.4 Lethal and sub-lethal effects of nickel

Nickel interacts with numerous inorganic and organic compounds. Some of these interactions are additive or synergistic in producing adverse effects, and some are antagonistic (Eisler, 1998).

In mammalian blood, absorbed nickel is present as free hydrated \(\text{Ni}^{2+}\) ions, as protein complexes, and as nickel bound to blood cells. Partitioning of nickel among these components varies according to the metal-binding properties of serum albumin, which is highly variable between species (Kasprzak, 1987; USEPA, 1986).

Inside the mammalian cell, nickel accumulates in the nucleus and nucleolus (IARC, 1984), disrupting DNA metabolism and causing cross links and
strand breaks (Kasprzak, 1987). Experimental evidence support the conclusion that the nickel-dependent formation of an activated oxygen species (superoxide ion, hydrogen peroxide, and a hydroxy radical) is a primary molecular event in acute nickel toxicity and carcinogenicity of Ni$^{2+}$ in mice (Hausinger, 1993; Novelli et al., 1995; Rodriguez et al., 1996; Stohs and Bagchi, 1995; Tkeshelashvili et al., 1993; WHO, 1991).

Signs of nickel poisoning in fishes include surfacing, rapid mouth and opercular movements and, convulsions and loss of equilibrium (Khangarot and Ray, 1990). Ionic nickel destroys gill lamellae, resulting in a decrease in the ventilation rate which may lead to blood hypoxia and death (Ellgaard et al., 1995). Other signs of nickel poisoning in fishes include decreased concentrations of glycogen in muscle and liver with simultaneous increases in levels of lactic acid and glucose in blood (Ghazaly, 1992), depressed hydrogen peroxide production in tissues, reduction in superoxide dismutase (Bowser et al., 1994), and contractions of vascular smooth muscle (Evans et al., 1990).

Nickel toxicity is modified by many variables (Eisler, 1998). Nickel is most lethal to freshwater crustaceans and fishes at pH 8.3 and least lethal at pH 6.3. The presence of chelating agents also reduces nickel toxicity (Lee and Lustigman, 1996). Nickel is less toxic to algae when copper is absent or present at low concentration. Tests have shown nickel to be more toxic to estuarine amphipods and clams under conditions of decreased salinity and increased temperature (WHO, 1991).

Nickel accumulates in fish tissues and cause alterations in gill structure, including hypertrophy mucus cells, separation of the epithelial layer from the pillar cell system, cauterization and sloughing, and necrosis of the epithelium (Nath and Kumar, 1989).

Rainbow trout exposed to nickel showed growth inhibition and altered immunoregulatory mechanisms. Nickel exposure cause reproduction
inhibition of daphnids at 30 $\mu g/\ell$, growth inhibition of freshwater and marine algae at 30–125 $\mu g/\ell$, and abnormal development of sea urchin embryos at 58 $\mu g/\ell$ (NRCC, 1981; Outridge and Scheuhammer, 1993; WHO, 1991).

Nickel is a reproductive toxicant in animals (Eisler, 1998). Specific effects of nickel on reproduction include degenerative changes in the testes, epididymis, and spermatozoa of rats; adverse effects on embryo viability of rats and hamsters; and delayed embryonic development of rodents (Smialowicz et al., 1984; USEPA, 1986). Male rats given 2.32 mgNi/kg body weight nickel acetate via intramuscular injection, showed inhibition of testosterone production and reduced growth. Females given the same treatment had increased uterine weights (USPHS, 1977).

Three generations of rats given 5 mg/\ell nickel in their drinking water, were intolerable for normal reproduction. All generations of rats given nickel in drinking water had increased proportions of runts and increased neonatal mortality when compared to controls. The third generation of nickel-treated rats, showed reductions in litter size and a reduction in the number of males (Schroeder and Mitchener, 1971). See Table 2.4 for a summary of sub-lethal effects of nickel on selected fish species.
### Table 2.4: Sub-lethal effects of nickel on selected fish species (adapted from Eisler, 1998).

<table>
<thead>
<tr>
<th>Species</th>
<th>Metal compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Physiological alterations</td>
<td></td>
</tr>
<tr>
<td><strong>Anabas testudineus</strong></td>
<td>146.0 mg/ℓ</td>
<td>30 days</td>
<td>▪ Depletion of glycogen and total proteins in liver and gonads.</td>
<td>Jha and Jha, 1995.</td>
</tr>
<tr>
<td><strong>Cyprinus carpio</strong></td>
<td>8.0 mg/ℓ</td>
<td>15 days</td>
<td>▪ Disrupted protein metabolism in gills and kidney.</td>
<td>Sreedevi et al., 1992.</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cyprinus carpio communis</strong></td>
<td>2.5; 5.0; 7.0 and 10.0 mgNi/ℓ</td>
<td>30 days</td>
<td>▪ Protein content decrease over time.</td>
<td>Thatheyus et al., 1992.</td>
</tr>
<tr>
<td>Fingerlings</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oncorhynchus mykiss</strong></td>
<td>116.0 mgNi/ℓ</td>
<td>-</td>
<td>▪ Cytotoxic.</td>
<td>Segner et al., 1994.</td>
</tr>
</tbody>
</table>
Table 2.4 continues...

<table>
<thead>
<tr>
<th>Species</th>
<th>Metal compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physiological alterations cont.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oreochromis niloticus</td>
<td>1.5 or 3.0 mg/l</td>
<td>10 days</td>
<td>▪ Glycogen depletion of liver and muscle.</td>
<td>Alkahem, 1995.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Increase in plasma glucose.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Alterations more pronounced at higher concentrations.</td>
<td></td>
</tr>
<tr>
<td>Tilapia nilotica</td>
<td>19.0, 32.0 or 51.0 mg/l</td>
<td>96 hours</td>
<td>▪ Time and dose dependant increase in blood glucose and lactic acid concentrations.</td>
<td>Ghazaly, 1992.</td>
</tr>
<tr>
<td></td>
<td>32.0 or 51.0 mg/l</td>
<td>96 hours</td>
<td>▪ Liver glycogen decrease</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Muscle glycogen decrease</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Elevated erythrocyte number.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Increase in haematocrit and haemoglobin.</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4 continues...

<table>
<thead>
<tr>
<th>Species</th>
<th>Metal compound/concentration</th>
<th>Exposure period</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histological alterations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Colisa fasciata</em></td>
<td>64.0 mg/t</td>
<td>96 hours</td>
<td>▪ Testicular degeneration.</td>
<td>Nath and Kumar, 1989.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Spermatogonial activity reduced.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Germ-cell degeneration.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Congested blood vessels.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Ovaries histological different.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>▪ Oocytes are reabsorbed.</td>
<td></td>
</tr>
<tr>
<td><strong>Reproductive alterations</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>
Studies have shown that nickel also affect endocrine and enzymatic processes in mammals. Nickel-induced endocrine effects include inhibition of insulin production in pancreas, prolactin in hypothalamus, amylase excretion in parotid gland, and iodine uptake in thyroid, disruption of hepatic mono-oxygenases. Inhibition of enzyme activity by nickel is reported for RNA polymerase, ATPase, dialkyl fluorophosphate, and aspartase. Nickel increases the duration of the action potential of excitable membranes of nerve and muscle tissues; this effect is competitive with and imitative of those of calcium (Eisler, 1998).

Nickel has also been reported to activate various enzymes, including bovine pancreatic ribonuclease, pancreatic deoxyribonuclease, carboxypeptidase, arginase, phosphoglucomutase and calcineurin (calmodulin-dependent phosphoprotein phosphatase). Nickel affects the activity of heme oxygenase, thereby affecting the absorption of haemoglobin iron. Nickel induces heme oxygenase activity in tissues of mice (Eisler, 1998).

2.6.5 Bioaccumulation and biomagnification of nickel

Nickel concentrations are elevated in aquatic plants and animals in the vicinity of nickel smelters, nickel-cadmium battery plants, electroplating plants, sewage outfalls, coal ash disposal basins, and heavily populated areas (Eisler, 1998).

The bioaccumulation of nickel under field conditions varies greatly among groups. Bioconcentration factors for aquatic macrophytes range from 6 in pristine areas to 690 near a nickel smelter; for crustaceans these values are 10-39; for mollusks, 2-191; and for fishes, 2-52 (Sigel and Sigel, 1988). This variability can be attributed to differential tissue uptake and retention of nickel, age of organism, and metal-tolerant strains (Bryan et al., 1977; Bryan and Hummerstone, 1978; USEPA, 1980b; Eisler, 1981; Chau and Kulikovsky-Cordeiro, 1995).
Fish can accumulate nickel from food and water (Eisler, 1998). Bioconcentration factors for rainbow trout after exposure for 6 months to 1.0 mgNi/l were 0.8 for muscle, 2.9 for liver, and 4.0 for kidneys (Calamari et al., 1982). Common carp (Cyprinus carpio) and tilapia (Tilapia nilotica) exposed for 16 days to 1.0 mgNi/l, had elevated concentrations in livers of 49-77 mgNi/kg dry weight (Canli and Kargin, 1995).

Although aquatic organisms can accumulate nickel from their surroundings, there is little evidence of significant biomagnification of nickel levels along food chains (NRCC, 1981; Sigel and Sigel, 1988; WHO, 1991).

Cadmium, chromium and nickel do cause adverse effects in fish as proven by the literature review. The aim of this research is to study the histological changes in fish caused by cadmium, chromium and nickel after acute exposure. The next chapter will discuss biomarkers as well as the use of histology as a biomarker in more detail.

2.8 References


Chapter 3

Literature review: Biomarkers

3.1 Biological markers of contaminant exposure

In the past, chemical analysis of water has proven to be of great use for the detection of pollutants within the environment (Peakall, 1992). The reliance on chemical data alone is becoming obsolete. There is now a trend to incorporate biological monitoring, or biomonitoring, into standard monitoring practices (Roux, 1994). Biomonitoring allows analysis of the effect of pollution on biological systems, providing an indication of both the past and the present water quality (Grant, 2004).

Biomonitoring at the lower levels of biological organization allows for an “early warning system” whereby stressors can be detected at an early stage, and dealt with before they exert their effects in higher levels of biological organization, e.g. at individual or community level (Grant, 2004).

According to Meyer et al. (1992) the term biomarker has been adopted to refer to the use of physiological, biochemical and histological changes as indicators of exposure and/or effects of xenobiotics at the organismal level. A more precise description of a biomarker is given the definition provided by the National Research Council (1989):

“A biomarker is a xenobiotically-induced variation in cellular or biochemical components or processes, structures or functions that is measurable in a biological system or sample.”
For the analysis of organism exposure to complex chemical mixtures, biomarkers offer the potential for integrating various interactions within the exposed organisms and expressing the accumulative impact of the toxicant exposure as a biomarker response measured at the site of toxicant action (Shugart et al., 1992).

According to the NRC (1987) and WHO (1993), biomarkers can be subdivided into three main classes:

i. Biomarkers of exposure, which indicate exposure to various chemicals.

ii. Biomarkers of effect, which indicate any adverse biochemical physiological alterations brought about by exposure to toxicants.

iii. Biomarkers of susceptibility, indicating the inherited or acquired ability of an organism to deal with a specific toxicant.

The responses of biomarkers can be regarded as biological or biochemical effect after exposure to a certain toxicant, which makes them theoretically useful as indicators of both exposure and effect. Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance, providing a link between exposure and effect. Biomarkers of exposure can be cost effective and sensitive tools for broad-spectrum screening studies to determine the absence or presence of toxicants. If these biomarkers respond in such a manner as to suggest a problem, a second study using more specific biomarkers (biomarkers of effect) may be performed (Van der Oost et al., 2003).

In order to assess the exposure or the effect of toxicants on the aquatic environment, the following biological and biochemical parameters may be examined in fish (Van der Oost et al., 2003):

- biotransformation enzymes (phase I and phase II);
- oxidative stress parameters;
- biotransformation products;
- stress proteins (metallothioneins and heat shock proteins);
- haematological parameters;
- immunological parameters;
- reproductive and endocrine parameters;
- genotoxic parameters;
- neuromuscular parameters;
- physiological, histological and morphological parameters.

Biomarker responses are powerful because they integrate a wide array of environmental, toxicological and ecological factors that control and modulate exposure to, as-well-as the effects of environmental contaminants. These same factors complicate the interpretation and the significance of the biomarker responses. Many non-pollution-related variables may have an additional impact on the various enzyme systems, and may thus interfere with the biomarker responses when experimental conditions are not thoroughly analyzed or controlled. Non-pollution related variables include factors such as the organism’s health, condition, sex, age, nutritional status, metabolic activity, reproductive and developmental status; as well as factors like season, ambient temperature and heterogeneity towards the environmental pollution (Van der Oost et al., 2003).

According to De Zwart (1995) there are several advantages to using biomonitoring methods in the control of chemical pollution. Firstly, these methods measure effects in which the bioavailability of the compounds of interest is integrated with the concentration and the compounds intrinsic toxicity. Secondly, biological measurements form the only way of integrating the effects of toxicants on a large number of individual and integrative processes. A disadvantage however is that it may prove difficult to relate the observed effects to specific aspects of pollution or to effects on the level of populations, communities or ecosystems. Biomarkers should therefore not be considered as a replacement for current chemical analysis, but rather as a supplement to be incorporated within environmental monitoring programs utilising many different indices (Deplege and Fossi, 1994).
It is important to establish the relationship between responses at the lower levels of biological organization (sub-cellular and cellular levels) to responses at higher levels such as populations, communities and ecosystems (Bayne et al., 1985; Capuzzo et al., 1985; Sasry and Miller, 1981; Sindermann, 1985). According to Bayne et al. (1985) it is essential to understand the causal relationships that link biological responses to stress with physiological attributes of animal fitness before effects on individuals can be extrapolated to consequent effect on population and communities. By measuring stress responses at various levels of biological organization one can monitor a spectrum of sensitivities to stress, a variety of specific effects, and several points of ecological relevance simultaneously. These will provide empirical evidence of linkages, the mechanism of which can be determined by controlled experimentation (Adams et al., 1993).

Figure 3.1 shows the theoretical relationships between ecological relevance and time-scales of pollutant-induced biomarker responses as proposed by Adams et al. (1993). The following biomarkers were applied:

- Physiological biomarker responses (which included various blood parameters and the necropsy based fish health assessment index).
- Condition indices (determination of Fulton’s condition factor as well as the determination of various organo-somatic indices).
- Histopathological biomarker responses (determining toxicant induces histopathological changes in the liver, gills and gonads of exposed fish; as well as determining the gonadal maturity index).

A study was done in conjunction with this study by Basson (2006) which focused on the changes in heat shock protein induction in the exposed fish, as well as the observed changes in blood cortisol levels (biochemical biomarker response).
The remainder of this chapter will focus on the advantages and disadvantages of the biomarkers as applied during this study. The following section (Section 3.2) will focus on the use of the necropsy-based fish health assessment index (NFHAI), calculation of the condition factor (CF) and the use of blood variables as effective biomarkers of contaminant exposure. Section 3.3 discusses the use and interpretation of various organo-somatic indices as biomarkers, and Section 3.4 discuss the use of histology as a biomarker.
3.2 Necropsy based fish health assessment index as biomarker (NFHAI)

Goede (1988) developed a systematic necropsy based fish health assessment for use by fisheries. It was developed to provide a rapid method to detect trends in the general health and condition of fish populations (Goede and Barton, 1990). The necropsy method consists of 16 variables that can be grouped into the following categories:

i. Length, weight and calculation of a condition factor.
ii. Blood parameters including haematocrit, leucocrit and plasma proteins.
iii. Macroscopic appearance of external organs.
iv. Macroscopic appearance of internal organs.

Adams et al. (1993) refined the original necropsy based fish health system by quantifying the alterations found in the examined organs, thus allowing for comparisons between different populations.

When using a system like the necropsy based fish health assessment index (NFHAI), several assumptions are made (Goede and Barton, 1990):

- When all organs and tissues appear normal according to the criteria, there is a good probability that fish are healthy.
- When fish are exposed to elevated levels of contaminants, tissue and organ function will change in order to maintain homeostasis.
- If a change in function persists in response to continuing stress, there will be a gross change in the structure of organs and tissues.
- If the appearance of an organ or tissue system depart from the normal or from a control condition, the fish are responding to changes brought about by the environmental stressor.

A limitation of this system is that the necropsy data do not provide evidence of the factors responsible for causing particular changes. Lesions observed at this level suggests that adaptive mechanisms (immunological, physiological and biochemical mechanisms) have been overwhelmed. To
obtain definite information about causal mechanisms, relevant biochemical, physiological, histological and toxicological studies are required. There can be gross microscopic alterations of histological changes without gross manifestations. Gross change may be predicted from histological change, but not the converse (Goede and Barton, 1990). The necropsy based fish health assessment index can thus be considered as a biomarker of exposure.

In this study the necropsy based fish health assessment index was applied to determine the general health of the exposure groups compared to the control group.

3.2.1 Condition factor (CF)

Fulton’s condition factor (Carlander, 1969), as calculated for the necropsy based fish health assessment index, is an organism-level response. Factors such as nutritional status, pathogen effects and toxic chemical exposures can cause an increase or decrease in the condition factor. Because the condition factor (CF) integrates different levels of sub-organismal processes, it is therefore able to signify the overall health and nutritional status of an individual fish (Adams et al., 1993).

In general, CF varies directly with nutrition (Tyler and Dunn, 1976). According to Adams et al. (1993) a value of one is indicative of a very good health status. A negative correlation exists between disease and condition in fishes (Möller, 1985). CF may vary in either direction outside the normal range in response to chemical exposure as shown in Table 3.1.

When using CF to assess fish health and condition it is important to consider the following (Schmitt et al., 2000):

- CF vary seasonally (Griffiths and Kirkwood, 1995; Saborowski and Buchholz, 1996), due to changes in food availability, metabolism, and with changes in gonadal status (Chellappa et al., 1995).
• CF varies greatly among fish taxa owing to their differential architecture, but condition indices can also vary from location to location within a species (Doyon et al., 1988; Fisher et al. 1996).
• A decrease in weight due to loss of energy stores can be offset by an increase in body water (Goede and Barton, 1990).

Table 3.1: Effects of contaminant exposures on the condition of exposed fish (adapted from Schmitt et al., 2000).

<table>
<thead>
<tr>
<th>Species</th>
<th>Contaminant / stressors</th>
<th>CF</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepomis auritus</td>
<td>Paper mill effluent</td>
<td>Increase</td>
<td>McMaster et al., 1991.</td>
</tr>
<tr>
<td>Catostomus commersoni</td>
<td>Metal mix</td>
<td>Decrease</td>
<td>Miller et al., 1992.</td>
</tr>
</tbody>
</table>

A limitation to using CF is that the results obtained do not provide evidence of the factors responsible for causing a change in the condition of the fish. The determination of CF is a useful tool when used as a first level screen to determine possible contaminant exposures (Van der Oost et al., 2003).

3.2.2 Haematological parameters

The use of haematological parameters in fish is becoming increasingly more prevalent in the assessment of environmentally stressful conditions because the properties of blood are sensitive to physiological as well as pathological changes in fish (Alexander et al., 1980; Blaxhall, 1972; Hickey, 1976). This is a result of the close association of the circulatory system with the external environment (Casillas and Smith, 1977). Because fish are cold-blooded animals, external factors modify their haematological constants (Blaxhall, 1972; Douellou and Guillaume, 1986). According to Van Vuren (1986), if the
water is affected by toxicants to such an extent that acute stress is obtained, then it will be reflected in haematological parameters.

There is controversy regarding the influence of sex on fish haematological variables. According to Douellou and Guillaume (1986) haematological constants are independent of sex, but according to findings by Blaxhall (1972), male fish always gives higher haematological values than female fish. Seasonal differences due to changing environmental conditions are well known from the literature (Hickey, 1976) and can be explained by the differences in water quality variables such as pH, oxygen saturation, conductivity and phosphates (Van Vuren and Hattingh, 1978a,b).

Haematological parameters are non-specific in their responses towards chemical stressors (Van der Oost et al., 2003). Nevertheless, they provide important information in effect assessment studies, by providing an indication as to the general physiology and health status of the organism under investigation (Beyer, 1996).

Haematological parameters, such as haematocrit, leucocrit and total protein, are generally less specific than the serum enzymes, and may also be influenced by natural factors such as bacterial challenges (Iwama et al., 1986). However, these parameters proofed to be useful biomarkers of toxicant effects in fish (Allen, 1993; Boon et al., 1992; Ghazali, 1992; Reddy et al., 1991; Tort et al., 1987; Van Vuren, 1986; Wedemayer and Yasutake, 1977) and are used to determine the oxygen capacity of the blood (Larsson, et al., 1985).

3.2.2.1 Haematocrit (H)

When a capillary tube filled with whole blood is centrifuged to separate the cells from the serum, the ratio of the cellular fraction to the total blood volume is called the haematocrit (Anderson, 1974; Hickey, 1976).
Red blood cells are predominant cells in the whole blood sample. A haematocrit value in the range of 30 - 45% can be considered as normal for tilapia species (Robinson, 1996). According to studies done by Houston and De Wilde (1968), the haematocrit were directly proportional to a rise in temperature in rainbow trout and carp. Stress conditions are accompanied by an increase in the haematocrit (Douellou and Guillaume, 1986). Higher haematocrit readings were recorded for rainbow trout from water contaminated with chromium salts (Schiffman and Fromm, 1959).

According to Cyriac et al. (1989), haematocrit values increase when fish are exposed to metals for longer than 24 hours. A high haematocrit value can result from acute stress (Barton et al., 1985; Soivio and Oikari, 1976; Casillas and Smith, 1977), while a low haematocrit value may indicate a diseased state (Cardwell and Smith, 1971).

A high haematocrit reading could imply polycythaemia induced by stress and a high haemoglobin concentration due to gill damage and impaired osmoregulation. The change in haematocrit values illustrates the significant decrease in erythrocytes under stress conditions (Van Vuren, 1986).

It is important to note that the haematocrit value can increase rapidly when kept under anaerobic conditions in the haematocrit tubes. The increase may be as a result of cellular swelling, caused by an increase in the carbon dioxide tension of the blood due to respiration (Soivio et al., 1974). Haematocrit readings should therefore be done immediately after blood has been drawn to prevent above mentioned influences on results.

3.2.2.2 Leucocrit (L)

The leucocytes include lymphocytes, monocytes, eosinophils, basophils and neutrophils. Leucocytes play an important role in the immune responses and defence systems of fish (Anderson, 1974; Jurd, 1985). An increase in the number of circulation leucocytes (leucocytosis) in fish is a normal reaction.
against attacks of foreign substances which can alter their normal physiological processes. The release of neutrophils is a non-specific response to a variety of stress stimuli in fish (Ellis et al., 1978).

The leucocrit is a gross measurement of white blood cell abundance and provides an indication of the fish health (Mcleay and Gordon, 1977, Wedemayer et al., 1983). According to Wedemayer et al. (1990), an abnormally high leucocrit (e.g. >5%) may indicate a sub-clinical infection. An extremely low leucocrit indicates either that circulating lymphocytes have been suppressed by elevated corticosteroid which is a characteristic acute stress response (Donaldson, 1981; Schreck, 1981) or that an active bacterial infection has induced leucocytolysis (Wedemeyer et al., 1983).

3.2.2.3 Total plasma protein concentration ([TP])

The protein content is important as the formation of antibodies and therefore immunity are dependant on it. The total plasma protein levels may vary with respect to the sex, size and state of maturity of the fish and it may be influence by environmental factors such as temperature or food availability. The low concentration of plasma protein influences the colloid osmotic pressure and is indicative of a haemodilution caused by kidney and liver damage, infectious diseases, starvation, depletion of energy stores, and impaired water balance (Cunjak, 1988; Lockhart and Metner, 1984; Wedemeyer and Yasukake, 1977).

3.3 Organo-somatic indices as biomarkers

Organo-somatic indices have been used extensively in fish health and population assessments as a first level screen to determine possible contaminant exposures (Ghosh et al., 2006; Goede and Barton, 1990; Larsson et al., 1984; Schmitt et al., 2000; Sloof et al., 1983). Organo-somatic indices generally express organ weight as a percentage of total body
weight. These indices reflect the status of organ systems, which may change in size due to environmental factors and stressors more rapidly than the organism weights and lengths change (Goede and Barton, 1990).

Organo-somatic indices are useful indicators of general organ and fish health, however these indices should be interpreted with caution. These parameters are not sensitive or specific, and may be affected by non-pollutant factors (Schmitt et al., 2000; Van der Oost et al., 2003). Organo-somatic indices serve as an initial screening biomarker to indicate exposure and effects (Mayer et al., 1992). The following organo-somatic indices were used in this study: hepato-somatic index (HSI), spleno-somatic index (SSI), gonadal-somatic index (GSI) and the fat-tissue index (FTI).

### 3.3.1 Hepato-somatic index (HSI)

Because of the energy storage and metabolic functions of the liver, alterations in liver size due to environmental stressors are of interest. The hepato-somatic index (HSI) is the weight of the liver expressed as a percentage of body weight (Slooff et al., 1983).

According to Gingerich (1982) the normal HSI values range between 1% to 2%. Values lower than 1% indicates possible atrophy of hepatocytes. Values higher than 2% indicate possible hypertrophy of hepatocytes. Alteration in liver size may reflect changes in the metabolism and energy reserves of an individual fish (Busacker et al., 1990).

Evaluation of the HSI must consider the role of both endogenous and exogenous factors (Schmitt et al., 2000):
- The HSI varies with seasonal cycles (Beamish et al., 1996; Delahunty and de Vlaming, 1980; Saborowski and Buchholz, 1996; Slooff et al., 1983).
Nutritional quality and regimes affect relative liver size (Daniels and Robinson, 1986; Foster et al., 1993; Heidinger and Crawford, 1977; Swallow and Fleming, 1969).

HSI shows variation with sex and changes in gonadal status (Fabacher and Baumann, 1985; Förlin and Haux, 1990; Grady et al., 1992). The HSI for females may change as the gonado-somatic index (GSI) changes due to the liver’s role in vitellogenesis (Scott and Pankhurst, 1992).

HSI is constrained by the allometry of the population, the species, or both (Grady et al., 1992).

Fish liver may also store blood during periods of quiescence (Gingerich, 1982), which suggests that the activity of the fish immediately prior to capture, and the protocol used to procure the liver, may affect relative liver size.

Of the organo-somatic indices, the HSI is the one most often associated with contaminant exposure (Adams and McLean, 1985). Table 3.2 summarizes results of previous laboratory studies showing both an increase and a decrease in the HSI upon contaminant exposure (Schmitt et al., 2000).
Table 3.2: Summary of studies done in which the hepato-somatic index (HSI) was applied as a biomarker of contaminant exposure (adapted from Schmitt et al., 2000).

<table>
<thead>
<tr>
<th>Species</th>
<th>Contaminants / stressors</th>
<th>HSI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncorhynchus mykiss</td>
<td>Acidity</td>
<td>Decrease</td>
<td>Lee et al., 1983.</td>
</tr>
<tr>
<td>Oncorhynchus mykiss</td>
<td>Sodium pentachlorophenate</td>
<td>Decrease</td>
<td>Hickey and Dickson, 1987.</td>
</tr>
<tr>
<td>Salmo salar</td>
<td>Cyanide</td>
<td>Decrease</td>
<td>Ruby et al., 1987.</td>
</tr>
<tr>
<td>Perca fluviatilis</td>
<td>Mixture of metals</td>
<td>Decrease</td>
<td>Larson et al., 1984.</td>
</tr>
<tr>
<td>Pleuronectes americanus</td>
<td>Crude oil</td>
<td>Increase</td>
<td>Fletcher et al., 1982.</td>
</tr>
<tr>
<td>Mystus nemurus</td>
<td>Hydrogen sulphide</td>
<td>Decrease</td>
<td>Hoque et al., 1998.</td>
</tr>
<tr>
<td>Mugil cephalus</td>
<td>Crude oil</td>
<td>Decrease</td>
<td>Chambers, 1979.</td>
</tr>
<tr>
<td>Oreochromis mossambicus</td>
<td>Low concentrations of cadmium and zinc</td>
<td>No change</td>
<td>Van Dyk et al., 2007.</td>
</tr>
</tbody>
</table>

3.3.2 Spleno-somatic index (SSI)

Because the spleen is a haematopoietic organ, spleen size is considered a useful diagnostic factor. Dysfunction of the spleen could have effects at the whole-organism level (Anderson, 1990). Histological data show cellular changes occurring in the spleen with exposure to contaminants, supporting the use of the spleno-somatic indices (SSI) as a relevant indicator of spleen dysfunction (Schmitt et al., 2000).

Certain endogenous and exogenous factors are known to affect the SSI (Schmitt et al., 2000):

- Spleen size varies among fishes, and among populations of the same species (Anderson et al., 1982; Ruklov, 1979).
Relative spleen weight may differ with gender, age, size, gonadal development, growth rate and seasonal changes (Krykhtin, 1976; Ruklov, 1979; White and Fletcher, 1985).

Contaminants can affect spleen directly (size and function), or they can suppress immune system functions to cause increasing disease prevalence and thus causing enlargement of the spleen (Goede and Barton, 1990). Decrease in the SSI might also be due to a decrease in haematopoietic function of the spleen or necrosis (Schmitt et al., 2000). Table 3.3 provides a summary of results obtained for the SSI in previous studies. Little work has been done on SSI of endemic fish and normal values have not been determined.

Table 3.3: Summary of studies done in which the spleno-somatic index (SSI) was applied as a biomarker of contaminant exposure (adapted from Schmitt et al., 2000).

<table>
<thead>
<tr>
<th>Species</th>
<th>Contaminants / stressors</th>
<th>SSI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicentrarchus labrax</td>
<td>Temperature increase</td>
<td>Decrease</td>
<td>Hadj-Kacem et al., 1987.</td>
</tr>
<tr>
<td>Tautogolabrus adspersus</td>
<td>Petroleum</td>
<td>Decrease</td>
<td>Payne et al., 1978.</td>
</tr>
<tr>
<td>Gadus morrhus</td>
<td>PCBs, PAHs, metals</td>
<td>Decrease</td>
<td>Pulsford et al., 1995.</td>
</tr>
</tbody>
</table>

### 3.3.3 Gonadal-somatic index (GSI)

Gonadal-somatic index (GSI) is routinely used to determine reproductive maturity, as well as to assess gonadal changes in response to environmental dynamics (e.g. seasonal changes) or exogenous stresses (e.g. contaminant exposure).
Natural variables affecting GSI:

- Age (Patnaik et al., 1994).
- Season and reproductive cycling (Schmitt et al., 2000).
- Gender; males experiencing less gondal weight gain during recrudescence than females (Schmitt et al., 2000).

Previous studies proved that exposure to various environmental pollutants can result in gonadal alterations such as a decreased GSI, morphological changes, or both (Schmitt et al., 2000). Fish used during this study are all from the same breeding stock and are thus of similar age and maturity, which allowed for comparison between the exposure groups and the control group.

**Table 3.4:** Studies in which the gonadal-somatic index (GSI) was applied as a biomarker of metal exposure.

<table>
<thead>
<tr>
<th>Species</th>
<th>Contaminants / stressors</th>
<th>GSI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stizostedion vitreum</td>
<td>Hg</td>
<td>Decrease</td>
<td>Friedman et al., 1996.</td>
</tr>
</tbody>
</table>

**3.3.4 Fat-tissue index (FTI)**

The fat-tissue index (FTI) is the total mesesentric fat (g) expressed as a percentage of total body weight (Abascal, 2004). FTI is dependant on several natural variables, such as fish size, sex time of year, stress level and food availability (Adams, 1993).
Linear regression analysis done on pooled data from previous studies on the migrant and spawning bluefin tuna revealed a negative correlation between the gonadal-somatic index (GSI) and the fat-tissue index (FTI), and a positive correlation between the gonadal-somatic index (GSI) and the hepato-somatic index (HSI) (Abascal, 2004).

### 3.4 Histology as biomarker

Cells respond to metabolic demands of the body, energy supply, and various physiologic and pathological stimuli (e.g. as result of exposure to xenobiotics) by adaptation. Adaptation is a reversible adjustment to environmental conditions that includes changes in cell function, morphology, or both. These changes can be identified with a light microscope (Figure 3.2, Damjanov, 1996). Figure 3.2 provides a diagram of cellular adaptations that can result due to the exposure to physiological stimuli and shows were light microscopy can be applied as a biomarker.

Histopathological alterations in fish tissues are biomarkers of effect of exposure to environmental stressors, and can be defined as any contaminant-induced physiological or biochemical change in an organism that leads to the formation of a lesion in cells, tissues or organs (Hinton et al., 1990).
Figure 3.2: Cell adaptation upon stimulus and the use of light microscopy as a biomarker (adapted from Damjanov, 1996).

There are advantages to using histopathology as biomarker:

- Histopathological responses can indicate potential problems before the effects appear at higher organizational level, so alterations at the cell and tissue levels provide early warnings of pollution effect (Hinton and Laurén, 1990).
- No geographical or ecosystem limitations (Van der Oost et al., 2003).
- Histology sections retain in situ relationships of different cell types and tissues in organs (Hinton and Laurén, 1990).
Acute changes are seen when contact levels are sufficiently high, while chronic duration is required to determine sub lethal aspects of change (Hinton and Laurén, 1990; Van der Oost et al., 2003).

Many alterations persist even after exposure to a toxicant has ceased so that host response to prior toxicity can also be used to determine effects (Hinton and Laurén, 1990; Van der Oost et al., 2003).

Disadvantages to using histopathology as a biomarker:

- The ability to detect alterations depends on the investigators' expertise in proper fixation, processing, and staining of preparations and experience in different alterations in different tissues (Hinton and Laurén, 1990).

These disadvantages were addressed in this study by standardizing procedures for fixation, processing, and staining. Specific criteria for classification of lesions were developed and applied uniformly, thus minimizing variation amongst investigators.

- The subjective nature of morphological studies makes correlations with other quantitative studies difficult. Previous studies only report alterations in quantitative terms (e.g. absent or present), or semi-quantitative terms (e.g. mild, moderate or severe)(Newman and Jagoe, 1996).

Incorporation of quantitative methods is essential to the continued development of histopathological indices of pollution exposure, and to the interpretation of histological responses (Newman and Jagoe, 1996). Bernet et al. (1999) proposed a histopathological assessment protocol which leads to standardized quantification thus allowing the comparison between different studies. The histopathological assessment protocol was successfully applied in various studies (Bernet et al., 1999; Bernet et al., 2004; Schmidt et al., 1999; Schmidt-Posthaus et al., 2001). The methodology of the
histopathological assessment protocol as applied during this study is described in Chapter 5.

### 3.4.1 Toxicant-induced pathological changes

The following section focuses on the original classification of pathological changes as described by Hibiya (1982). This original classification was slightly adapted by Bernet et al. (1999) for use in the histopathological assessment protocol as discussed later in Chapter 5.

#### 3.4.1.1 Circulatory disturbances (CD)

Circulatory disturbances are a pathological condition of blood and tissue fluid flow. Disturbances considered are:

- Haemorrhage, blood leaking from blood vessels.
- Hyperaemia, congestion of an organ caused by venous as well as arterial processes.
- Aneurism, well-outlined dilations of arterial blood vessels.
- Intercellular oedema (dropsy), stagnant tissue fluid which has leaked from capillaries into tissue, body cavity, and eye orbit. Oedema is caused by congestion, kidney lesions, and failure of osmoregulation, excitation of vascular motor neurons, toxic substances, bacterial infection and severe inflammation. All the above-mentioned causes induce permeability changes in capillaries.

#### 3.4.1.2 Regressive changes (RC)

Regressive changes are classified as changes which result in loss of an organ or reduction in organ function. These involve atrophy, degeneration (malformation or dysfunction of cellular structures as result of cell damage) and necrosis:
Atrophy is the state in which the number and volume of the cells and the amount of intercellular substances, tissues and organs are reduced. Atrophy is caused by deficiency in the local supply of nutrients and can also be an effect of toxic substances.

Degeneration is the state in which the physiological substances present in tissues increase or appear in other places. Various types of degeneration can be classified:

- Granular degeneration is the state in which proteinous granules form in the cells, cloudy swelling and hyaline droplets are distinguishable. Cloudy swelling is the result of distended cells with fine eosinophillic granules in the cytoplasm. Marked changes of the nucleus are not usually observed. Fine granules are formed by the breakdown of swollen mitochondria, and eosinophillic hyaline-like droplets appear in the cytoplasm. Relatively small hyaline droplets which appear in the epithelia cells of the renal proximal tubes are considered to be a physiological condition indicating the re-absorption of proteins.

- Hydropic degeneration is the state in which cells are swollen because of the accumulation of large amounts of colloidal proteins in the cytoplasm. Cytoplasm stains weakly and indicates a reticular or vacuolar shape. This degeneration is also called vacuolar degeneration when the vacuolar structures are marked. Vacuolar degeneration may also appear in the nucleus.

- Colloid degeneration is caused by the accumulation of dense colloidal proteins in the cytoplasm.

- Hyaline degradation occurs in connective tissue. Fine fibers gradually thicken and finally become homogenous eosinophillic substances. Fibrocytes usually disappears. The parenchyma cells atrophy when connective tissue undergoes this alteration. Hyalinization indicates cellular change and is characterized by glassy homogenous eosinophillic material seen as droplets within cell cytoplasm.
- Amyloid degeneration (amyloidosis) is caused by hyaline-like substances appearing in the cytoplasm. This degeneration is also called amyloidosis and the hyaline-like droplets are complexes of protein and chondroitin sulphate.

- Keratin degeneration is caused by eosinophilic keratin in cytoplasm. The nucleus becomes pyknotic and then disappears.

- Fatty degeneration occurs in organs following ischemia, chemical or physical injury, or metabolic disturbances caused by infections and other systemic diseases. Fat is stored in the cytoplasm in the form of triglyceride-rich droplets. Small droplets eventually coalesce into large vacuoles, which can fill the entire cytoplasm and displace the nucleus. Fatty degeneration is accompanied by pyknosis and necrosis (Damjanov, 1996).

- Glycogen degeneration is caused by a large accumulation of glycogen in cytoplasm.

- Calcareous degeneration results when calcium salts precipitate within the cells and intercellular spaces. It is sometimes accompanied by pyknosis and necrosis.

- Necrosis is the state in which the cells and tissues lower activity and eventually die. The nucleus undergoes pyknosis, karyorrhexis and karyolysis. The cytoplasm becomes homogenous and stains evenly with eosin. Necrotic cells are eventually absorbed and destroyed, but can lead to calcification.

It is not always possible to determine exactly when a particular cell becomes necrotic. Most of the changes by which necrosis is recognised, occur after cell death and are due to secondary release of lytic enzymes normally sequestrated within the cell. In the liver necrosis is usually recognised by secondary changes seen on histological examination. In preparations stained with haematoxylin and eosin (H & E) the nuclei may gradually lose their characteristic
staining with haematoxylin so that the whole cell stains uniformly with eosin (Pieterse, 2004).

- Nuclear alterations used to identify necrotic cells (Damjanov, 1996):
  - Pyknosis is the necrotic change in cell nuclei and is characterized by compaction of chromatin that causes hyperchromatic staining.
  - Karyorrhexis is marked by the fragmentation of the nucleus.
  - Karyolysis is the necrotic change characterized by dissolution of chromatin in the nucleus, and incomplete stages can be identified where only nuclear outlines are visible. Destruction of the nucleus is marked by swelling and dissolution of the nucleus.

### 3.4.1.3 Progressive changes (PC)

Progressive changes indicate hyperplasia or hypertrophy of cells and tissues, which leads to an increase in organ function (Damjanov, 1996).

- Hyperplasia is an increase in cell number and can only occur in organs composed of mitotic cells that divide and multiply in response to stimuli.
- Hypertrophy is characterized by an increase in cell size.

### 3.4.1.4 Inflammation (I)

Inflammation is the protective reaction of living animals when physical and chemical stimuli and parasites strongly affect local tissues. In severe cases necrosis, cloudy swelling and atrophy of cells are observed. Blood circulation is affected and fluid exudates from the body. Macrophage and monocytes infiltrate the infected site and hyperplasia of fibroblasts.
3.4.1.5 Tumours (T)

Tumours develop when cells and tissue undergo uncontrolled autonomous proliferation. According to the original tissue and anticipated biological behaviour of the tumour cells, tumours can be divided into two groups:

- Benign tumour cells resemble the tissue from which it was formed.
- Malignant tumour cells show no resemblance to the original cells.

These pathological cellular changes in reaction to metal exposures are described in more detail in Chapter 6 and 7, which investigate and discuss the histopathological changes in the gills, gonads and the liver of *O. mossambicus* after exposure to sub-lethal concentrations of cadmium, chromium and nickel.

3.4.2 Gonad maturity index

Many biomarkers vary seasonally with the reproductive cycle. To correctly interpret these biomarkers, one must know the reproductive status of each fish, which can be achieved by determining its gonadal stage or condition histologically.

To evaluate the reproductive maturity of the fish several histologically based classification schemes have been described. The classification system adopted for this study; as implemented by Schmit *et al.* (2000); is adapted from Goodbred *et al.* (1997), Nagahama (1983), Rodriguez *et al.* (1995), and Treasurer and Holiday (1981). A summary of the classification system and the different developmental stages are given in Table 5.5.

Histological structures of the organs of fishes are fundamentally the same as those of higher vertebrates. However, since fish are aquatic, morphologically and physiologically they possess specific characteristics lacking in land animals. Tissues of fishes are sometimes slightly different from those of
higher vertebrates (Takashima and Hibiya, 1995). Chapter 4 provide a summary of the general organization and structure of the fish liver, gill, ovary and testis. Chapter 4 also provides a summary of the functions of these organs as well as their application as biomarkers for toxicant exposure.

### 3.5 References


4.1 Target organ selection

When selecting a target organ for histological analysis it is important to consider organs which are sensitive to environmental pollution. By selecting the appropriate target organ for histological analysis, the investigator may use a variety of morphological manifestations of chronic toxicity as biomarkers indicative of prior exposure.

When conducting a proper histological investigation on specific organs of an exposed specimen, it is important to first examine and investigate the morphology and histology of the same organ of healthy specimens, assumed to reflect the normal histological structure of that organ. This will allow the investigator to easily identify deviations or abnormal occurrences in the histology (Van Dyk, 2003). The following sections consist of a literature review on the normal morphology and histology of the liver, gills and gonads, as well as the specialized functions which render these organs ideal target organs for this study.
4.2 Liver

4.2.1 Macroscopic anatomy of the fish liver

Fish liver is a dense organ located ventrally in the cranial region of the body cavity. The size, shape, and volume of the liver are adapted to the space available between other visceral organs (Bruslé and González i Anadon, 1996; Vincentini et al., 2005).

Figure 4.1: Macroscopic anatomy of the liver of *O. mossambicus*. A) Liver is located ventrally. B) G - gallbladder; LL - left lobe; RL - right lobe.
The liver of *Oreochromis mossambicus*, as shown in Figure 4.1, is a large bila
ted organ. The left lobe is bigger and spreads throughout almost the entire corporeal cavity. The visceral face of the liver has the impression of the intestine. The gallbladder is well developed and has a round shape.

Fish liver is generally reddish-brown because of its rich supply of blood. The liver is nourished by a dual blood supply via two afferent blood vessels (hepatic artery and portal vein) and a single efferent vessel (hepatic vein) located at the helium (Munshi and Dutta, 1996).

### 4.2.2 Normal histology of the fish liver

According to Hinton and Laurén (1990), the liver comprises two tissue compartments:

- parenchyma (the epithelial cells that perform the organ’s major functions), and
- Non-parenchyma or stroma (blood vessels and connective tissue).

The parenchyma includes the various cells situated within the liver as well as the respective extra-cellular spaces. Figure 4.2 provides a structural stratification of the teleost liver.
Figure 4.2: Structural stratification of the teleost liver. The organ comprises of two compartments: parenchyma (epithelial cells which performs the organs major functions) and non-parenchyma (stroma comprising of blood vessels and connective tissue) (Adapted from Hinton and Laurén, 1990).

4.2.2.1 Parenchyma

The liver of vertebrates is a digestive gland of endodermic origin. It’s made up of cellular plates, each of which separates several lacunae: the vascular (sinusoids) and biliary (canaliculi) network. Figure 4.3 shows the classical vertebrate liver. Fish liver belongs to the lower vertebrate category (Ellias and Benglesdorf, 1952) and consists of highly anatomised tubules that originate from the blind tubules organization of the Cyclostomata (Figure 4.4).
Figure 4.3: Classical vertebrate liver. Arrangement of hepatocytes and sinusoids in the classical liver lobule, showing the directions of blood flow and bile drainage (Ross et al., 1989).

A) Cells

a) Hepatocytes

Hepatocytes constitute about 80% of the cell population of the liver. Most of the functions of the liver are performed by the hepatocytes. Light-microscope observations show that it is not possible to distinguish hepatic lobules in fish livers (Munshi and Dutta, 1996).

Between the sinusoids, hepatocytes are arranged as plates, usually two cells thick. Each plate shows polarized hepatocytes with a sinusoidal face for absorption and a biliary face for excretion (Munshi and Dutta, 1996).
Branching of cords can result in four or more cell layers per plate (Figure 4.4). This cord-like structure is not always clearly visible (Geyer, 1989).

**Figure 4.4:** Structural organization of the hepatic parenchyma of fish (Biagianti-Risbourg, 1990) (adapted from Munshi and Dutta, 1996).

The hepatic parenchyma of fish is very homogeneous with the polygonal shaped hepatocytes appearing hexagonal (Geyer, 1989), which are often weakly basophilic (poor in organelles). The nucleus is spherical with a single, central nucleolus. Mitochondria, Golgi apparatus, endoplasmic reticulum and other basic organelles are present in the cytoplasm. Regional or zonal enzymatic activity is not present in the teleost liver (Hampton *et al.*, 1985; Robertson and Bradley, 1991; Schär *et al.*, 1985). The cell membrane of the individual hepatocytes is clearly visible through light microscopy analysis (Munshi and Dutta, 1996).

b) **Biliary epithelial**

In many teleost species the biliary tree originates as an intercellular canaliculus, formed by the close apposition of two hepatocytes. The bile
ducts consist of a simple cuboidal epithelium with a PAS-positive brush border. Rodlet cells are sometimes scattered among the epithelial cells. The biliary epithelium rests on a basal lamina and a wall consisting of both collagen and muscular fibres (Bruslé and Gonzàlez I Anadon, 1996). Fibroblasts are present in the wall, and hematopoietic tissue can sometimes be found at this location depending on the species (Ferguson, 1989).

c) **Endothelial**

Cells that line the sinusoids include endothelial cells (Figure 4.3). These endothelial cells have thin, indistinct cytoplasm and small, elongated, darkly stained nuclei without nucleoli.

d) **Ito cells**

Ito cells are cells that contain a large amount of lipid droplets that are the storage site of vitamin A (Fujita et al., 1986; Robertson and Bradley, 1992; Sakano and Fujita, 1982; Wake et al., 1987). Ito cells are variable in shape, mostly elongated, and rich in free ribosomes (Ross et al., 1989).

e) **Macrophage**

According to Munshi and Dutta (1996), Kupffer cells, which are typical of the mammalian liver, have not been described in many Teleostei. The presence of Kupffer cells have been described in *O.mossambicus* by Van Dyk (2003). Kupffer cells line the sinusoids and contain a large amount of cytoplasm with organelles typical of those found in phagocytic cells. Often the cytoplasm contains fragments of red blood cells and iron in the form of ferritin, indicative of the cells role in red blood cell breakdown (Ross et al., 1989). Kupffer cells have a bean-shaped nucleus and plump cytoplasm with star-shaped extensions (Wisse and Knook, 1977). A special category of
macrophage, the melano-macrophage (MM), is very common in fish (Munshi and Dutta, 1996), and will be discussed in more detail in section 4.2.2.2 a.

B) Extra cellular spaces

a) Sinusoids

Between the cords of hepatocytes is a network of cylindrical blood sinusoids (Elias and Bengelsdorf, 1952). The sinusoid is an irregularly dilated vessel whose diameter is larger than the diameter of regular capillaries (Ross et al., 1989).

The sinusoidal lumen is lined by fenestrated and continuous squamous endothelium where no basal lamina occurs (Figure 4.3). These cells consist of flattened cells with an ovoid, flat nucleus and a cytoplasm very poor in organelles but rich in microfilaments. Endothelial cells display fenestrations (Bruslé and González I Anadon, 1996). The presence of fenestrae in the endothelium and the lack of basal lamina allow metabolites an easy crossing through the blood barrier to the hepatocytes (Ferri and Sesso, 1981).

b) Space of Disse

The space of Disse is the peri-sinusoidal space between the sinusoidal endothelium and the hepatocytes (Figure 4.3). It is large and well developed in fish, in contrast to higher vertebrates. The hepatocyte microvilli project into this space and greatly amplify the blood-hepatocyte exchange surface area (Munshi and Dutta, 1996). The space of Disse is a zone of rapid intercellular exchange and contains plasma, small amount of connective tissue that constitutes the normal framework of the liver, and perisinusoidal cells such as Ito cells. The space of Disse is not always distinguishable in well-fixed, normal liver biopsy material. However, in post-mortem liver, the
hepatocytes shrink, and the space becomes more conspicuous (Sternberg, 1997).

4.2.2.2. Non-parenchyma (stroma)

a) Macrophage aggregates (MA)

Pigment-bearing macrophages are a prominent feature in fish spleen, kidney and sometimes liver (Agius, 1980). In advanced teleosts they form discrete aggregations called macrophage aggregates (MA) or melano-macrophage centres (MMC). Macrophage aggregates are believed to be functional equivalents of the germinal centres, active in the centralization of foreign material and cellular debris for destruction, detoxification or reuse, the storage of exogenous and endogenous waste products, the immune response, and iron storage and recycling (Ellis et al., 1976; Ferguson, 1976).

MMC’s are highly variable in size, number, and content, depending on the species, age, and health status (Agius, 1980). They are usually located in the vicinity of the hepatic arteries, portal veins, or bile ducts. They concentrate heterogeneous materials such as lipofuscin (natural yellowish colour), melanin (natural brown or black colour), ceroid or hemosiderin (Munshi and Dutta, 1996).

b) Large bile ducts (BD)

Bile ducts accompany the hepatic artery and portal vein while coursing through the liver (Sternberg, 1997). The ramification of these three structures is usually referred to as the portal triad in mammals. However, the triads are indistinct, if not absent, in almost all teleostei. The term ‘portal region’ is more correct when referring to fish liver (Munshi and Dutta, 1996). Bile ducts are nourished by the hepatic arteries via a complex peri-biliary plexus of capillaries, which supply all structures within the portal tracts. Bile
is formed in hepatocytes, steadily secreted into bile canaliculi and then to the intra- and extra-hepatic bile ducts (Sternberg, 1997).

c) Arteries (A) and veins (V)

The hepatic artery and portal vein enter the liver at the porta hepatica and then branches into smaller vessels (Damjanov, 1996). From the small branches of the portal vein, the venous and arterial blood enters into the sinusoids where exchanges between the blood and the hepatocytes occur. From the sinusoids, the blood drains into a thin-walled terminal or central vein (Ross et al., 1989). The central vein enters into the sub-lobular vein which finally drains into the hepatic vein.

The portal vessels are clearly distinguishable due to the pancreatic acini that surround it (Geyer, 1989). The lobules are build up of cords of hepatocytes which radiate out from a centrally located central vein. Central veins are found randomly throughout the hepatic parenchyma of fish. The hepatic arteries differ from the veins by its narrow lumen, and thick wall with more elastic fibres and its endothelial cells that are generally more voluminous than in veins (Munshi and Dutta, 1996).

d) Hepatopancreas

The pancreatic exocrine tissue develops around the portal vein during ontogenesis. It remains extra-hepatic or penetrates more or less deeply into the liver parenchyma depending on the species. Pancreatic tissue in fish liver which occurs around the major portal vessels are collectively called the hepatopancreas (Ferguson, 1989). Pancreatic tissue can be differentiated from hepatic tissue by its acinar arrangement and its characteristic stain with H & E (basophilic basal pole and cytoplasm rich in eosinophilic zymogen granules). Thin septa of connective tissue separate the hepatocytes from the exocrine pancreatic cells (Munshi and Dutta, 1996). For the purpose of this
study the hepatopancreas was not considered as part of the liver parenchyma, and were therefore not histologically analysed.

### 4.2.3 Function of the fish liver

Some major functions of the liver include protein synthesis and secretion, bile formation and secretion, metabolism of lipid-soluble drugs (including detoxification), lipoprotein synthesis and secretion and urea formation from ammonium ions (Ross et al., 1989). The structural organization of the liver is based on its many functions and its position between the digestive tract and the rest of the body (Sternberg, 1997).

According to Ross et al. (1989), both exocrine and endocrine functions are attributed to the liver by many authors. Although this is true, it should not be overlooked that the liver is also an organ engaging in metabolic conversions. The liver is classified as exocrine because one of its products (bile), is secreted through a system of ducts into the duodenum, and also classified endocrine because most of its products are secreted directly into the bloodstream.

Summary of the major functions of the fish liver include (Heath, 1991; Munshi and Dutta, 1996)

- Inter-conversion of foodstuffs;
- Storage (lipids, carbohydrates, Vitamin A, iron);
- Removal and metabolism of foreign chemicals in the blood;
- Nitrogen catabolism;
- Bile formation;
- Synthesis of plasma proteins and cholesterol;
- Metabolism of hormones;
- Immune defence (imuno-globulin synthesis);
- Blood turn-over.
The liver is important in the metabolism and excretion of xenobiotic substances. Previous studies have proven that the concentration of such substances may at any time be higher in the liver than any other organ or the environment. Hepatic cells are therefore exceptional models for the study of the effect of such xenobiotic substances on the tissues of fish.

The liver receives arterial blood from the hepatic artery as well as blood from veins from the digestive system and spleen via the hepatic portal vein. The liver is situated directly in the way of blood vessels that convey substances absorbed from the digestive system. This position gives the liver the first chance to metabolize these substances and it is also the first organ to be exposed to toxic compounds that have been ingested. The liver has the ability to degrade toxic compounds, but can be overwhelmed by elevated levels of these compounds and can subsequently be damaged (Ross et al., 1989).

4.2.3.1 Detoxification

The liver can be regarded as the body’s detoxification organ and hence a target organ of various xenobiotic substances. The liver accomplishes these detoxification actions by means of three mechanisms as summarised by Cabot (2003):

- Kupffer cells
- Phase I detoxification pathway,
- Phase II detoxification pathway.

Kupffer cells

Unwanted particulate matter metabolic waste products are filtered and ingested by specialized cells lining the sinusoids called "Kupffer cells". These cells break down and destroy potentially harmful substances, rendering them harmless. Refer to section 4.2.2.1 for discussion on the presence of Kupffer cells in telostei.
Phase I pathway
This pathway is also known as the cytochrome P-450 enzyme system. The liver cell detoxifies a toxic substance by utilising the processes of oxidation, reduction and hydrolysis. These processes lead to the generation of oxygen free radicals. A need for antioxidants arises to prevent cellular damage. For efficient phase I detoxification to occur, the liver requires adequate amounts of the nutrient selenium, folic acid, vitamins B2, B3, B6, phosphatidyl choline and bioflavonoids.

Phase II pathway
This pathway are primarily concerned with the conjugation of endogenous compounds with metabolites produced by phase I reactions. During the phase II pathways, the liver cells add either a glycine or sulphate molecule to a chemical rendering it water-soluble. These water-soluble molecules are then deposited into the bile. Bile acts as a carrier whereby toxic substances are dumped into the intestines and later excreted. Through conjugation, the liver is thus able to turn xenobiotics and hormones, and neurotransmitters into excretable substances.

If filtering and/or detoxification systems become inefficient, it will cause a build-up of toxicants in the blood stream, increasing the load placed on other organs and systems which will ultimately result in death.

4.2.4 Liver as biomarker of contaminant exposure

Constant exposure to xenobiotic substances, for example heavy metals, may overwhelm the liver’s detoxification capability and cause some degree of structural damage within the liver parenchyma. The liver may thus be expected to be the primary target of toxic substances, providing an excellent biomarker of aquatic pollution (Braunbeck and Völkl, 1993).
According to Hinton and Laurén (1990), there are several important reasons for selecting the liver for the detection of biomarkers:

- the liver is the major site for the enzymatic detoxification of xenobiotics;
- nutrients derived from gut absorption are stored in the hepatocytes and released for further catabolism by other tissues;
- bile synthesised within hepatic cells aids in the carrying of conjugated metabolites of pollutants to the intestine for excretion; and
- vitellogenin, a major yolk protein, is exclusively synthesised within the liver.

**Table 4.1:** Summary of the functions of fish liver favouring its use as a biomarker (adapted from Hinton and Laurén, 1990; Van Dyk, 2003).

<table>
<thead>
<tr>
<th>Function</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrition</td>
<td>Uptake, storage and release of fats and carbohydrates.</td>
</tr>
<tr>
<td>Metabolic activation</td>
<td>Conversion of pro-carcinogen to carcinogen.</td>
</tr>
<tr>
<td>Detoxification</td>
<td>Glucuronide formation.</td>
</tr>
<tr>
<td>Endocrine / reproduction</td>
<td>Synthesis of vitellogenin.</td>
</tr>
<tr>
<td>Digestive</td>
<td>Zymogen from exocrine pancreas / hepatopancreas.</td>
</tr>
</tbody>
</table>

Biomarker responses in liver tissue may involve all levels of biological organization. Changes may be seen in the distribution of molecules on the cell surface, the organelle number, volume, shape or distribution, cell volume, morphology, distribution or number; and organ volume or relative weight (Hinton and Laurén, 1990).

Enzymes and co-factors related to both Phase I and Phase II pathways can be used as biological markers of exposure to xenobiotic substances. Phase I reactions are catalysed by mixed-function oxidase (i.e. cytochrome P450 [cyt
P450, cytochrome \( b_5 \) [cyt \( b_5 \)], and NADPH cytochrome P450 reductase [P450 RED]) (Van der Oost et al., 2003).

Cytochromes P450, comprising a large family of heme proteins, are membrane-bound proteins which are located predominantly in the endoplasmic reticulum of the liver. Hepatic cytochrome P450 isozymes (CYP1A) protein levels seem to be a very sensitive biomarker of exposure. Elevation of the CYP1A protein levels due to exposure to environmental pollutants is preceded by an increase in CYP1A mRNA levels (Stegeman and Hahn, 1994). Measurement of CYP1A mRNA by Northern blots is becoming an integral part of investigations on CYP1A regulation, while several field trials have shown its suitability as a biomarker (Bucheli and Fent, 1995).

Phase II enzymes and co-factor levels may also be affected by exposure to environmental pollutants. Examples of phase II enzymes and co-factors used as biomarkers of pollution include reduced and oxidised glutathione (GSH and GSSG), glutathione-S-transferase (GST), and UDP-glucuronyl transferase (UDPGTs) (Van der Oost et al., 2003).

The synthesis of glucuronides by microsomal UDP-glucuronyl transferases (UDPGTs) is a major pathway for the inactivation and subsequent excretion of both endogenous and xenobiotic organic compounds. These enzymes exhibit a broad specificity for structurally diverse compounds and multiple iso-enzymes belonging to a number of multi-gene families are found (George, 1994). The liver is quantitatively the most important site for glucuronidation of xenobiotics in fish. UDPGT activity in fish is reported to be influenced by sex, season, pH and temperature differences. Although not as sensitive as phase I enzymes, the UDPGT activity appears to be the phase II parameter which is most responsive to pollutant exposure (Stegeman et al., 1992).
Stress proteins found in liver, such as heat shock proteins and metallothioneins, are also used as biomarkers. The stress proteins comprise a set of abundant, inducible proteins which are actively involved in the protection and repair of the cell in response to stress and harmful conditions (Di Giulio et al., 1995; Stegeman et al., 1992). The syntheses of heat shock proteins have been shown to increase as a result of exposure to chemical stresses, such as metals, and heat (Stegeman et al., 1992).

Metallothioneins are inducible proteins functioning in the regulation of essential metals (such as copper and zinc), and the detoxification of these and other, non-essential metals (Roesijadi and Robinson, 1994). The cellular interaction involving metallothioneins are expected to follow two general lines: interception and binding of metal ions that are initially taken up by the cell; removal of metals form non-thionein ligands that include cellular targets of toxicity. The latter may represent a detoxification function for structures, which have been reversibly impaired by inappropriate metal binding (Van der Oost et al., 2003). Metallothionein induction by exposure to a variety of metals (e.g. Cd, Cu and Ni) is associated with their protective function (Stegeman et al., 1992). Metallothioneins play a key role in the accumulation of metals in the liver of fish (Heath, 1991). Studies on several fish species have demonstrated a dose-responsive increase in metallothionein induction (George and Young, 1986; George, 1989; Hogstrand and Haux, 1991; George et al., 1992) as well as a time-responsive increase (Beyer et al., 1997) after exposure to metals.

The hepato-somatic index (HSI), as discussed previously in section 3.3.1, is readily used as a biomarker of contaminant exposure.

4.2.4.1 Fish liver histology as a biomarker of contaminant exposure

With regard to fish liver histology, various changes can be expected upon contaminant exposure. Table 4.3 provides selected examples of liver toxic
responses. Further studies need to be done on the liver histological lesions as a result of cadmium, chromium and nickel exposures.

Macrophage aggregates have long been recognized as potentially useful biomarkers of fish health (Wolke et al., 1985a) but intrinsic and extrinsic factors may confound investigations into the role of contaminants. Occurrence of macrophage aggregates may vary depending on the size, nutritional status, or health of a particular fish species (Agius, 1979; Agius, 1980; Agius and Roberts, 1981; Wolke et al., 1985b). However, numerous studies had documented an increase in their number, size or hemosiderin content in fish collected at contaminated sites when compared to those collected at reference sites (Bucke et al., 1984; Poels et al., 1980; Khan and Kiceniuk, 1984; Kranz and Peters, 1984; Spazier et al., 1992; Wolke et al., 1985b). Macrophage aggregates have been suggested as potentially sensitive histological biomarkers and/or immunological biomarker of contaminant exposure (Schmitt et al., 2000).
**Table 4.2:** Hepatic responses to contaminant exposure (adapted from Hinton and Laurèn, 1990; Takashima and Hibiya, 1995; Damjanov, 1996).

<table>
<thead>
<tr>
<th>Level of organization</th>
<th>Histological identifiable responses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organ</strong></td>
<td></td>
</tr>
<tr>
<td>Hepatitis</td>
<td>(lymphocyte and leucocyte infiltration around blood vessels).</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>(proliferation of connective tissue).</td>
</tr>
<tr>
<td>Tumour</td>
<td></td>
</tr>
<tr>
<td><strong>Tissue</strong></td>
<td></td>
</tr>
<tr>
<td>Parenchyma</td>
<td>Dilatation and/or congestion of sinusoids.</td>
</tr>
<tr>
<td></td>
<td>Structural alteration (chord disarray)</td>
</tr>
<tr>
<td></td>
<td>Proliferation of biliary ductules.</td>
</tr>
<tr>
<td>Stroma</td>
<td>Increase in macrophage aggregates.</td>
</tr>
<tr>
<td></td>
<td>Congestion of arteries and veins.</td>
</tr>
<tr>
<td><strong>Cell</strong></td>
<td></td>
</tr>
<tr>
<td>Hepatoctes</td>
<td>Necrosis.</td>
</tr>
<tr>
<td>Biliary epithelia</td>
<td>Atrophy / hypertrophy.</td>
</tr>
<tr>
<td></td>
<td>Plasma alterations. (Including: hydropic change, fatty degeneration, vacuolar degradation.)</td>
</tr>
<tr>
<td></td>
<td>Enhanced phagocytosis.</td>
</tr>
<tr>
<td>Macrophage</td>
<td>(Increase in phagocyte number.)</td>
</tr>
<tr>
<td></td>
<td>Increase in macrophage aggregates.</td>
</tr>
<tr>
<td><strong>Organelle</strong></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Swollen / rupture.</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>Flattened.</td>
</tr>
<tr>
<td>Vesicle</td>
<td>Enhanced secretions.</td>
</tr>
<tr>
<td>Endoplasmic reticulum (ER)</td>
<td>Swollen.</td>
</tr>
<tr>
<td></td>
<td>Proliferation of smooth ER.</td>
</tr>
<tr>
<td></td>
<td>Degranulation of ribosomes of rough ER</td>
</tr>
<tr>
<td>Lysosome</td>
<td>Increased numbers.</td>
</tr>
<tr>
<td>Peroxisome</td>
<td>Formation of auto phagosomes</td>
</tr>
<tr>
<td><strong>Nucleus</strong></td>
<td>Pyknosis / karyorrhexis / karyolysis/ Pleomorphic.</td>
</tr>
</tbody>
</table>

Chapter 4 Literature review: Target organs
4.3 Gill

4.3.1 Macroscopic anatomy of the gill

Teleosts have five pairs of gill arches. The last pair of gill arches generally transforms into the pharyngeal bone and does not play a role in respiration (Hibiya, 1982). Figure 4.5 shows the macroscopic view of the gills of *O. mossambicus*. Each arch consists of a bony support skeleton attached anteroventrally to the lower jaw and posterodorsally to the roof of the buccopharyngeal chamber (Figure 4.5 A). Two rows of gill filaments, (also known as primary lamellae), radiate posteroventrally from the arch (Figure 4.5 B). The tips of the filaments from adjacent arches are closely positioned to form a gill curtain. Figure 4.6 illustrates how this curtain causes water to sieve between the filaments when water is pumped from the buccal chamber to the opercular chambers (Olson, 1996).

*Figure 4.5:* Macroscopic anatomy of the fish gill. A) Position and orientation of the gill in *O. mossambicus*. B) Gill arch showing two pairs of filaments (primary lamellae).
As shown in Figure 4.6, each paired filament has a series of lamellae located perpendicular to the filament (Yasutake and Wales, 1983). Normal gill tissue has a reddish appearance because of the rich capillary network and blood supply.

**Figure 4.6:** Paired gill arches are located on either side of the pharynx with large numbers of filaments (primary lamellae) arranged in rows along the arches (Gilmour, 1998). The secondary lamellae (flattened, plate like structures extending outwards from both the upper and lower surfaces of the filament) form a sieve through which water must pass with the counter-current flow of blood perusing in them creating a highly efficient system for gaseous exchange (Schmidt-Nielsen, 1997).
4.3.2 Normal histology of the gill

A) Gill arches

The gill arches and filaments are supported by a branching system of cartilaginous rods. Two sets of striated muscles (abductor and adductor) are present in the gill arch. These muscles spread the filaments apart or draw them together. Embedded in the superficial tissue of the gill arches are numerous taste buds, supplementing those scattered abundantly throughout the buccal cavity (Yasutake and Wales, 1983).

B) Filament (Primary lamellae)

The filament (also known as the primary lamellae) consists of blood vessels, cartilaginous gill rays, melanocytes, lymphocytes, macrophage, coarse eosinophilic leucocytes, undifferentiated cells, endothelial cells and epithelial cells. Undifferentiated cells in the central part of the primary lamellar epithelium are thought to be the stem cells of all the other types of cells (Dang, 2000). Neuro-epithelial cells are located primarily on the distal half of the primary lamellae. These cells may perform a paracrine function in gill regulation (Laurent, 1984) but some may also function as chemo-receptors (Bailly et al., 1992; Zaccone et al., 1992).

The primary lamellae serve more as a support for the secondary lamellae (Groman, 1982). Figure 4.7 gives a diagrammatical representation of the normal histological structure of the primary and secondary lamellae.

C) Lamellae (Secondary lamella)

The Lamellae (oaslo known as the secondary lamellae) consist of mucus cells, rodlet cells, chloride cells, pavement cells, pillar cells with lacunae, and blood vessels (Groman, 1982).
**Figure 4.7:** Normal histological structure of the filament with lamella. CC - chloride cell; L - lacuna; MC - mucus cell; PC - pavement cells; Pi - pillar cell; RBC - red blood cell; UC - undifferentiated cells. (Adapted from Mallatt, 1985).

**a) Pavement cells (PC)**
Pavement cells also known respiratory cells or simple squamous cells are the predominant cell type found in gill tissue. Pavement cells can be identified by their broad pentagonal or hexagonal shape and a large spherical centrally located nucleus (Figure 4.7).

**b) Mucus cells (MC)**
Mucus cells are abundant on the cell surface of the lamellae, appearing as granular domes or vacuolated cells in light microscopy (Yasutake and Wales, 1983; Figure 4.7). The mucus cells are of two different types; the ordinary mucus cells and the rodlet cells. Interposition of the mucus coat between the environment and epithelium suggests that the primary function of mucus is isolative in nature (Olson, 1996).
c) Chloride cell (CC)

Chloride rich cells are also called ionocytes or mitochondria rich cells. In most teleost species that have been examined under conditions accepted as normal, the chloride cell occupies only a small fraction (generally less than 15%) of the total surface area of all epithelial cells exposed to the environment. Chloride cells are concentrated on the trailing edges of the filaments and the lamellae. This location however differs from specie to specie and is effected by contaminant exposure (Perry, 1997). Chloride cells can be identified by their granular appearance, large basal nuclei, numerous cytoplasmic vesicles and mitochondria with enlarged separated cristae (Groman, 1982; Figure 4.7). Chloride cells are larger and more spherical than other epithelial cells; and tend to project from the gill surface (Yasutake and Wales, 1983).

d) Lacunae (L)

Each lamella is composed of a network of interconnected spaces separated and supported by pillar (pilaster) cells (Figure 4.7). These spaces, also called lacuna, are lined with endothelial cells. The endothelial cells in turn are separated from the double layer of epithelial cells by a basement membrane (Yasutake and Wales, 1983)

e) Pillar cells (Pi)

Contractile pillar cells separate one side of the secondary lamellae from the other (Figure 4.7). Pillar cells has a fine fibrous cytoplasm containing abundant mitochondria (Groman, 1982)

f) Blood vessels (BV)

Capillaries are found throughout the secondary lamellae (Figure 4.5). The surface of the secondary gill lamellae is covered by many small capillaries running parallel to each other and separated by pillar cells (Hibiya, 1982).
4.3.3 Function of gills

The gill epithelia perform a variety of critical physiological functions including:

- Gaseous exchange, acid-base balance and regulation, ionic (Ca\(^+\), Cl\(^-\) and Na\(^+\)) transport activities and osmotic homeostasis of extra-cellular body fluids.
- Water exchange, toxicant uptake / detoxification / excretion, metabolic transformations, metabolism of circulating hormones and nitrogenous waste excretion.

The gill epithelium thus provides vital functions for quality of life and the very existence of the animal (Chu et al., 2001).

The gill is fundamentally a system for bringing blood haemoglobin into close contact with the water so that oxygen can be absorbed and carbon dioxide be released. Fish employ a method known as the counter-current system to extract oxygen from the water (Wedemeyer, 1976). Figure 4.6 diagrammatically explains how the counter-current system works.

The direction of blood flow in the capillaries is opposite that of respiratory water flow between the secondary gill lamellae. This counter-current system enhances remarkably the gas exchanging ability (Figure 4.6). The venous blood passes through the afferent branchial artery diverging from the ventral aorta to the capillaries of the secondary gill lamellae, exchanges gas from the respiratory water, and becomes arterial blood. Most arterial blood flows into the dorsal aorta through the efferent brachial artery. This method removes almost all of the oxygen (80-90%) from the water that passes over the gills and then transfers it to the blood (Wedemeyer, 1976).

Pavement cells are also be the site of Na\(^+\) and Ca\(^{2+}\) uptake, especially if the number of chloride cells is reduced (Goss et al., 1995; Perry, 1997). The mitochondria-rich chloride cells are believed to be the principal site of trans-
epithelial influxes of ions, and \( \text{Na}^+/\text{K}^+ \)-ATPase is an important enzyme in the regulation of the ion balance (Flik et al., 1995; Laurent and Perry, 1995; Perry, 1997).

Mucus cells are typical unicellular glands, which form a thin layer on the gill surface and separate the epithelium membranes from the water. Mucus cells are essential for fish respiration and osmoregulation and also play a protective role:

- Physical, prevent mechanical abrasion of limit parasitic, bacterial, or viral access.
- Biochemical through an ability to sequester or precipitate specific molecules, e.g. heavy metals (Olson and Fromm, 1973; Varanasi and Markey, 1978).
- Immunological through secretion of immunoglobulin (Itami et al., 1988; Ourth, 1980).
- Premselective through an ability to affect oxygen (Ultsch and Gross, 1979) or electrolyte diffusion (Handy, 1989; Pärt and Lock, 1983; Shephard, 1984, Simonneaux et al., 1987).

**4.3.4 Gill as biomarker of contaminant exposure**

The function of the gills necessitates the exposure of a system of capillaries with sufficient surface area to the water to facilitate the required gas exchange. Gills of fish constitute up to 90% of the total body surface area and are in intimate contact with ambient water (Mayer-Gostan et al., 1987; Perry and Laurent, 1993; Yasutake and Wales, 1983). The respiratory system therefore provides the most extensive interface of a fish with the aquatic environment (Heath, 1987). This exposure in turn makes the tissue vulnerable to the external environment and water born toxicants (Yasutake and Wales, 1983).
Fish gills are the primary uptake site of toxicologically effective concentrations of waterborne metals (Pelgrom et al., 1995; Sprague, 1987) and are considered the crucial organ when it comes to the induction of compensatory responses, whether adaptive or pathological of the organism (Pelgrom et al., 1995; Tao et al., 2001; Taylor et al., 2002). Gills are not only the primary receptor surface for all changes in the natural environment, but perpetually determine the extent of homeostatic regulation of the internal environment (Wood and Soivio, 1991). All stressors affect, directly or indirectly the branchial structure as well as various physiological and biochemical characteristics of gills, often with negative consequences to the hydromineral balance of fish (Wendelaar Bonga, 1997).

Chemical stressors, such as metals, influence the gill epithelium in three ways:

- direct damage, including necrosis of the cells (McDonald and Wood, 1993; Wendelaar Bonga, 1997);
- indirectly by evoking local responses in these tissues (McDonald and Wood, 1993; Wendelaar Bonga, 1997);
- indirectly via primary stress hormones, producing stress responses in the gills (Wendelaar Bonga, 1997).

Although the liver is the most important organ with respect to cytochromes P450, immunohistochemical staining revealed that CYP1A was expressed in the gills as well (Van Veld et al., 1997). Levine and Oris (1999) suggested that CYP1A expression due to exposure to rapidly metabolized substances should preferably be measured in tissues that make direct contact with the environment, such as the gill.

The presence of cytochrome P450E (P451A1) in pillar cells is a potentially useful biomarker in the gills (Hinton et al., 1992). This enzyme is inducible and detectable (Miller et al., 1989) and may serve as a biomarker of
exposure to a variety of contaminants in the environment (Hinton et al., 1992).

While the liver is quantitatively the most important site for glucuronidation of xenobiotics in fish, significant UDP-glucuronyl transferases (UDPGTs) activities have also been detected the gills (George, 1994). UDPGT activities in gill tissue can thus also be used as a biomarker of toxicant exposure.

The capacity for methelothionein (MT) induction is greatest in tissues that are active in uptake, storage and excretion of substances such as the gills of fish (Roesijadi and Robinson, 1994). MT immunopositivity can be found in all branchial cell types (except mucus cells). Mature and pre-existing gill cells (chloride cells, pavement cells) can apparently not induce the expression of MT and require exposure to metals in an earlier stage of their development. Old cells have to be replaced by new cells before the protective action of MT can become effective and, as soon as expression is initiated in undifferentiated cells, MT remains expressed throughout the life cycle of the cell (Dang et al., 1999).

Enzymes that regulate ionic (Ca\(^+\), Cl\(^-\) and Na\(^+\)) transport activities and osmotic homeostasis of extracellular body fluids in gill tissue are used as biomarkers. Na\(^+\)/K\(^+\)-ATPase in fish gills is a highly conserved membrane enzyme essential for ion homeostasis at the cellular and organismal level. Na\(^+\)/K\(^+\)-ATPase enzyme is located mainly in gill chloride cells of freshwater-adapted teleost (Dang et al., 2000; Lin et al., 2003). Na\(^+\)/K\(^+\)-ATPase is important, not only as a driving force for many transporting systems in a variety of osmoregulatory epithelia including gills (which play a crucial role in branchial epithelial ion transport), but also for sustaining Na\(^+\) and K\(^+\) ratios and the osmotic environment inside the cells (Dang, 2000; Lin et al., 2003). Studies on copper (Laurén and McDonald, 1985; MacDonald and Wood, 1993; Pelgrom et al., 1995) showed a reduction in Na\(^+\)/K\(^+\)-ATPase activity and an increase in chloride cell proliferation. The direct mechanism is not
known (Dang, 2000). Ca\(^{2+}\) -ATPase enzyme is located mainly in gill chloride cells and regulates the influx of Ca\(^{2+}\). Exposure to ambient cadmium inhibits this enzyme as well as Na\(^+\)/K\(^+\)-ATPase (discussed in section 2.5.4).

Studies done by Fu et al. (1989) on cadmium have shown an increase in the release of the internal hormone cortisol. This hormone potentially plays a role in the restoration of metal induced disrupted ion-balance, by decreasing the branchial efflux and increase the uptake capacity of ions partly by means of proliferation of ion-transporting cells, namely chloride cells, in the gills (McDonald and Wood, 1993; Perry et al., 1992). Handling of the specimens prior to dissection, however, affects cortisol levels.

**4.3.4.1 Gill histology as biomarker of contaminant exposure**

Mallatt (1985) reviewed the most common gill lesions found under several stressful conditions, and concluded that the most commonly reported changes (epithelial lifting, necrosis, hyperplasia, hypertrophy and haemorrhage) were results of lethal conditions. Figure 4.8 diagrammatically shows the common irritant induced lesions as recorded by Mallatt (1985).

At sub-lethal concentrations a variety of toxicants have been shown to induce chloride cell hyperplasia (Avella et al., 1987; Laurent et al., 1985: Perry and Wood, 1985). The proliferation of chloride cells are thought to be a compensatory response to ion loss, and therefore chloride cell hyperplasia may therefore be a good biomarker of adaptation (Hinton et al., 1992). Hyperplasia of undifferentiated epithelial cells which results in clubbing and lamellar fusion is a much less specific lesion associated with a wide variety of unrelated insults (Hinton et al., 1992). Table 4.3 summarises a few of the expected histological responses to contaminant exposure.
**Table 4.3:** Histological responses to contaminant exposure in the gill (compiled from Hinton *et al.*, 1992 and Mallatt, 1985).

<table>
<thead>
<tr>
<th>Level of organization</th>
<th>Histological identifiable responses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organ</strong></td>
<td>Necrosis</td>
</tr>
<tr>
<td></td>
<td>Tumour</td>
</tr>
<tr>
<td><strong>Primary lamellae</strong></td>
<td>Hyperplasia</td>
</tr>
<tr>
<td></td>
<td>Necrosis</td>
</tr>
<tr>
<td><strong>Secondary lamellae</strong></td>
<td>Branching</td>
</tr>
<tr>
<td></td>
<td>Fusion</td>
</tr>
<tr>
<td></td>
<td>Necrosis</td>
</tr>
<tr>
<td><strong>Cells</strong></td>
<td>Hyperplasia/necrosis</td>
</tr>
<tr>
<td>Undifferentiated cells</td>
<td></td>
</tr>
<tr>
<td>Chloride cells</td>
<td>Hyperplasia/necrosis</td>
</tr>
<tr>
<td>Mucus cells</td>
<td>Hyperplasia/increased secretions</td>
</tr>
<tr>
<td>Pavement cells</td>
<td>Hyperplasia/hypertrophy/necrosis/oedema</td>
</tr>
<tr>
<td></td>
<td>vacuolization</td>
</tr>
<tr>
<td>Pillar cells</td>
<td>Collapse</td>
</tr>
</tbody>
</table>
Figure 4.8: Diagrammatic representation of the common irritant induced gill lesions. Six lamellae are shown (a-f) with a) as the normal lamellae. 1 = epithelial lifting. 2 = necrosis. 3 = lamellar fusion. 4 = hypertrophy. 5 = hyperplasia. 6 = epithelial rupture. 7 = mucus secretion. 8 = aneurism. 9 = congestion. 10 = mucus cell proliferation. 11 = chloride cell damage. 12 = chloride cell proliferation. 13 = leucocyte infiltration. 14A = dilated blood sinus. 14B = Constricted blood sinus. (From Mallatt, 1985).
4.4 Gonads

The vertebrate gonad is composed of two basic cell types: the germ cells, giving rise to mature gametes (eggs or sperm), and non-germ cells or somatic cells which support, nourish and help regulate the activities and development of the germ cells. Acellular supportive elements, such as basement membranes, are also present in the gonads. The gonads of adult *O. mossambicus* are paired, elongated structures attached to the body cavity on either side of the dorsal mesentery, suspended directly from the ventral surface of the gas bladder by peritoneal mesenteries. The peritoneal mesenteries are continuations of the parietal peritoneum that extends from either side of the gas bladder. This peritoneum continues laterally around the ventral surface of each gonad. Gonadal arteries, veins, lymphatic ducts and nerves enter each gonad via the mesenteric attachments.

Histological studies of tilapia gonads have been reported by Hyder (1969), Katz *et al.* (1976), Nakamura (1975), Nakamura and Takahashi (1973), Yoshikawa and Oguri (1978), and Chmilevskii (1996). Hibiya (1982) and Groman (1982) described histological structures in great detail. The following sections (4.4.1 - 4.4.2) will therefore discusses the histological structures found in fish gonads by referring to Hibiya (1982) and Groman (1982).

4.4.1 Macroscopic anatomy of the ovary

The ovary of fish is generally a pair of sac-shaped organs covered with an ovarian wall and consisting of an ovarian cavity and numerous ovarian lamellae where oogenesis takes place. The ovarian cavity connects with the oviduct, and the oviduct form each bilateral ovary join together to lead to the genital pore. An ovary with such a structure is called a cyst-ovarian type ovary.
4.4.2 Histology of the ovary

General oocyte growth and developments have been summarized by Wallace and Selman (1981) and Tyler and Sumpter (1996) for all teleosts. Hibiya (1982) and Groman (1982) described the histological structures found in the cystovarian ovary. Histological structures found in *O. mossambicus* were therefore described by referencing to Hibiya (1982) and Groman (1982).

In order to understand the normal histology of the ovary it is important to understand oogenisis. The normal histology of the ovary will therefore be described by discussing the normal development of the ovary.

Oogenisis occurs within the ovigerous lamellae with mitosis of primary germ cells or oogonia that subsequently undergo meiosis to form oocytes. Oogonia are released from the germinal epithelium of the lamellae and mature within the dendric fold of the follicular epithelium. Juvenile females are easily identified histologically by the presence of numerous immature ova within the ovigerous lamellae. A mature female contains oogonia and oocytes in several stages of maturation prior to spawning (Groman, 1982). The classification adopted during this study recognizes six developmental stages for females (Goodbred *et al.*, 1997; Nagahama, 1983; Rodriguez *et al.*, 1995; Treasurer and Holiday, 1981). Classification of the developmental stages is discussed in detail in section 5.6.3.

4.4.2.1 Oogenesis

Oogenisis starts with the proliferation of oogonia on the ovarian lamella. The oogonia in the early stage are large cells with a large nucleus including a nucleolus. These cells occur in nests in the interstitial tissue and contain lightly acidophilic-staining cytoplasm. At a later stage of development these cells enlarge, and stain basophilic and contains a large central nuclei with diffuse chromatin (Groman, 1982).
After multiplication the oogonium develops into the primary oocyte. The size of the cell stays similar, but the nuclear cytoplasm appears thread-like with the chromosomes distributed throughout the nucleus. The chromosomes then assemble at one side of the nucleus and the nucleolus adjacent to the nuclear membrane localizes at the opposite end of the nucleus. The nucleolus moves to the central part of the nucleus, increase in size and become a germinal vesicle. The chromosomes become slender and distributed throughout the nucleus, and chromatic nucleoli begin to appear. The nucleoli become smaller, move the periphery of the nucleus and arrange themselves on the inner side of the nuclear membrane. The cell body enlarge and the cytoplasm becomes strongly basophilic (Hibiya, 1982).

Follicle cells surrounding the oocyte become clearly distinguishable. With the accumulation of yolk substances vitellogenesis become morphologically conspicuous, the basophilic cytoplasm becomes acidophilic as the growth of the oocyte proceeds. The essential yolk substances are yolk vesicle, yolk globules and oil droplets (Hibiya, 1982).

When accumulation of yolk substances becomes conspicuous, hyperplasia of follicle cells is recognized, and the squamous theca cells line up in two layers to form an outer and inner theca membrane outside the follicle cell layer. When vitellogenesis is about to begin, the egg membrane becomes clear, and in the course of vitellogenesis becomes hyperplasic and differentiate to form inner and outer layers. Radial striation also becomes clear and this stratum is called the zona radiata. The thickness of the egg membrane decreases just before full maturation (Hibiya, 1982).

With the completion of vitellogenisis, movement of the germinal vesicle, fusion of yolk globule and grouping of oil droplets occur and the egg diameter increases sharply. A marked increase in organ weight occurs due to water absorption. After movement of the germinal vesicle to a animal pole, the fist meiotic division occurs and the fist polar body is released. The second
meiotic division starts and division is arrested at the metaphase upon ovulation (Hibiya, 1982).

After spawning the ovary is composed of postovulatory follicles, immature oocytes and mature eggs left un-spawned. The follicle cells in the postovulatory follicles, undergo hypertrophy and multiply, showing phagocytosis. Some oocytes become atretic during maturation. Follicle cells and wondering cells take part in re-absorption of the denatured and un-spawned cells (Hibiya, 1982).

4.4.3 Macroscopic anatomy of the testis

Little work has been done on the histology of the testis of *O. mossambicus*. An extensive study and literature review was however done by Pieterse (2004) on the expected normal histology as well as the effect of copper exposure on the histology of the testes of *O. mossambicus*. The section 4.4.4 and section 4.4.4.1 will focus on the normal morphology and histology as described by Pieterse (2004).

The testes *O. mossambicus* are soft, creamy white bilateral glands. The gonoduct (the main sperm duct) leaves the posterior end of each testis and unite to form a common duct, which opens in the urogenital orifice. The testis is covered by a thin peritoneal epithelium membrane. The underlying tunica albuginea is composed of dense fibrous connective tissue and a few muscle fibres. The tunica extends around the major blood vessels and continues as the external lining of the sperm duct.

The testes vary in size from small strands in juvenile fish to large creamy, flabby organs in mature males. The size of the testes appears to be correlated with the size and stage of reproductive maturity (Pieterse, 2004). Juvenile males are easily identified histologically by the absence of spermatozoa and the presence of immature stages of spermatogenesis.
(largely spermatocytes). All stages of development are represented in the mature male, but spermatozoa predominate (Groman, 1982). For the purpose of this study, four developmental stages are recognized for males (Goodbred et al., 1997; Nagahama, 1983; Rodriguez et al., 1995; Treasurer and Holiday, 1981). Classification of the developmental stages is discussed in detail in section 5.6.3.

4.4.4 **Histology of the testis**

The functional unit of the testis is the lobule which originates in the interstitial tissue. The interstitial tissue in *O. mossambicus* is divided into two groups known as peripheral interstitium and interlobular interstitium. The interstitium constitutes of loose connective tissue in which Leydig cells and blood cells are found. Leydig cells are large ovoid cells and appear in groups in between contiguous lobules.

In order to understand the normal histology of the testis it is important to understand spermatogenesis. The normal histology of the testis will therefore be described by discussing the different cells that can be identified during spermatogenesis.

4.4.4.1 **Spermatogenesis**

The unit of testicular development is the lobule, within which a variable number of spermatogenic cysts at different stages of development are present. The lobules are lined in the inside by Sertoli cells which are important for the support and nutrition of developing spermatozoa. Sertoli cells are recognizable because of their angular contour and irregular shaped nucleus. The unit of spermatogenic activity is the cyst for all the stages beyond the primary spermatogonia. The lobules are filled with cysts, which contain developing germ cells.
O. mossambicus is a continuous breeder and all the stages of spermatogenesis, namely primary spermatogonia, secondary spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa are present throughout the year. The process of spermatogenesis and spermiogenesis occur within the different cysts of a lobule.

Primary spermatogonia develop from the germinal epithelium. Spermatogonia, always found singly, are large cells (12 -13 µm) with a large nucleus (6.5 µm), cytoplasm is clear and variably in quantity. Cell membrane is distinct, though faint. Only one nucleolus is found in nucleus. Greatest concentration of spermatogonia is found at the periphery of the lobule of the testis.

Secondary spermatogonia develop from the primary spermatogonia. Secondary spermatogonia are smaller than primary spermatogonia with a deep staining nucleus and a prominent nucleolus. Secondary spermatogonia cysts consist of four to eight cells within a cyst. This distinguishing feature is used to separate the secondary spermatogonia from the primary spermatogonia.

After the proliferation stage the spermatogonia develops into a primary spermatocyte. Primary spermatocytes can be distinguished from secondary spermatogonia by the absence of nuclear membrane and indistinct nucleolus. Deeply staining chromatin occupies practically the entire cell.

Primary spermatocytes develop in to secondary spermatocytes. Secondary spermatocytes can be identified by the chromatin matter which is characteristically found in a dense clumped state. Secondary spermatocytes are smaller than the primary spermatocytes.
Spermatids develop from the secondary spermatocytes and are even smaller than the spermatocytes. Many of these cells eventually undergo a meiotic division to become haploid spermatozoa. Light microscopy analysis does not show clearly how complete their development is before they are released into the lumen.

### 4.4.5 Hormonal regulation

Fish display strict hormonal and environmental regulation of the reproductive processes. Hormonal regulation is the function of the endocrine system composed of a collection of cells, tissues, and organs that produce and secrete hormones that influence virtually every stage of the lifecycle (reviewed by Norris 1997; Van der Kraak et al., 1998). The coordinated reproductive efforts of the hypothalamus, pituitary, and gonads have led to the designation of the hypothalamo-pituitary-gonadal axis.

The hypothalamus exerts ultimate control over the reproductive process by synthesizing and releasing gonadotropin-releasing hormones (GnRHs) that stimulate the release of several gonadotropin hormones from the pituitary.

In non-mammalian vertebrates, including several orders of teleosts, two gonadotropin hormones, GTH-I and GTH-II, have been identified (Kawauchi et al., 1989; Swanson et al., 1991). In fish, GTH-I is important for vitellogenesis, the process of yolk protein synthesis, and early gonadal development. GTH-II is secreted late in gonadal development and plays a role in the final maturation and release of mature gametes (ovulation in females, spermiation in males).

The sex steroids (androgens, estrogens and progesterone), is a class of hormones derived from cholesterol and synthesized by the gonads in response to circulating levels of GTH-I and GTH-II. These sex steroids
collectively control the development of the gonads and gametes, secondary sexual characteristics, and reproductive behavior (Liley and Stacey, 1983).

Circulating sex steroid levels are subject to normal variation due to differences in sex, age, geographical location, species, and season (Barry et al., 1990; Bromage et al., 1982; Chang and Chen, 1990; Denslow et al., 1997; Down et al., 1990; Goodbred et al., 1997; So et al., 1989).

### 4.4.6 Vitellogenesis

Vitellogenin is a glycolipophosphoprotein egg yolk precursor. GTH-I, released from the pituitary, stimulates the production of 17b-estradiol in the ovaries (reviewed by Redding and Patino, 1993). High levels of circulating 17b-estradiol stimulate the liver to synthesize and release vitellogenin. After being released by the liver into the bloodstream, vitellogenin is delivered to the ovaries where it is recognized by high-affinity receptors on the surface of the oocyte and internalized by receptor-mediated endocytosis (Specker and Sullivan, 1994). Finally, vitellogenin is enzymatically cleaved within the oocyte to form the yolk proteins that serve to nourish the developing embryo.

The vitellogenic response is dependent on a number of intrinsic factors, including species, sex, and maturation/reproductive stage, as well as extrinsic factors, such as water temperature, season, and chemical composition of the aquatic environment (Korsgaard et al., 1986, Wallaert and Babin, 1994). There is evidence for the regulation of vitellogenin synthesis by circadian rhythms in catfish (Lamba et al., 1983), photoperiod in rainbow trout (Bromage et al., 1982), and winter sea temperatures in small-spotted catshark (Craik, 1978).
4.4.7 Use of gonads as biomarker of contaminant exposure

Decreased reproductive capability in organisms may be considered as one of the most damaging effects of pollutants released by man. A number of pollutants, with widespread distribution in the environment, are reported to have endocrine activity which might affect reproduction and thus might threaten the existence of susceptible species (Colborn et al., 1993; Peterson et al., 1993; White et al., 1994). Animals at high trophic levels, generally having limited reproduction rates, are likely to be the most vulnerable (Van der Oost, 2003).

Effects on reproductive competence as a response to pollution stress have been demonstrated in fish. There is increasing evidence that low-level pollution may decrease the fecundity of fish populations, leading to a long-term decline and eventually extinction of important natural resources (Kime, 1995).

According to (Schmitt, 2000) reproductive biomarkers are useful for:

i. examining the effects of environmental stressors, such as chemical contaminants, eutrophication, and temperature fluctuations, on an individual or population,

ii. predicting future reproductive trends and population abundance by serving as early indicators of sub-lethal effects of environmental stressors, and

iii. providing insight into the causal relationships between reproductive failure and environmental stressors.

Pollutant effects may occur at multiple sites of the reproductive system. They may cause lesions, haemorrhage, or malformations in the gonads, pituitary, liver and the brain (Kime, 1995). Pollutants may reduce reproductive success of fish by interacting directly with the germ cells (Armstrong, 1990), resulting in a high rate of mitotic chromosome abnormalities (Longwell et al.,
1992). Production and secretion of hormones of the hypothalamus, pituitary, and gonads is usually inhibited and their metabolism by the liver can be altered (Kime, 1995).

Measuring reproductive hormones in plasma yield biochemical information concerning the reproductive status of an individual, as well as provide a method for detecting potential reproductive injury. Although this technique has been used to gain information regarding early development and the reproductive cycles of healthy individuals (Freund et al., 1995; Johnson and Casillas, 1991), monitoring sex steroid concentrations has primarily been utilized to study the endocrine-disrupting effects of various environmental chemicals (Schmitt, 2000).

There is evidence that the stress of collecting, holding, and obtaining blood from fish may affect hormone concentrations (Barton and Iwama, 1991; Jardine et al., 1996; Magri et al., 1982; McMaster et al., 1994; Van den Heuvel et al., 1995).

Numerous studies demonstrate that exposure to a variety of contaminants, including bleached kraft mill effluent (McMaster et al., 1991; Munkittrick et al., 1991; Munkittrick et al., 1992; Munkittrick et al., 1994), agricultural pesticides (Goodbred et al., 1997; Gross et al., 1997; Singh et al., 1994; Singh and Singh, 1987; Singh and Singh, 1991), industrial chemicals (Sivarajah et al., 1978; Spies et al., 1996), and metals (Allen-Gil et al., 1993; Thomas, 1988), can lead to alterations in plasma sex steroid concentrations in a variety of fish species. There is substantial evidence to suggest that pollutants alter the sex steroid levels by interfering at multiple sites along the hypothalamo-pituitary-gonadal axis (Mukherjee et al., 1991). However, metals are thought to reduce sex steroid levels by stimulating the production of cortisol, which subsequently accelerates the metabolism of steroids in the liver (Hansson, 1981).
The discovery that many structurally diverse chemicals (e.g., chlorobenzene and dichlorodiphenyltrichloroethane (DDT) as well as cadmium) that are released into the environment possess estrogenic properties (Le Guével et al., 2000; McLachlan, 1993) has encouraged the development and utilization of bio-assays that evaluate estrogenicity. The development of a universal assay has however been challenging because some chemicals that bind the estrogen receptor do not elicit an estrogenic response and, conversely, some compounds evoke an estrogenic response without interacting with the receptor (Schmitt, 2000). This unpredictable structure-function relationship demands an assay based on the bio-activity of potential environmental estrogens. The fact that vitellogenin synthesis is primarily regulated by circulating estrogens has made vitellogenin an attractive indicator of potential estrogen action (Palmer and Palmer, 1995; Palmer and Selcer, 1996).

The measurement of vitellogenin levels in female and male fish provides an additional biomarker for determining the stage of maturation, assessing reproductive health, and predicting the estrogenicity of various compounds. A number of studies have reported considerable variability in both sexes. Although female vitellogenin production can be affected by contaminants, plasma concentrations are so variable that it is difficult to determine the significance. Vitellogenin production by male fish became the focus of a number of investigations (Schmitt, 2000). Various studies led to the assumption that healthy males would not produce vitellogenin and, therefore, the detection of plasma vitellogenin in males would serve as a reliable biomarker of exposure to environmental estrogens (Heppell et al., 1995; Palmer and Selcer, 1996; Sumpter and Jobling, 1995). Most males do not produce vitellogenin in measurable quantities; nevertheless, vitellogenin receptors have been detected in testes, muscle, and spermatocytes (Bidwell and Carlson, 1995; Tao et al., 1996). Because the biological significance of vitellogenin in males is currently unknown, correlating vitellogenin production
with endocrine disruption or feminization is difficult in the absence of data corroborating such conditions (e.g. histopathology).

Gonadal-somatic index (GSI), as discussed in Section 3.3.3, is routinely used to determine reproductive maturity, as well as to assess gonadal changes in response to environmental conditions or the effect of contaminant exposure on the reproductive system.

### 4.4.8 Gonadal histopathology as biomarker of contaminant exposure

Gonadal histopathology is often utilized alone, or in conjunction with the GSI, to confirm gonadal phenotype, determine the state of sexual development, and investigate reproductive impairment (Schmitt, 2000). Although gonadal histopathology is routinely used to detect higher level responses expressed as morphological abnormalities, histopathology is capable of providing information at multiple levels of biological organization (i.e., distribution of molecules; distribution, number, volume, morphology of organelles, cells, and organs). Observed alterations in cells and tissues are often reflective of previous biochemical and physiological modifications.

Only one histopathological reproductive biomarker has yet been identified, namely oocyte atresia (Hinton et al., 1992). Oocyte atresia, as defined by an involution or resorption of unfertilized eggs by the ovaries, is a normal physiological event in all fish, but it has become a pathological condition noted in fish after exposure to certain environmental contaminants (Cross and Hose, 1988; Johnson et al., 1988; Kirubagaran and Joy, 1988). Oocyte atresia is characterised by a degeneration and necrosis of developing ova, and subsequent infiltration by macrophages (Meyers and Hendricks, 1985). The ability to detect increased degeneration or necrosis of developing oocytes by histological examination has inspired the use of oocyte atresia as a biomarker of reproductive impairment.
Response to a variety of toxicants and conditions results in relatively few histopathological changes in the gonads for the exposed fish (Glasier, 1986). Histopathological biomarkers found in the ovaria include:

- ovarian atresia,
- failure to ovulate associated with fibrosis, and
- inflammation due to bacteria, protozoa and metazoan.

Histopathological biomarkers found in the testis include:

- testicular atrophy (age / toxicants)
- sperm reduction (toxicants that reduces mitosis), and
- inflammation due to bacteria, protozoa and metazoan.

The ovary of the female includes the following cellular components that are of histopathological concern:

- germinal epithelium, produces oogonia that become oocytes,
- and the follicular epithelium which supports oogenesis

The testes of the male include the following cellular components that are of histopathological concern:

- Sertoli cells, constituting the blood -testis barrier;
- Leydig cells which are interstitial cells involved in the male hormone production;
- developing germ cells including in order of differentiation, spermatogonia,
- spermatocytes, spermatids, spermatozoa; and
- secretory cells involved in the production of spermatic fluid are part of the testes of a few fish.

According to Hinton et al., 1990; to little data exist to consider reproductive neoplasm in fish a reliable biomarker of chemical exposure.

The purpose of this chapter was to provide background information on the biology and histology of the target organs selected for this study, and their
use as a biomarker. The following chapter will focus on the methodology followed during this study including the collection, acclimation and the exposure of *O. mossambicus*. Chapter 5 will also give a detailed account of the methodology followed during tissue collection and the preparation of micro-slides and how the histopathological assessment tool was applied.

### 4.5 References


George, S.G. (1994). Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. In: *Aquatic Toxicology; Molecular, Biochemical*


5.1 Test organism

For the purpose of this study *Oreochromis mossambicus* (Mozambique tilapia, Figure 5.1) was chosen as test organism. *O. mossambicus* is endemic to Southern Africa and shows a wide distribution in rivers and lakes. *O. mossambicus* is found in east costal rivers from the lower Zambezi system, south to the Bushman’s system, Eastern Cape Province, south of the Phongolo system. *O. mossambicus* is wildly dispersed beyond this range to inland regions and to the south-west and west coastal rivers, including the lower Orange River and rivers of Namibia. Because of their ability to tolerate pH variance and high salinities, they often reach into closed estuaries and coastal reaches of rivers (Skelton, 1993).

*O. mossambicus* are remarkably hardy (Table 5.1), and have relatively high resistance to diseases (bacterial, parasitic and viral) at high stocking densities (Wong and Chiu, 1993; Bainy et al., 1996; Popma and Masser, 1999).

**Table 5.1:** Selected environmental requirements for optimum growth and survival for Tilapia species (adapted from Hubbard, 2005.)

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>Oxygen (mg/l)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optimum</strong></td>
<td>27 - 33</td>
<td>&gt; 5.0</td>
<td>6 - 9, Ideal 7.5</td>
</tr>
<tr>
<td><strong>Survive</strong></td>
<td>6 - 42</td>
<td>&lt; 0.3</td>
<td>5 - 10</td>
</tr>
</tbody>
</table>
The Mozambique tilapia feeds on algae, diatoms and detritus but adults also eat insects and other invertebrates like crustaceans (Skelton, 1993). Mozambique tilapia are readily available, abundant, and inexpensive, can grow to more than 36 cm, are capable of reaching sexual maturity in 5 months, reproduce well in captivity, adapt readily to new environments and are able to live for up to 13 years (DPI, 2003; Nikonenko, 1986; Sell, 1993; Vonck et al., 1998). Widely used in aquaculture, commercial and subsistence farming, *O. mossambicus* makes for an ideal test organism with which to conduct exposure studies (Skelton, 1993).

**Figure 5.1:**  A) *Oreochromis mossambicus*. B) Breeding male. C) Distribution throughout South Africa (adapted from Skelton, 1993).

Crossbreeding between *O. mossambicus* and *O. niloticus* is well documented (Moralee et al., 2000). If crossbreeding did occur in test organisms it will affect the credibility of the obtained results. Before the onset of this study a genetic study was done to determine the purity of the strain of the fish to be used in this study. Samples of muscle tissue were analyzed using starch gel electrophoresis (Moralee et al., 2000).
5.1.2 Collection and acclimation of fish

*O. mossambicus* was obtained from the University of Zululand, Empangeni, Kwazulu-Natal, South Africa in November 2004. Fish were removed from the general holding tanks using a net, and placed in a 1 000 ℓ plastic transport tank (Figure 5.2) filled with water from the facility from which the fish were collected. 2-phenoxy ethanol (40 ml/500 ℓ) was added to the water to reduce stress (Hattingh *et al.*, 1975). The fish were transported to the University of Johannesburg by road. The water was kept at a constant temperature of 23°C (± 1°C) and aerated with pure oxygen (O₂), obtained from Afrox, during the transport. No mortalities were noted.

*Figure 5.2:* 1 000 ℓ plastic transport tank.
On arrival at the University of Johannesburg, fish were transferred into 1000 ℓ aerated general holding tanks (Figure 5.3) containing aged tap water (23°C, ± 1°C). The general holding tanks were maintained using a biological filter (Figure 5.3). The fish underwent a week-long daily treatment of course salts to prevent bacterial infection, and were not fed for 72 hours after arrival (reducing build up of organic pollution). Thereafter feeding resumed on a daily basis using commercial trout pellets (protein = 39.9%; lipid = 5.3%; ash = 9.6%; carbohydrate = 45.2%; energy = 22.8kJ/g; Nussey, 1998). Fish were allowed to acclimate for 6 weeks prior to experimentation.

Figure 5.3: General holding tanks connected to a biological filter.

5.2 Experimental design

Fish were exposed to 10% (n = 20) and 20% (n = 20) of the LC50 concentration of nickel, cadmium and chromium, respectively under controlled conditions (23°C ± 1°C) for 96 hours in an environmental room. A control group of fish (n = 5) for each medium type was acclimated in a separate flow-through system in the same room as the exposure fish.
The control group was therefore subjected to the same physical conditions as the exposure group of fish, with the exception of the addition of the selected metals to the water during the exposure period. The results obtained from the control group were therefore accepted to represent the normal condition.

### 5.2.1 Exposure medium

Previous studies done at the Randse Afrikaanse University to compare the effect of borehole water and tap water on heat shock protein production in *O. mossambicus*, showed tap water to be a better exposure medium (Grant, 2004). Tap water was therefore used during this study. Monthly water quality reports were acquired from Rand Water for tap water for the duration of the exposures (Addendum A).

### 5.2.2 Exposure method

A flow-through system was chosen for this study because it provides an ideal environment when exposing fish to a chosen chemical. Oxygen levels and constant concentration of the exposure chemical can easily be maintained (Nussey, 1998).

The flow-through system, illustrated in Figure 5.4, consisted of a 900 ℓ reservoir, four 90 ℓ exposure tanks (4) and a biological filter. During the acclimation period water was pumped from the biological filter to the exposure tanks, from where water flowed back to the biological filter (Figure 5.4 A). This allowed for removal of excretory products from the recirculation water by the biological filter (3). The flow rate of the system was controlled by a tap. The flow of water increased the oxygen concentration in the water. The water flowed out of the experimental tanks (1a-d) through the outlet pipe (6). A wider screening pipe (7) was placed over the outlet pipe. The screening pipe had indentations at the bottom, which caused a slight
suction. Water, excretion and waste products were therefore transported quickly out of the exposure tanks thought the outlet pipe (6) which drained (8) into the filter. During the exposure period water was pumped from the 900 ℓ reservoir tank to the exposure tanks (Figure 5.4 B).

5.2.3 Preparation of flow-through system

Four systems were prepared in the same environmental room which allowed for accurate temperature control. Three of these systems were used to expose fish to the desired metal concentration. No metals were added to the forth system which were used as a control system throughout the exposures. To prevent contamination from previous studies the systems were washed with phosphate free soap and left to stand with commercial pool acid for 24 hours. The biological filter was also rinsed with tap water. This procedure was followed before each exposure. After cleaning, tanks were filled with tap water and allowed to age for two weeks before the fish was added.

5.2.4 Exposure

Each 90 ℓ tank accommodated 3 test organisms separated from one another by a mesh divider. Tanks were covered with mesh, as shown in Figure 5.5, to keep fish from jumping out of the tanks. Fish were acclimated for a further 2 weeks in the flow-through system and fed daily. The photoperiod (12 hour light: 12 hour dark) and temperature (23°C ± 1°C) were kept constant throughout the experiment, as fluctuating light periods, and temperature, may have negative influences on the fish’s metabolism (Van Vuren, 1986). Each system was connected to a biological filter, shown in Figure 5.6, which removed any excretory products. Tanks were regularly cleaned of any solid waste or uneaten food. Water quality readings were recorded for each individual holding tank as well as for each reservoir tank on a regular basis before, and during the exposure period. Five physical parameters namely: temperature; conductivity; total dissolved solids (TDS);
oxygen concentration and pH, were recorded for each tank using the Cyberscan series of water quality meters.

**Figure 5.5:** 90 ℓ exposure tanks accommodated 3 test organisms separated from one another by a mesh divider. Tanks were also covered with mesh.

**Figure 5.6:** Each exposure system was connected to a biological filter.
Feeding was terminated 48 hours before the onset of exposure. This minimizes the production of wastes in the exposure tanks which may also complex the test substance and affect its toxicity. It also avoids the addition of possible chemical interference by components in the food (Mance, 1987). Solid waste or uneaten food was removed in the morning prior to the exposure.

The biological filter was removed during exposure to ensure that metals do not accumulate on the filter. The 900 ℓ reservoir tank replaced the biological filter during exposure as illustrated in Figure 5.4 B. Water quality tests were performed continuously to ensure good water quality throughout the exposures. Test concentrations for the specific metals, listed in Table 5.2, were determined by taking the volume of the specific tanks into account.

Table 5.2: LC₅₀ values and the representative metal concentrations used in exposures.

<table>
<thead>
<tr>
<th>Metal compounds (abbreviations used during study)</th>
<th>Species</th>
<th>96-hour LC₅₀ (mg/ℓ)</th>
<th>90 ℓ* exposure tanks (g/90 ℓ)</th>
<th>900 ℓ* reservoir tanks (g/900 ℓ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10% Metal solution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CdCl₂ (Cd10)</td>
<td>Cyprinus carpio</td>
<td>0.240</td>
<td>0.0035</td>
<td>0.0350</td>
</tr>
<tr>
<td>K₂Cr₂O₇ (Cr10)</td>
<td>Cyprinus carpio</td>
<td>0.108</td>
<td>0.0055</td>
<td>0.0550</td>
</tr>
<tr>
<td>NiCl₂ (Ni10)</td>
<td>Cyprinus carpio</td>
<td>10.600</td>
<td>0.3862</td>
<td>3.8625</td>
</tr>
<tr>
<td><strong>20% Metal solution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CdCl₂ (Cd20)</td>
<td>Cyprinus carpio</td>
<td>0.240</td>
<td>0.0070</td>
<td>0.0705</td>
</tr>
<tr>
<td>K₂Cr₂O₇ (Cr20)</td>
<td>Cyprinus carpio</td>
<td>0.108</td>
<td>0.0110</td>
<td>0.1110</td>
</tr>
<tr>
<td>NiCl₂ (Ni20)</td>
<td>Cyprinus carpio</td>
<td>10.600</td>
<td>0.7725</td>
<td>7.7250</td>
</tr>
</tbody>
</table>

(See Addendum B for calculations)
The 96 hour LC$_{50}$ concentration is the concentration that is calculated to cause the mortality of 50% of the test population after a 96 hour exposure. These values were obtained from Mance (1987) for CdCl$_2$ and NiCl$_2$, and the ECOTOX database of the USEPA (2001) for K$_2$Cr$_2$O$_7$. The chemically pure metals were supplied by Saarchem (Pty) Ltd (South Africa) and were dissolved in the water in which the fish was acclimated.

Fish were exposed to the desired metal concentration for 96 hours, during which the flow tempo of the system was maintained at 60 ml/min.

5.3 Water quality

Water quality is a term used to describe the chemical, physical and biological characteristics of water (DWAF, 2002). When exposing fish the condition in the test system must be stable and unlikely to stress the test organisms during the exposure. The exposing substance should ultimately be the only variable in the system that will exert a possible stressful effect on the test organisms (Mance, 1987).

5.3.1 Physical water quality parameters

pH

pH is a general measure of the acidity or alkalinity of a water sample and is indicated on a scale of 0 to 14 (acidic (1-6.9), neutral (7), alkaline (7.1-14)). pH is a measure of the hydrogen ion (H$^+$) concentration in a water sample. The hydrogen ion concentration plays a significant role in the toxicity and availability of a test substance as well as all reactions related to water quality. A direct effect of pH on an organism includes alterations in the rate and type of ion exchange across the body’s surface (Grant, 2004). The pH of natural waters is determined largely by geological and atmospheric influences. Freshwater resources in South Africa are relatively well buffered.
However, acidification from industrial effluents, mine drainage and acid precipitation cause a lowering of the pH, leading to the mobilization of elements such as iron, aluminium, cadmium, cobalt, copper, nickel, lead and zinc (DEAT, 1999).

For the purpose of this study, the pH values were kept constant.

**Temperature (T)**

The temperature of water is a measure of its internal energy content expressed as degrees Celsius (ºC) (Boyd, 2000). Temperature plays an important role in exposure studies because it affect the rates at which chemical reactions take place, and therefore the metabolic rate of the organism under study (DWAF, 1996a). This will have an effect on the organism’s tempo of metal uptake and its ability to cope with the stressor. Temperature also affects the amount of dissolved oxygen present in the water. The toxicity of a particular test substance may also be affected by temperature, but for the majority of metals it is not possible to establish any relationship between toxicity and water temperature (Mance; 1987).

For the purpose of this study water temperature was kept at ± 23ºC. In order to conserve the credibility of the results, temperatures were kept as constant as possible, not varying by more than 1ºC.

**Oxygen concentration (O₂)**

Dissolved oxygen within a solution is the amount of oxygen dissolved in the solution at any given time, with a theoretical equilibration concentration of 100% saturation. The maintenance of adequate dissolved oxygen is an important criterion when exposing organisms to a toxic substance, as the depletion of dissolved oxygen combined with the addition of a toxicant will compound the stress placed on an organism (Grant, 2004).
This parameter is commonly expressed as a concentration in terms of milligrams per litre (mg/l) or as a percentage (%). Dissolved oxygen concentrations for surface water ranges from 0 mg/l (in extremely poor water conditions), to as high as 15 mg/l in 0°C water. Levels below 5 mg/l are however stressful to most aquatic organisms.

**Conductivity (σ) and total dissolved solids (TDS)**

Conductivity is a numerical expression of the ability of water to conduct an electrical current, resulting from the presence of charged species in solution (DWAF, 1993). Conductivity is expressed in micro Siemens per centimetre (µS/cm).

The total dissolved solids (TDS) concentration is a direct measure of the quantity of all compounds dissolved in the water, both organic and inorganic, and ionized or un-ionized (Dallas and Day, 1993). This parameter is usually expressed as parts per million (ppm) and is directly related to conductivity as follows:

\[
\text{TDS (mg/l)} = \text{EC (mS/M at 25°C)} \times 6.5
\]

Conductivity and total dissolved solids is influenced by an array of factors, for example the concentration and nature of the solutes, their degree of dissociation into ions, their electric charge, the mobility of the ions, and the temperature of the solution (DWAF, 1993). The concentration of various dissolved ions may influence the ionic balance of an organism. Should the ionic balance of the organism under study be influenced, the results obtained will not be a true reflection of the effects of the test chemical being tested (Grant, 2004).
5.3.2 Metal speciation

Visual MINTEQ version 2.5 (Gustafsson, 2006) is a computer software program designed to calculate the percentage of free metal ion and the degree of speciation from existing water quality data. Theoretical concentrations of free metal ions (Cd$^{2+}$, CrO$_4^{2-}$ and Ni$^{2+}$) present in the exposure media were calculated by using Visual MINTEQ.

5.4 Necropsy

5.4.1 Condition factor (CF)

After 96 hours of exposure the fish were individually removed from the tanks using a hand net. Each fish was weighted (g) and the total length (from the tip of the snout to furthest tip of the caudal fin) was recorded in mm. The condition factor (CF) for each fish was calculated (Carlander, 1969):

$$\text{Fulton's CF} = \frac{\text{Weight (g)} \times 10^5}{\text{Length (mm)}^3}$$

5.4.2 Blood variables

5.4.2.1 Blood collection

A wet cloth was placed over the fish eyes to reduce stress. 1 ml disposable syringe fitted with 26G needles was washed with sodium heparin (5000 i.u/ml). Blood was drawn from the dorsal aorta through the lateral line system as illustrated in Figure 5.7. Blood was stored in 5 ml lithium/heparin vacutainers and kept on ice for the duration of the dissection.
5.4.2.2 Haematocrit (Hct) and leucocrit (L)

Blood analysis was done immediately after dissection to ensure the accuracy of the results (as discussed in section 3.2.2.2). Micro-haematocrit tubes were filled with blood and one end was plugged with clay. Tubes were then centrifuged for 3 minutes at 15 000 revolutions per minute. The haematocrit
was determined by reading the total blood volume and both the packed red blood cell volume as well as the buffy layer (packed leucocytes) by using a micro-haematocrit tube reader. The haematocrit and leucocrit was determined by expressing the reading from the micro-haematocrit tube reader (mm) as a percentage.

5.4.2.3 **Total protein determination ([TP])**

The vacutainers containing the remainder of the whole blood was centrifuged for 10 minutes at 3 000 revolutions per minute. The plasma was removed and placed in eppendophs (supplied by Merck) and frozen until further analysis could be done.

A standard total protein determination kit was used (Roche No 1553836) with a 51,7 g/ℓ CFAS protein standard (Roch No 759350). A sample blank was prepared for each sample to eliminate the effect of haemolysis on the absorbance of the sample. All samples were analyzed in duplicate. Samples were read on a Bio-Tec micro plate reader at 490λ.

5.4.2.4 **Blood sample analysis**

Haematocrit and leucocrit were graded according to the criteria stipulated by Adams *et al.* (1993). Total protein concentration was however graded according to criteria determined for *O. mossambicus* (Robinson, 1996). Table 5.3 gives a summary of the grading system used in this study.

5.4.3 **External examination**

Fish was examined externally before dissection. All observations were recorded according to the variables as proposed by Adams *et al.* (1993). The following variables were considered during the external examination (Luus-Powell, 1997):
Skin: Any damage to the skin were noted, e.g. lesions and open wounds. Other abnormalities noted under this criterion were: crooked spine and tumours.

Fins: Conditions of fins were recorded according to the degree of erosion, e.g. no erosion, mild or severe erosion. Previously eroded fins which were healing were considered as normal. If one fin displayed erosion it was record as mild erosion. If however more than one fin displayed different degrees of erosion, the worst condition was ranked.

Eyes: The condition of the eyes was recorded as normal, blind (including cataracts) exophthalmic, haemorrhagic or missing.

Opercula: The opercula were considered normal when no shortening were observed and the gills were completely covered by them. Other conditions noted were the degree of shortening.

Parasites: Any ecto-parasites were removed before dissection. Parasites were only recorded as no observed parasite or few observed parasites.

5.4.4 Internal examination

After blood collection and the external examination, fish were sacrificed by spinal dissection. Dissection of the fish was done by cutting from the anal vent forward to the pectoral girdle. Care was taken not to damage the internal organs. Figure 5.8 shows the internal anatomy of *O. mossambicus*. All macroscopic alterations to internal organs/structures were noted and graded based on the criteria as stipulated by Adams *et al.* (1993). The following variables were considered during the internal examination (Luus-Powell, 1997):

Gills: Condition of gills were recorded as normal, frayed (eroded on the tip of the gill lamellae), pale, clubbed or marinate.
Hindgut: The condition of the hindgut was recorded as normal (no reddening or inflammation), slight inflammation, moderate inflammation or severe inflammation.

Spleen: Spleen was considered normal if the colour was red or black; or organ appeared to be granular. Enlargement of the spleen and the presence of nodules were considered as abnormal.

Liver: The liver might be red, pale red, light brown (fatty) or dark brown. Liver was considered normal if it was red, pale red or was slightly discoloured. The presence of nodules and focal discolouration was considered as abnormal. Blotchy appearance might be a result of blood-pooling. This condition must not be mistaken for focal discolouration, but should rather be noted as general discoloration.

Bile: Bile colour ranges form yellow straw colour to a light or dark green. Bile colour depends on the feeding regime (Adams, 1993) e.g. quality and quantity of food, time since last meal. For this study bile colour were noted, but not graded or included in the calculation of the necropsy health assessment index (Luus-Powell, 1997; Robinson, 1996).

Mesenteric fat:
The internal body fat was expressed as a percentage covering the cecum. This parameter was recorded, but not used in the calculation of the necropsy-based health assessment index (HAI) as it can vary wildly depending on several factors. The mesenteric fat was however weighed and the fat tissue index was determined (see paragraph 3.3.4 and 5.5.2).

Kidney: The kidney was graded as normal, swollen, granular or mottled. Mottled or patchy conditions range from scattered patches of grey to total grey discolouration.

*O. mossambicus* do not possess a pseudobranch and the thymus is reduced in adult fish. For the purpose of this study the assessment of both the
pseudobranch and the thymus was not included in necropsy-based health assessment index (Robinson, 1996).


5.4.5 Fish health assessment index calculation

All macroscopic alterations noted to internal and external organs/structures were graded based on the criteria as stipulated by Adams *et al.* (1993). Table 5.3 summarizes all the variables considered during this study, as well as the grading system.
**Table 5.3:** Criteria used to grade macroscopic alterations to external and internal organs/structures (adapted from Adams *et al.*, 1993).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Variable condition</th>
<th>NFHAI value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External examination</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eyes</td>
<td>Normal.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Exophthalmia/haemorrhage/blind and other.</td>
<td>30</td>
</tr>
<tr>
<td>Skin</td>
<td>Normal.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild aberration.</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Moderate aberration.</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Severe aberration.</td>
<td>30</td>
</tr>
<tr>
<td>Fins</td>
<td>Normal.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Light erosion.</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Mild erosion.</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Severe erosion.</td>
<td>30</td>
</tr>
<tr>
<td>Opercula</td>
<td>Normal.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild shortening.</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Severe shortening.</td>
<td>20</td>
</tr>
<tr>
<td><strong>Internal examination</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gills</td>
<td>Normal.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Frayed/clubbed/discoloured/pale/and other.</td>
<td>30</td>
</tr>
<tr>
<td>Hind gut</td>
<td>Normal.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild inflammation.</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Moderate inflammation.</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Severe inflammation.</td>
<td>30</td>
</tr>
<tr>
<td>Bile</td>
<td>Straw, partly full.</td>
<td>Noted but not considered for NFHAI calculation.</td>
</tr>
<tr>
<td></td>
<td>Straw, full.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Light green.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dark/ blue green.</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.3 continues...

<table>
<thead>
<tr>
<th>Variables</th>
<th>Variable condition</th>
<th>HAI value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mesenteric fat</strong></td>
<td>No fat. &lt; 50% of cecum covered. &gt; 50% of cecum covered. 100% of cecum covered.</td>
<td>Noted but not considered for NFHAI calculation.</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td>Normal /slight discoloration. Fatty/nodules/cists/focal discoloration and other.</td>
<td>0 30</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>Dark red/red/ Nodular, enlarged, other.</td>
<td>0 30</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td>Normal. Mottled/swollen/ granular and other.</td>
<td>0 30</td>
</tr>
<tr>
<td><strong>Parasites</strong></td>
<td>None observed. Few observed. Moderate observed. Numerous observed.</td>
<td>0 10 20 30</td>
</tr>
</tbody>
</table>

**Blood variables**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Variable condition</th>
<th>HAI value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haematocrit</strong></td>
<td>30 - 45%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&gt; 45%</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>19 - 29%</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>&lt;18%</td>
<td>30</td>
</tr>
<tr>
<td><strong>Leucocrit</strong></td>
<td>&lt; 4%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&gt; 4%</td>
<td>30</td>
</tr>
<tr>
<td><strong>Plasma proteins</strong></td>
<td>&gt; 6 g/100 ml</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt; 3 g/100 ml</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3 - 6 g/100 ml</td>
<td>30</td>
</tr>
</tbody>
</table>

*Graded according to Robinson, 1996
A NFHAI for each fish within an exposure group were calculated by summing the numerical values obtained for all the considered variables. The NFHAI for the exposure group was then calculated by averaging all the NFHAI values of the individual fish from the exposure group. The standard deviation (SD) for each sample was calculated.

5.4.6 Tissue collection

The gills (shown in Figure 5.8; B) were immediately section out and placed in 10% BNF (neutrally buffered formalin). The liver (C) and spleen (E) were carefully removed from the rest of the visceral organs and weighed for the determination of the hepato-somatic index (HSI), and the spleno-somatic index (SSI). Length and weight of both gonads (F) were measured separately for determination of the gonadal-somatic index (GSI). Mesenteric fat was removed and weight for determination of the fat tissue index.

Tissue samples collected for histological analysis were sampled at the same morphological region of each organ to allow for comparative analysis. These samples were immediately fixed in 10% neutrally buffered formalin (tissue: BNF ratio of 1:10), to ensure that stressor-dependent pre-mortem cell death and necrosis can be differentiated from post-mortem changes (Trump et al., 1980).

5.5 Tissue and data processing

5.5.1 Histology sample processing

Tissue samples were properly fixed for 3 days in 10% BNF (neutrally buffered formalin). After fixation, samples were washed in running tap water for 12 hours and then dehydrated using rising concentrations of ethanol. The samples were then transferred to xylene, a transitional solvent miscible with both dehydrating agent and embedding medium, but inert with regards to
the specimen (Woods and Ellis, 1994). After 5 minutes tissue samples were transferred to fresh xylene and allowed to clear. The cleared tissue samples were then transferred to a 60°C oven for infiltration purposes. Proper infiltration was attained by transferring samples through a series of rising concentrations of paraffin wax to xylene. Samples were imbedded in paraffin wax blocks, allowed to set and sectioned (4 µm thick, using a Leica RM 2125 RT wax microtome). The sample sections were stretched with an albumin and distilled water solution, mounted on glass microscope slides and dried. Dried sections were stained with haematoxylin and eosin (H & E) following a standardized protocol (Van Dyk, 2007).

10 sections were made of each organ investigated, resulting in 200 sections per organ for each exposure group and 300 sections per organ for the control group.

5.5.2 Calculation of the somatic indices

Hepato-somatic index (HSI)

The hepato-somatic index was determined by expressing liver weight as a percentage of total body weight (Schmitt et al., 2000):

\[
\text{HSI} = \frac{\text{Liver weight (g)}}{\text{Total body weight (g)}} \times 100
\]

Spleno-somatic index (SSI)

The spleno-somatic index is the weight of the spleen expressed as a percentage of total body weight (Schmitt et al., 2000):
Gonadal-somatic index (GSI)

The gonadal-somatic index is the weight of the gonads expressed as a percentage of total body weight (Schmitt et al., 2000):

\[
GSI = \left( \frac{\text{Gonads (g)}}{\text{Total Body Weight (g)}} \right) \times 100
\]

Fat-tissue index (FTI)

The fat-tissue index is the total mesenteric fat (g) expressed as a percentage of total body weight (Abascal, 2004):

\[
FTI = \left( \frac{\text{Mesenteric fat (g)}}{\text{Total body weight (g)}} \right) \times 100
\]

5.6 Histological assessment

The histological assessment was done by applying the histopathological assessment tool as proposed by Bernet et al. (1999). This tool was previously applied during both laboratory (Van Dyk, 2006), and field investigations (Marchand, 2006). By applying the histopathological assessment tool, histological results can be expressed both qualitatively and quantitatively, thus allowing for statistical comparisons between the different exposure groups without discarding qualitative results.
5.6.1 Histopathological assessment tool

5.6.1.1 Qualitative assessment

Histological description

For each organ investigated, the respective pathological changes are classified into five reaction patterns (Rp). This classification is a slight modification of the original classification as described by Hibiya (1982). Each reaction pattern includes several histological changes which concern either the functional units of the organ or the entire organ (Table 5.4).

A detailed description of the original classification and the histological changes was already discussed in section 3.4.1. This section will thus focus on the classification as used in the histopathological assessment tool.

Reaction pattern 1 (Rp 1): Circulatory disturbances (CD)
Only circulatory disturbances as a result of a pathological condition of blood and tissue fluid flow are considered under reaction pattern 1. Changes in tissue fluid content related to inflammatory processes are considered in reaction pattern 4. Changes considered here are haemorrhage, hyperaemia, aneurism, and intercellular oedema.

Reaction pattern 2 (Rp 2): Regressive changes (RC)
- Architectural and structural alterations: changes in tissue structure as well as in shape and arrangement of cells.
- Plasma alterations: changes in cytoplasm caused by hyaline droplets (granular degeneration), colloidal droplets (colloid degeneration), degenerative fatty vacuolization of hydropic glycogen droplets (glycogen degeneration), calcareous degeneration, and thickening of the fine fibres of connective tissue (hyaline degeneration).
- Deposits: intercellular accumulations of substances primarily as a result of degenerative processes.
- Nuclear alterations: changes in the nuclear shape and structure of chromatin (e.g. karyolysis, karyorrhexis and pyknosis).
- Atrophy.
- Necrosis.

**Reaction pattern 3 (Rp 3): Progressive changes (PC)**
Histological changes considered here are hypertrophy and hyperplasia.

**Reaction pattern 4 (Rp 4): Inflammation (I)**
Inflammatory processes, such as oedema, are often associated with processes belonging to other reaction patterns. Histological changes considered, in this index, as inflammatory changes are:
- Exudates: fluid containing a high protein concentration, and a large amount of cellular debris exuded from blood and lymph vessels.
- Activation of the reticulo-endothelial system (RES): hypertrophy of the RES, which consists of endothelial cells and macrophages that line small blood vessels.
- Infiltration: leucocytes penetrating the walls of blood vessels and infiltrating the surrounding tissue.

**Reaction pattern 5 (Rp 5): Tumour (T)**
Tumours are classified as benign or malignant tumours.

### 5.6.1.2 Quantitative assessment

**Importance factor (ω)**
The relevance of a lesion depends on how it affects organ function and the ability of the fish to survive. This is taken into account by assigning an importance factor to every alteration. The changes, as listed in the histological description, are classified into three importance factors:

1. Minimal importance: lesion easily reversible.
2. Moderate importance: lesion is reversible in most cases if irritant is removed.
3. Marked importance: lesion is generally irreversible, leading to partial or total loss of organ function.

**Score value (a)**

Every histological change is assessed and assigned a score value ranging from 0 - 6, depending on the degree and extent of the alteration:

- **0** - Unchanged
- **2** - Mild occurrence
- **4** - Moderate occurrence
- **6** - Severe occurrences
Table 5.4: Histopathological assessment tools for fish liver, gills and gonads.

<table>
<thead>
<tr>
<th>Reaction pattern</th>
<th>Functional units / cells analyzed</th>
<th>Histological change</th>
<th>Importance factor (ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rp 1: CD</strong></td>
<td>Blood vessels</td>
<td>Haemorrhage / intercellular oedema</td>
<td>1</td>
</tr>
<tr>
<td><strong>Rp 2: RC</strong></td>
<td><em>Liver tissue</em></td>
<td>Structural alterations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Hepatocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Inter cellular</em></td>
<td>Plasma alterations:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Connective tissue</td>
<td>- Intra cellular deposits</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Bile ducts</em></td>
<td>- Fatty degeneration</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Biliary epithelia</td>
<td>Inter cellular deposits</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increase in MMC</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nuclear alterations</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Atrophy</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Necrosis</td>
<td>3</td>
</tr>
<tr>
<td><strong>Rp 3: PC</strong></td>
<td><em>Liver tissue</em></td>
<td>Hypertrophy</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Hepatocytes</td>
<td>Hyperplasia</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Interstitial tissue</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Connective tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Bile duct</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Biliary epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rp 4: I</strong></td>
<td>Including:</td>
<td>Exudate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Macrophage</td>
<td>Activation of RES</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Lymphocytes</td>
<td>Infiltration</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Leucocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rp 5: T</strong></td>
<td>Whole section.</td>
<td>Benign tumour</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Malignant tumour</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 5.4 continues....

<table>
<thead>
<tr>
<th>Reaction pattern</th>
<th>Functional units / cells analyzed</th>
<th>Histological changes</th>
<th>Importance factor ($\omega$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gills</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rp 1: CD</em></td>
<td>(Blood vessels)</td>
<td>Haemorrhage/hyperaemia/aneurysm /intercellular oedema</td>
<td>1</td>
</tr>
<tr>
<td><em>Rp 2: RC</em></td>
<td><em>Epithelium</em></td>
<td>Structural alterations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>(Secondary lamellae)</em></td>
<td>Plasma alterations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>including:</td>
<td>Inter cellular deposits</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Pavement cells</td>
<td>Nuclear alterations</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Mucus cells</td>
<td>Atrophy</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Chloride cells</td>
<td>Necrosis</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>- Pillar cells with lacunae</td>
<td>Rapture of pillar cells</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Supporting tissue</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>(Primary lamellae)</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>including:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Endothelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Epithelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Undifferentiated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Connective tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Cartilage tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Muscle tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rp 3: PC</em></td>
<td><em>Epithelium</em></td>
<td>Hypertrophy</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Supporting tissue</em></td>
<td>Hyperplasia</td>
<td>2</td>
</tr>
<tr>
<td><em>Rp 4: I</em></td>
<td>Including:</td>
<td>Exudate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>Activation of RES</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Lymphocytes</td>
<td>Infiltration</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Leucocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rp 5: T</em></td>
<td>Whole section.</td>
<td>Benign tumour</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Malignant tumour</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 5.4 continues...

<table>
<thead>
<tr>
<th>Reaction pattern</th>
<th>Functional units / cells analyzed</th>
<th>Histological changes</th>
<th>Importance factor (ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rp 1: CD</td>
<td>(Blood vessels)</td>
<td>Haemorrhage / hyper anaemia aneurysm / intercellular oedema</td>
<td>1</td>
</tr>
<tr>
<td>Rp 2: RC</td>
<td>*Lobule cysts</td>
<td>Disorganization of lobules Detachement of basal membrane Inhibition of spermatogenesis</td>
<td>1 1 3</td>
</tr>
<tr>
<td></td>
<td>*Interstitial tissue including:</td>
<td>Structural alterations Plasma alterations Inter cellular deposits Nuclear alterations Atrophy Necrosis</td>
<td>1 1 2 2 3</td>
</tr>
<tr>
<td></td>
<td>- Sertoli cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Connective tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Blood vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Spermatogonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Spermatocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Spermatids</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Spermatozoa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rp 3: PC</td>
<td>*Lobule cysts</td>
<td>Hypertrophy</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>*Interstitial tissue</td>
<td>Hyperplasia</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>*Spermatogonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Spermatocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Spermatids</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*Spermatozoa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rp 4: I</td>
<td>Including:</td>
<td>Exudate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Macrophage</td>
<td>Activation of RES</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Lymphocytes</td>
<td>Infiltration</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Leucocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rp 5: T</td>
<td>Whole section.</td>
<td>Benign tumour</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Malignant tumour</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 5.4 continues...

<table>
<thead>
<tr>
<th>Reaction pattern</th>
<th>Functional units / cells analyzed</th>
<th>Histological changes</th>
<th>Importance factor (ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovaries</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rp 1: CD</td>
<td>(Blood vessels)</td>
<td>Haemorrhage / hyper anaemia aneurysm / intercellular oedema</td>
<td>1</td>
</tr>
<tr>
<td>Rp 2: RC</td>
<td>* Ovary</td>
<td>Inhibition of oogenesis</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(whole section)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Oogonia</td>
<td>Structural alterations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>* Oocytes</td>
<td>Plasma alterations</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>* Interstitial tissue</td>
<td>Inter cellular deposits</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>including:</td>
<td>Nuclear alterations</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Connective tissue</td>
<td>Atrophy</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Blood vessels</td>
<td>Necrosis</td>
<td>3</td>
</tr>
<tr>
<td>Rp 3: PC</td>
<td>* Oogonia</td>
<td>Hypertrophy</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>* Oocytes</td>
<td>Hyperplasia</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>* Interstitial tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rp 4: I</td>
<td>Including:</td>
<td>Exudate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Macrophage</td>
<td>Activation of RES</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>- Lymphocytes</td>
<td>Infiltration</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>- Leucocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rp 5: T</td>
<td>Whole section.</td>
<td>Benign tumour</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Malignant tumour</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5.4 summarize the systematic procure followed during this study while analyzing sections, specifying the functional units and cells examined as well as the importance factor for each alteration observed. To minimize bias, slides were randomly analysed without the histologists knowing which exposure groups the slide represented. All slides were analyzed by 3 independent histologists.
Using importance factors and score values, the following indices can be calculated:

**Reaction index of an organ (I_{org \ rp}):**

\[
I_{org \ rp} = \sum (a_{org \ rp \ alt} \times \omega_{org \ rp \ alt})
\]

Where:
- \( \text{org} = \) organ (constant)
- \( \text{rp} = \) reaction pattern (constant)
- \( \text{alt} = \) alteration
- \( a = \) score value
- \( \omega = \) importance factor

The quality of the lesions in an organ is expressed by the reaction index. It is calculated by the sum of the multiplies importance factors and score values of the histological changes of the corresponding reaction pattern. The sum of the five reaction indices of an organ is equivalent to the organ index (\(I_{org}\)). Respective reaction indices of an organ from different individuals can be compared.

**Organ index (I_{org}):**

\[
I_{org} = \sum_{rp} \sum_{alt} (a_{org \ rp \ alt} \times \omega_{org \ rp \ alt})
\]

Where:
- \( \text{org} = \) organ (constant)
- \( \text{rp} = \) reaction pattern
- \( \text{alt} = \) alteration
- \( a = \) score value
- \( \omega = \) importance factor

This index represents the degree of damage to an organ. It’s the sum of the multiplied importance factors and score values of all changes found within the examined organ. A high index indicates a high degree of damage.
Calculating the organ index allows a comparison between the degrees of damage of the same organ in different individuals.

**Total index (Tot-I):**

\[
\text{Tot-I} = \sum_{\text{org}} \sum_{\text{rp}} \sum_{\text{alt}} (a_{\text{org rp alt}} \times \omega_{\text{org rp alt}})
\]

Where: \( \text{org} \) = organ (constant)  
\( \text{rp} \) = reaction pattern (constant)  
\( \text{alt} \) = alteration  
\( a \) = score value  
\( \omega \) = importance factor

This index represents a measure of the overall health status based on the histological lesions. It is calculated by adding up all organ indices of an individual fish. As the total index is calculated in the same way for every fish, a comparison between individuals is possible.

**Total reaction index (I_{rp}):**

\[
I_{rp} = \sum_{\text{org}} \sum_{\text{alt}} (a_{\text{org rp alt}} \times \omega_{\text{org rp alt}})
\]

Where: \( \text{org} \) = organ (constant)  
\( \text{rp} \) = reaction pattern (constant)  
\( \text{alt} \) = alteration  
\( a \) = score value  
\( \omega \) = importance factor

This index represents the quality of the histological lesions in all examined organs of an individual fish. It is the sum of the corresponding reaction indices of all examined organs of a fish. Using this index allows a comparison between different individuals.
5.6.2 Gonad maturity index

The developmental stages of the gonads were graded according to the classification as adopted by Schmitt et al. (2000). This classification recognizes six developmental stages for females and four maturational stages for males. The stages are based on the size and developmental status of the oocytes (ovaries) and spermatozoa (testes) contained within the reproductive organs. Table 5.5 provides the histological characteristics of the maturational stages in both the ovaries and testis.

**Table 5.5:** Histological characteristics of gonadal stages as defined by Schmitt et al., 2000 (adapted from Goodbred et al., 1997; Nagahama, 1983; Rodriguez et al., 1995; Treasurer and Holiday, 1981).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Testis characteristics</th>
<th>Ovary characteristics</th>
</tr>
</thead>
</table>
| 0     | **Undeveloped (immature):**  
Little or no spermatogenic activity in geminal epithelium.  
Immature stages of spermatogenesis (largely spermatocytes).  
No spermatozoa observed. | **Undeveloped:**  
Pre-vitellogenic oocytes observed exclusively.  
Oocyte diameter <250 µm.  
Oocyte cytoplasm stains basophilic with H&E. |
Table 5.5 continues...

<table>
<thead>
<tr>
<th>Stage</th>
<th>Testis characteristics</th>
<th>Ovary characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Early spermatogenic:</strong> Mostly thin geminal epithelium with scattered spermatogenic activity. Spermatocytes to spermatids predominate. Few spermatozoa observed.</td>
<td><strong>Early development:</strong> &gt;90% Pre-vitellogenic, remaining oocytes early to mid-vitellogenic. Oocytes slightly larger (up to 300 µm). Late perinucleolus through cortical alveolar stages.</td>
</tr>
<tr>
<td>2</td>
<td><strong>Mid –spermatogenic:</strong> Geminal epithelia are of moderate thickness. Moderate proliferation and maturation of spermatozoa. An equal mix of spermatocytes, spermatids and spermatozoa present.</td>
<td><strong>Mid development:</strong> Majority of observed follicles are early and mid-vitellogenic. Oocytes larger (300–600 µm diameter) and contains peripheral yolk vesicles. Globular and uniformly thick chorion. Cytoplasm is basophilic, yolk globules eosinophilic when stained with H&amp;E.</td>
</tr>
<tr>
<td>3</td>
<td><strong>Late spermatogenic:</strong> Thick geminal epithelium. Diffuse regions of proliferation and maturation of spermatozoa. All stages of development are represented, but spermatozoa predominate.</td>
<td><strong>Late development:</strong> Majority of developing follicles are late vitellogenic. Oocyte diameter is 600-1000 µm. Eosinophilic yolk globules distributed throughout the oocyte cytoplasm. Thick chorion present.</td>
</tr>
<tr>
<td>4</td>
<td>N/A</td>
<td><strong>Late development:</strong> Majority of developing follicles are late vitellogenic. Large follicles (&gt;1000 µm).</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td><strong>Post-ovululatory:</strong> Spent follicles. Remnants of the theca externa and granulosa.</td>
</tr>
</tbody>
</table>
5.7 Statistical analysis

Statistical analysis was done by Statcon, the statistical consultation services of the University of Johannesburg. A series of non-parametric (The Kruskal-Wallis) and parametric (ANOVA) tests were performed on the data sets using SPSS software (Version 12).

The non-parametric Kruskal-Wallis test analyzes variance by ranking the means. This test calculates the normal ANOVA, but tests for difference in ranks of three or more independent groups (Pallant, 2005).

Chi-square is a non-parametric test of statistical significance for bivariate tabular analysis (also known as cross-breaks). Test of statistical significance specifies the degree of confidence in accepting or rejecting a hypothesis. The hypothesis tested for with chi-square is whether or not two different samples are different enough in some characteristic that it can be generalized from the samples that the populations from which the samples are drawn are also different in the characteristic. If the significant value (p), calculated by the Chi-square, are smaller or equal to 0.05 there is a significant difference between the individual values from all the data sets. The Chi-square test is performed on nominal data sets and ultimately compares frequencies of distributions occurring in different categories and groups (Connor-Linton, 2003; Morgan et al., 2001).

Non-parametric test is a rough estimate of confidence and accepts weaker, less accurate data as input than parametric tests (e.g. t-test and analysis of variance) and therefore has less status in the pantheon of statistical tests. Nonetheless, its limitations are also its strengths; because Chi-square is more 'forgiving' in the data it will accept, it can be used in a wide variety of research contexts (Connor-Linton, 2003).
Analysis of variance (ANOVA) is a method of testing the null hypothesis that several group means are equal in the population, by comparing the sample variance estimated from the group means to that estimate within the group. The one-way ANOVA procedure produces a one-way analysis of variance for a quantitative dependent variable by a single factor (independent) variable. This technique is an extension of the two-sample t test (Pallant, 2005).

The ANOVA assume that sampling was random, the groups are homogeneous, distribution is normal, sample size are large enough to represent population and dependent variable data are represented on an interval or ratio scale (Pallant, 2005).

Data sets are first tested for homogeneity of variances by subjecting the data to the Levene statistic for the equality of group variance. This test determines if the variance in the values is the same for the different data groups. If the significance value (p) was greater than 0.05, the assumption of homogeneity of variances was not violated and this test was then followed by the one-way ANOVA. If however the significant value was less than 0.05, the assumption of homogeneity of variances was violated. The equality of group means was then determined by applying the Brown-Forsythe test (StatSoft Inc. 2006).

If the significant value (p), as determined by ANOVA, was smaller than or equal to 0.05 there is a significant difference between the data sets. However, the ANOVA does not distinguish between which data sets the difference lies. The Scheffe test of multi comparison, assuming equal variances, is then calculated to determine where the significant difference lies (Morgan et al., 2001).

The Brown-Forsythe test for the equality of group means is preferable to the ANOVA statistic when the assumption of equal variances does not hold. If significant values (p), as determined by the Brown-Forsythe test, were smaller than or equal to 0.05 there was a significant difference between data
sets. As with the ANOVA, the Brown-Forsythe test does not distinguish between which data sets the difference lay. To determine between which groups the significant difference lies, the Dunnett's T3 test of multi comparison, assuming unequal variances, is then calculated (StatSoft Inc. 2006).

To summarize, for the purpose of this study both non-parametric and parametric statistical tests were preformed on the data sets. The non-parametric statistical test (Kruskal-Wallis test which included the calculation of the Chi-square), compared all the values obtained from all the exposure groups for a specific biomarker. This was done in order to determine if there is a statistical significant difference between the individual values obtained for the biomarker from all the exposure groups. The parametric statistical test (one way ANOVA and the Brown-Forsythe test) compared the different exposure groups to the control group. This was done in order to determine if the exposure to the selected metals resulted in a significant change in the various biomarkers applied during this study.

The following chapter focus on the results obtained from the exposures, as well as the results obtained after statistical analysis. Chapter 7 will interpret and discuss these results which will lead to a final conclusion.

**5.8 References**


Chapter 5    Materials and methods


Chapter 6

Results

This chapter presents the results obtained from the different biomarkers applied during this study. Statistical analysis was done on all data obtained to determine statistical significant differences between the exposure groups and the control groups. Chapter 7 discusses the results obtained and compares these results to results from similar studies.

When conducting an exposure study it is important to keep physical water quality parameters stable. Not only can varied physical water quality parameters cause additional stress on the test organisms, but it can also affect the concentration of the free metal species available to the organisms. Section 6.1 focuses on the water quality results obtained for the control and exposure groups during the exposure period.

6.1 Exposure medium analysis

Physical water quality parameters were measured daily. Section 6.1.1 reports the average readings obtained during the exposures. Section 6.1.2 reports the theoretical free metal ions during the exposures. The percentages of free metal ions available during the exposures were calculated by taking the daily physical water quality parameters into account.
6.1.1 Physical water quality parameters

6.1.1.1 Cadmium exposure

pH
Initial pH readings of 8.3 ± 0.0 and 8.1 ± 0.1 were recorded for the 10% and 20% control groups respectively (Table 6.1). The 10% and 20% exposure groups had initial pH values of 8.3 ± 0.0 and 7.8 ± 0.1 respectively. Measurements of the pH of the water remained relatively constant for the duration of the exposure period.

Temperature (T)
Before the onset of the exposure period daily recordings of the water temperature of the 10% control group yielded an average of 23.5 ± 0.2°C, while an average water temperature of 23.8 ± 0.1°C was recorded for the 20% control group. An average water temperature of 23.2 ± 0.2°C and of 23.4 ± 0.2°C was recorded for the 10% and the 20% exposure groups respectively (Table 6.1). Daily measurements indicated little deviation (± 1°C) from the initial values, for the duration of the exposure period. As a result, all values fell within the target water quality range specified in Table 5.1.

Dissolved oxygen (DO)
Prior to the commencement of the exposure period, average dissolved oxygen concentrations in the water were recorded as 91.4 ± 2.2% and 7.8 ± 0.1 mg/l of the 10% control group, while the 20% control group yielded an average of 98.4 ± 1.3% and 8.4 ± 0.1 mg/l (Table 6.1). Similar results were obtained for the exposure groups, with little deviation obtained in daily measurements. As a result, all values fell within the target water quality range specified in Table 5.1.
**Total dissolved salts (TDS)**
Initial TDS readings of 117.0 ± 4.7 ppm, 102.0 ± 0.8 ppm, 113.3 ± 1.0 ppm and 93.0 ± 3.3 ppm were recorded for the 10% control group, 20% control group, 10% exposure group and the 20% exposure group respectively (Table 6.1). These values gradually decreased during the exposure period, reaching final values of 106.5 ± 0.7 ppm, 83.5 ± 1.6 ppm, 105.9 ± 0.7 ppm and 83.7 ± 1.7 ppm for the 10% control group, 20% control group, 10% exposure group and the 20% exposure group respectively (Table 6.1).

**Conductivity (ơ)**
A gradual decrease in the conductivity of the water was observed in both the control groups and the exposure groups during the exposure period of 96 hours. Initial conductivity readings of 253.8 ± 1.3 µS/cm, 216.0 ± 0.0 µS/cm, 240.8 ± 6.3 µS/cm and 216.0 ± 0.0 µS/cm were recorded for the 10% control group, 20% control group, 10% exposure group and the 20% exposure group respectively (Table 6.1). These values gradually decreased during the exposure period, reaching final values of 224.5 ± 2.2 µS/cm, 177.1 ± 1.6 µS/cm, 225.3 ± 1.4 µS/cm and 177.9 ± 3.3 µS/cm for the 10% control group, 20% control group, 10% exposure group and the 20% exposure group respectively (Table 6.1).
<table>
<thead>
<tr>
<th>Table</th>
<th>6.1</th>
<th>Cadmium</th>
</tr>
</thead>
</table>


6.1.1.2 Chromium exposure

**pH**
For the 10% control group, 20% control group, 10% exposure group and 20% exposure group initial pH readings were recorded as 7.8 ± 0.2, 8.3 ± 0.1, 8.2 ± 0.1 and 8.3 ± 0.1 respectively (Table 6.2). Daily measurements of the pH of the water remained relatively constant for the duration of the exposure period, deviating by no more than 0.5 pH units per time interval.

**Temperature (T)**
Prior to the commencement of the exposure period, daily recordings of the water temperature yielded an average of 21.6 ± 0.1°C and 22.7 ± 0.2°C for the 10% control group and 20% control group respectively. An average water temperature of 21.4 ± 0.1°C was recorded for the 10% exposure group, and 22.6 ± 0.3°C for the 20% exposure group (Table 6.2). Daily measurements taken indicated little deviation from the initial values obtained, for the duration of the exposure period. All values thus fell within the target water quality range specified in Table 5.1.

**Dissolved oxygen (DO)**
Prior to the commencement of the exposure period, average dissolved oxygen concentrations in the water were recorded as 84.1 ± 4.3% (7.7 ± 0.2 mg/l) for the 10% control group, 90.3 ± 4.0% (7.9 ± 0.4 mg/l) for the 20% control group. Average dissolved oxygen concentrations recorded for the 10% exposure group and the 20% exposure group were recorded as 85.0 ± 5.8% (7.6 ± 0.5 mg/l) and 92.4 ± 5.5 (8.1 ± 0.6 mg/l) respectively (Table 6.2). Little deviations were obtained in daily measurements. As a result, all values fell within the target water quality range specified in Table 5.1.

**Total dissolved salts (TDS)**
Initial TDS readings of 138.2 ± 5.9 mg/l, 134.0 ± 4.0 mg/l, 132.2 ± 2.1 mg/l and 102.8 ± 0.5 mg/l were recorded for the 10% control group, 20%
control group, the 10% exposure group and the 20% exposure group respectively (Table 6.2). All values showed a gradual decrease during the exposure period reaching final values of 131.8 ± 1.5 mg/ℓ, 131.8 ± 1.5 mg/ℓ, 131.6 ± 2.1 mg/ℓ and 87.9 ± 2.1 mg/ℓ for the 10% control group, 20% control group, the 10% exposure group and the 20% exposure group respectively (Table 6.2).

**Conductivity (σ)**

Average initial conductivity readings of 293.0 ± 12.7 μS/cm, 263.8 ± 1.7 μS/cm, 281.3 ± 4.5 μS/cm and 204.6 ± 0.6 μS/cm were obtained for the 10% control group, 20% control group, 10% exposure group and 20% exposure group respectively (Table 6.2). Final values of 280.0 ± 3.2 μS/cm (10% control group), 280.0 ± 3.2 μS/cm (20% control group), 280.2 ± 2.9 μS/cm (10% exposure group) and 175.5 ± 4.4 μS/cm (20% exposure group) were obtained, indicating a decrease in conductivity by the end of the exposure period (Table 6.2).
Table 6.2
6.1.1.3 **Nickel exposure**

**pH**
Initial pH readings for the control group, the 10% exposure group and 20% exposure group were recorded as 8.1 ± 0.1, 8.2 ± 0.1, 8.0 ± 0.2 and 8.1 ± 0.1 respectively (Table 6.3). These values deviated by no more than 0.5 pH units for the duration of the exposure period.

**Temperature (T)**
Average water temperature readings of 22.3 ± 0.3°C, 22.6 ± 0.3°C, 23.1 ± 0.1 and 22.4 ± 0.2°C were recorded for the 10% control group, 20% control group, 10% exposure group and the 20% exposure group respectively (Table 6.3). Consecutive daily measurements showed little deviation (< 1°C) from the initial values for the duration of the exposure period.

**Dissolved oxygen (DO)**
Prior to the commencement of the exposure period, average dissolved oxygen concentrations in the water were recorded as 134.3 ± 5.3% (11.1 ± 0.4 mg/l) for the 10% control group, 105.3 ± 7.0% (8.8 ± 0.6 mg/l) for the 20% control group. Average dissolved oxygen concentrations recorded for the 10% exposure group and the 20% exposure group were recorded as 110.0 ± 2.5% (9.3 ± 0.3 mg/l) and 107.2 ± 3.7 (9.3 ± 0.5 mg/l) respectively (Table 6.2). Oxygen levels fell within all the groups, reaching final values of 73.2 ± 0.3% (6.3 ± 0.0 mg/l), 98.5 ± 5.1% (8.6 ± 0.5 mg/l), 77.7 ± 2.4% (6.7 ± 0.2 mg/l) and 103.9 ± 4.8% (9.1 ± 0.1 mg/l) for the 10% control group, for the 20% control group, 10% exposure group and the 20% exposure group. All values therefore still fell within the target water quality range specified.

**Total dissolved salts (TDS)**
Initial TDS readings of 85.1 ± 0.7 ppm, 90.6 ± 1.9 ppm, 87.8 ± 3.8 ppm, and 105.1 ± 3.8 ppm were recorded for the 10% control group, 20% control...
group, the 10% exposure group and the 20% exposure group respectively (Table 6.3). The values for the 10% control group, the 10% exposure group as well as the 20% exposure group decreased gradually during the exposure period, reaching final values of 79.6 ± 1.7 ppm and 80.4 ± 1.2 ppm and 89.5 ± 0.5 ppm. Values for the 20% control group indicated a gradual increase, with a final value of 100.3 ± 9.1 ppm.

**Conductivity (σ)**

Average initial conductivity readings of 180.8 ± 1.7 µS/cm, 192.7 ± 4.0 µS/cm, 180.8 ± 8.2 µS/cm and 212.8 ± 9.3 µS/cm were obtained for the 10% control group, 20% control group, 10% exposure group and 20% exposure group respectively (Table 6.3). Final values of 168.9 ± 0.2 µS/cm (10% control group), 171.0 ± 2.2 µS/cm (10% exposure group) and 190.1 ± 0.8 µS/cm (20% exposure group) were obtained, indicating a decrease in conductivity by the end of the exposure period (Table 6.3). The 20% control group however showed a slight increase in conductivity during the exposure to reach the final value of 213.2 ± 19.2 µS/cm.
Table 6.3
6.1.2 Metal speciation

For each exposure group the free metal species available to the test organism were predicted by Visual MINTEQ based on available water data (Table 6.4). No metal was added to the control exposure groups, and therefore the percentage of free metal ions was 0%. In the 10% exposure groups 0.024 mg/l Cd\(^{2+}\), 0.0108 mg/l Cr\(^{6+}\) and 1.06 mg/l Ni\(^{2+}\) was added to the respective exposure groups, obtaining an average bioavailable percentage of 98.67%, 98.84% and 98.79% for Cd\(^{2+}\), Cr\(^{6+}\) and Ni\(^{2+}\) respectively. In the 20% exposure groups, the average bioavailable percentage of Cd\(^{2+}\), Cr\(^{6+}\) and Ni\(^{2+}\) determined as 99.33%, 98.38% and 98.07% respectively for 0.048 mg/l Cd\(^{2+}\), 0.0212 mg/l Cr\(^{6+}\) and 2.12 mg/l Ni\(^{2+}\) added to the respective exposure groups.

6.2 Necropsy

Water quality between the control exposure groups varied only slightly, summarized in the previous section. For the purpose of statistical analysis, control groups were therefore considered as one. In order to simplify the presentation of the results obtained during this study, the control group will also be considered as an exposure group. This exposure study therefore consisted of 7 exposure groups: the control group, 10% LC\(_{50}\) Cd exposure group, 20% LC\(_{50}\) Cd exposure group, 10% LC\(_{50}\) Cr exposure group, 20% LC\(_{50}\) Cr exposure group, 10% LC\(_{50}\) Ni exposure group and the 20% LC\(_{50}\) Ni exposure group.

6.2.1 Condition factor (CF)

Table 6.5 provides a summary of the average length, weight and condition factor (CF) calculated for the exposure groups.
Table 6.4 Metal speciation
6.2.1.1 Control specimens

Of the thirty nine specimens from the control group, eighteen specimens (46.2%) were recorded as female and twenty one (53.2%) as male. For this group of fish, an average weight of 46.6 ± 13.60 g and an average standard length of 15.12 ± 1.33 cm were recorded. An average CF of 1.33 ± 0.20 was calculated for the control group (Table 6.5).

6.2.1.2 Cadmium specimens

Of the twenty specimens used in the 10% LC$_{50}$ cadmium exposure, five specimens (21.1%) were recorded as female and fifteen (78.9%) as male. An average weight of 40.87 ± 7.20 g and an average standard length of 14.49 ± 0.81 cm were recorded for this exposure group. An average CF of 1.31 ± 0.11 was calculated for this exposure group (Table 6.5).

Within the 20% LC$_{50}$ cadmium exposure group, four (46.2%) of the nineteen specimens used in the exposure were recorded as female, and fifteen specimens (53.2%) were recorded as male. For this group of fish, an average weight of 46.6 ± 13.60 g and an average standard length of 15.12 ± 1.33 cm were recorded. The average CF of this exposure group was calculated as 1.34 ± 0.18 (Table 6.5).

6.2.1.3 Chromium specimens

In the 10% LC$_{50}$ chromium exposure group, eight specimens (44.4%) were recorded as female and ten (55.6%) as male. A total of eighteen specimens were sacrificed. For this group of fish, an average weight of 57.07 ± 16.16 g and an average standard length of 16.16 ± 1.48 cm were recorded. An average CF of 1.32 ± 0.10 was calculated for this exposure group (Table 6.5).
Of the twenty specimens used as the 20% LC$_{50}$ chromium exposure group, ten specimens (50.0%) were recorded as female and ten (50.0%) as male. For this group of fish, an average weight of 37.77 ± 5.25 g and an average standard length of 13.94 ± 0.71 cm were recorded. An average CF of 1.39 ± 0.11 was calculated (Table 6.5).

### 6.2.1.4 Nickel specimens

For the 10% LC$_{50}$ nickel exposure group seven of the nineteen specimens used was recorded as female (36.8%), and twelve specimens (46.2%) were recorded as male (63.2%). An average weight of 54.70 ± 13.10 g and an average standard length of 15.61 ± 1.36 cm were recorded, and an average CF was calculated as 1.42 ± 0.11 (Table 6.5).

Of the nineteen specimens used in the 20% LC$_{50}$ nickel exposure group, nine specimens (47.4%) were recorded as female and ten (52.6%) as male. An average weight of 52.11 ± 14.35 g and an average standard length of 15.27 ± 1.44 cm were recorded, this resulting in a calculated average CF of 1.44 ± 0.12 (Table 6.5).
Table 6.5: Average length, weight and condition factor (CF) calculated for the control group and exposure groups (n - number of specimens; SD - standard deviation; F - female; M - male; T - total = male + female).

<table>
<thead>
<tr>
<th>Exposure groups</th>
<th>Sex</th>
<th>n</th>
<th>Weight ± SD (g)</th>
<th>Length ± SD (cm)</th>
<th>Condition factor (CF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>F</td>
<td>18</td>
<td>42.56 ± 11.46</td>
<td>14.75 ± 1.02</td>
<td>1.31 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>21</td>
<td>50.06 ± 14.57</td>
<td>15.43 ± 1.50</td>
<td>1.34 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>39</td>
<td>46.60 ± 13.60</td>
<td>15.12 ± 1.33</td>
<td>1.33 ± 0.20</td>
</tr>
<tr>
<td>10% LC₅₀</td>
<td>F</td>
<td>5</td>
<td>49.61 ± 9.34</td>
<td>15.36 ± 0.92</td>
<td>1.29 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>15</td>
<td>54.80 ± 8.69</td>
<td>16.18 ± 1.11</td>
<td>1.35 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>20</td>
<td>53.50 ± 8.91</td>
<td>15.98 ± 1.10</td>
<td>1.31 ± 0.11</td>
</tr>
<tr>
<td>20% LC₅₀</td>
<td>F</td>
<td>4</td>
<td>38.77 ± 10.63</td>
<td>13.53 ± 0.59</td>
<td>1.29 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>15</td>
<td>41.43 ± 6.39</td>
<td>14.75 ± 0.66</td>
<td>1.55 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>40.87 ± 7.20</td>
<td>14.49 ± 0.81</td>
<td>1.34 ± 0.18</td>
</tr>
<tr>
<td>10% LC₅₀</td>
<td>F</td>
<td>8</td>
<td>52.02 ± 16.64</td>
<td>15.71 ± 1.31</td>
<td>1.31 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10</td>
<td>61.11 ± 15.40</td>
<td>16.52 ± 1.58</td>
<td>1.32 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>18</td>
<td>57.07 ± 16.16</td>
<td>16.16 ± 1.48</td>
<td>1.32 ± 0.10</td>
</tr>
<tr>
<td>20% LC₅₀</td>
<td>F</td>
<td>10</td>
<td>36.65 ± 4.31</td>
<td>13.72 ± 0.75</td>
<td>1.42 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10</td>
<td>38.88 ± 6.07</td>
<td>14.15 ± 0.63</td>
<td>1.37 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>20</td>
<td>37.77 ± 5.25</td>
<td>13.94 ± 0.71</td>
<td>1.39 ± 0.11</td>
</tr>
<tr>
<td>10% LC₅₀</td>
<td>F</td>
<td>7</td>
<td>51.43 ± 10.70</td>
<td>15.20 ± 0.93</td>
<td>1.45 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>12</td>
<td>56.61 ± 14.41</td>
<td>15.85 ± 1.58</td>
<td>1.41 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>54.70 ± 13.10</td>
<td>15.61 ± 1.38</td>
<td>1.42 ± 0.11</td>
</tr>
<tr>
<td>20% LC₅₀</td>
<td>F</td>
<td>9</td>
<td>55.06 ± 16.40</td>
<td>15.50 ± 1.58</td>
<td>1.45 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10</td>
<td>49.45 ± 12.42</td>
<td>15.06 ± 1.35</td>
<td>1.43 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>52.11 ± 14.35</td>
<td>15.27 ± 1.44</td>
<td>1.44 ± 0.12</td>
</tr>
</tbody>
</table>
6.2.1.5 Statistical analysis

Non-parametric statistical analysis

The Kruskal-Wallis test was preformed on the data obtained from all the exposure groups. After both the female and male CF data sets were ranked according to means in the different groups, the Chi-square test was preformed. Table 6.6 summarizes the results of the Chi-square test.

Table 6.6: Results obtained from the Chi-square test preformed on the CF as calculated for the females (F) males (M) from the different exposure groups.

<table>
<thead>
<tr>
<th></th>
<th>CF F</th>
<th>CF M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-square ($x^2$)</td>
<td>8.341</td>
<td>14.102</td>
</tr>
<tr>
<td>Degrees of freedom (df)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Significant value (p)</td>
<td>0.214</td>
<td>0.029</td>
</tr>
</tbody>
</table>

According to the results obtained from the Chi-square test, there are no significant differences between the CF of the females from the compared groups ($p > 0.005$). The results from the male data sets however points to a difference between the groups ($p \leq 0.005$). Figure 6.1 and Figure 6.2 graphically summarizes the median, quartiles, and extreme values calculated for the CF of the females and males respectively.
Parametric statistical analysis

**Figure 6.1:** Median, quartiles, and extreme values of the calculated condition factor (CF) of female *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant different from the control group as determined by non-parametric as well as parametric statistical tests (× - not significantly different; ✔ - significantly different).
Figure 6.2: Median, quartiles, and extreme values of the calculated condition factor (CF) of male *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant different from the control group as determined by non-parametric as well as parametric statistical tests (× - not significantly different; ✓ - significantly different).
- **Condition factor - Female (CF F)**
  The data set of the exposure groups did not pass the test of homogeneity of variances (Levene statistic), or the robust equality of means (Brown-Forsythe) test. No significant differences were therefore found between the females of the control group and the females of the exposure groups. Figure 6.1 summarizes results regarding the CF of all the females from the exposure groups.

- **Condition factor - Male (CF M)**
  The data set passed the test of homogeneity of variances (Levene statistic). The One-way ANOVA pointed to a significant difference between the data sets \((f(6.86)=2.913; \ p=0.012)\). The Scheffe test of multiple comparisons was then preformed to determine which exposure groups are significantly different from the control group. No significant differences were however found between the control group and the exposure groups. Figure 6.2 summarizes results regarding the CF of all the males from the exposure groups.

**6.2.2 Blood variables**

Table 6.7 list both female and male blood variables obtained for all the exposure groups. Because the frequency between the males and females in each exposure group are highly variable, statistical analysis were only preformed on each exposure group as a whole.

**6.2.2.1 Control specimens**

For the control group of fish, an average haematocrit of \(20.07 \pm 7.46\%\), and an average leucocrit of \(0.71 \pm 0.69\%\) were calculated. An average total plasma protein concentration of \(2.06 \pm 1.57 \text{ g/100 mℓ}\) was calculated (Table 6.7).
Table 6.7: Average blood variables as determined for the control group and exposure group  \( (n - \text{number of specimens}; \ H - \text{haematocrit}; \ L - \text{leucocrit}; \ [TP] - \text{total plasma protein concentration}; \ F - \text{female}; \ M - \text{male}; \ T - \text{total} = \text{male} + \text{female}).

<table>
<thead>
<tr>
<th>Exposure groups</th>
<th>Sex</th>
<th>n</th>
<th>H ± SD (%</th>
<th>L ± SD (%)</th>
<th>[TP] ± SD (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>F</td>
<td>18</td>
<td>21.55 ± 8.56</td>
<td>0.83 ± 0.96</td>
<td>2.17 ± 1.21</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>21</td>
<td>18.67 ± 6.16</td>
<td>0.60 ± 0.21</td>
<td>2.06 ± 1.57</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>39</td>
<td>20.07 ± 7.46</td>
<td>0.71 ± 0.69</td>
<td>2.11 ± 1.39</td>
</tr>
<tr>
<td>Cadmium 10% LC(_{50})</td>
<td>F</td>
<td>5</td>
<td>19.92 ± 3.32</td>
<td>0.30 ± 0.09</td>
<td>2.32 ± 0.90</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>15</td>
<td>20.40 ± 5.64</td>
<td>0.45 ± 0.40</td>
<td>2.48 ± 1.28</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>20</td>
<td>20.28 ± 5.08</td>
<td>0.41 ± 0.35</td>
<td>2.44 ± 1.18</td>
</tr>
<tr>
<td>Cadmium 20% LC(_{50})</td>
<td>F</td>
<td>4</td>
<td>15.54 ± 3.24</td>
<td>0.85 ± 0.12</td>
<td>1.40 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>15</td>
<td>17.19 ± 4.72</td>
<td>0.92 ± 0.68</td>
<td>1.20 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>16.92 ± 4.47</td>
<td>0.91 ± 0.62</td>
<td>1.24 ± 0.30</td>
</tr>
<tr>
<td>Chromium 10% LC(_{50})</td>
<td>F</td>
<td>8</td>
<td>27.78 ± 12.47</td>
<td>0.85 ± 0.51</td>
<td>2.97 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10</td>
<td>21.93 ± 4.91</td>
<td>0.91 ± 0.47</td>
<td>2.97 ± 1.56</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>18</td>
<td>24.34 ± 8.98</td>
<td>0.88 ± 0.47</td>
<td>2.97 ± 1.29</td>
</tr>
<tr>
<td>Chromium 20% LC(_{50})</td>
<td>F</td>
<td>10</td>
<td>21.39 ± 6.25</td>
<td>0.64 ± 0.24</td>
<td>1.79 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10</td>
<td>21.47 ± 9.85</td>
<td>0.67 ± 0.22</td>
<td>1.73 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>20</td>
<td>21.43 ± 7.91</td>
<td>0.65 ± 0.23</td>
<td>1.76 ± 0.57</td>
</tr>
<tr>
<td>Nickel 10% LC(_{50})</td>
<td>F</td>
<td>7</td>
<td>20.31 ± 4.92</td>
<td>0.30 ± 0.07</td>
<td>2.66 ± 0.71</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>12</td>
<td>28.05 ± 7.58</td>
<td>0.32 ± 0.12</td>
<td>3.46 ± 2.99</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>25.20 ± 7.61</td>
<td>0.31 ± 0.11</td>
<td>3.16 ± 2.41</td>
</tr>
<tr>
<td>Nickel 20% LC(_{50})</td>
<td>F</td>
<td>9</td>
<td>22.09 ± 5.27</td>
<td>0.55 ± 0.19</td>
<td>6.10 ± 4.96</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>10</td>
<td>21.27 ± 5.28</td>
<td>0.52 ± 0.12</td>
<td>3.62 ± 3.44</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>19</td>
<td>21.66 ± 5.14</td>
<td>0.54 ± 0.15</td>
<td>4.79 ± 4.30</td>
</tr>
</tbody>
</table>

6.2.2.2 Cadmium specimens

For the 10% LC\(_{50}\) cadmium exposure group an average haematocrit and leucocrit were determined as 20.28 ± 5.08% and 0.41 ± 0.35%,
respectively. An average total plasma protein concentration of 2.44 ± 1.18 g/100 ml was determined for this exposure group (Table 6.7).

An average haematocrit of 16.92 ± 4.47% and leucocrit of 0.91 ± 0.62% was determined for the 20% LC$_{50}$ cadmium exposure group (Table 6.7). An average total plasma protein concentration of 1.24 ± 0.30 g/100 ml was calculated for this exposure group (Table 6.7).

6.2.2.3 Chromium specimens

An average haematocrit of 24.34 ± 8.98% and an average leucocrit of 0.88 ± 0.47% were recorded for the 10% LC$_{50}$ chromium exposure. An average of 2.97 ± 1.29 g/100 ml was calculated for the total plasma protein concentration (Table 6.7).

For the 20% LC$_{50}$ chromium exposure group an average haematocrit and leucocrit were determined as 21.43 ± 7.91% and 0.65 ± 0.23%, respectively. An average total plasma protein concentration of 2.76 ± 0.57 g/100 ml was calculated for this exposure group (Table 6.7).

6.2.2.4 Nickel specimens

For the 10% LC$_{50}$ nickel exposure group an average haematocrit and leucocrit were determined as 25.20 ± 7.61% and 0.31 ± 0.11%, respectively. An average total protein concentration of 3.16 ± 2.41 g/100 ml was calculated for this exposure group as a whole (Table 6.7).

An average haematocrit of 21.66 ± 5.14% and leucocrit of 0.54 ± 0.15% was determined for the 20% LC$_{50}$ cadmium exposure group. An average total protein concentration of 4.79 ± 4.30 g/100 ml was determined for this exposure group as a whole (Table 6.7).
6.2.2.5 Statistical analysis

Non-parametric statistical analysis

The Kruskal-Wallis test was performed on the data obtained from all the exposure groups. All data sets were ranked according to the means of the different groups. The Chi-square test was performed on the ranked data. Table 6.8 summarizes the results obtained from the Chi-square test.

Table 6.8: Results obtained from the Chi-square test performed on the blood variables of the different exposure groups. (H - haematocrit; L - leucocrit; [TP] - total plasma protein concentration).

<table>
<thead>
<tr>
<th></th>
<th>H</th>
<th>L</th>
<th>[TP]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-square ($x^2$)</td>
<td>16.520</td>
<td>53.267</td>
<td>48.554</td>
</tr>
<tr>
<td>Degrees of freedom (df)</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Significant value ($p$)</td>
<td>0.011</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

According to the results obtained from the Chi-square test, there are significant differences ($p \leq 0.005$) between the exposure groups for the haematocrit, leucocrit as well as the total plasma protein concentration.
Parametric statistical test

Figure 6.3: Median, quartiles, and extreme values of the calculated haematocrit (Hct) of *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (× - not significantly different; ✓ - significantly different).
Figure 6.4: Median, quartiles, and extreme values of the calculated leucocrit (L) of *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (* - not significantly different; ✓ - significantly different).
Figure 6.5: Median, quartiles, and extreme values of the calculated total plasma protein concentration ([TP]) of *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (× - not significantly different; ✓ - significantly different).
• **Haematocrit (Hct)**
  The data sets passed the test of homogeneity of variances (Levene statistic). The One-way ANOVA pointed to a significant difference between the data sets ($f(6,142) = 3.052; p = 0.008$). The Scheffe test of multiple comparisons was then performed to determine which exposure groups are significantly different from the control group. No significant differences were however found between the control group and the exposure groups. Figure 6.3 graphically summarizes the median, quartiles, and extreme values as determined for the haematocrit from the different exposure groups.

• **Leucocrit (L)**
  The data sets passed the test of homogeneity of variances (Levene statistic). Results obtained from the One-way ANOVA, pointed to a significant difference between the data sets ($f(6.142) = 4.343; p = 0.000$). The Scheffe test of multiple comparisons was then performed to determine which exposure groups are significantly different from the control group. No significant differences were however found between the control group and the exposure groups. Figure 6.4 graphically summarizes the median, quartiles, and extreme values as determined for the leucocrit from the different exposure groups.

• **Total plasma protein concentration ([TP])**
  The data sets violated the test of homogeneity of variances (Levene statistic). The data sets were then subjected to the robust quality of means (Brown-Forsythe) test, which hinted at a difference between the data sets. The Dunnet T3 test was then performed to determine which exposure groups are significantly different from the control group. 20% LC$_{50}$ cadmium exposure was found to be significantly different from the control group. Figure 6.5 graphically summarizes the median, quartiles, and extreme values as determined for the total plasma proteins from the different exposure groups.
6.3 Necropsy based fish health assessment index (NFHAI)

All the fish from all the exposure groups was examined externally and internally. Alterations to the internal and external structures as well as alterations to the expected blood parameters were graded according to criteria stipulated in Table 5.3. Table 6.9 lists the average NFHAI scores determined for the different variables examined. Because the frequency of males and females in each exposure group are highly variable, statistical analysis were only preformed on the exposure group as a whole.

6.3.1 Control specimens

An external investigation of each specimen was executed and it was found that all control specimens were in a state of good health in terms of the condition of the fins, eyes, mouth, scales, opercula and behaviour, as well as their general external appearance. No structural abnormalities were recorded regarding the condition of the major visceral organs (including the spleen and kidney) of any of the specimens. No inflammation of the hindgut was found in any of the specimens. The group was also free of external or internal parasitic infections. No mortalities occurred within the specified exposure times.

Some deviation from the reddish to coffee brown liver colour specified as normal by NFHAI was seen in 33 specimens (84.6% of the control group). These livers were classified as discoloured and scored a 30 on the NFHAI scoring system.

Of the 39 haematocrit reading obtained for the control group, 15 (38.46%) specimens haematocrit fell below 18% and scored a 30 on the NFHAI score. A further 21 (53.8%) specimens haematocrit readings fell between 19% and 29% and scored a 20 on the NFHAI score. Only 3 (7.7%) specimens
haematocrit fell within the normal specified range of 30% to 45%. All of the 39 leucocrit readings determined for the control group fell within the normal accepted range of below 4%. Blood for total plasma protein analysis could not be collected from two specimens. All 37 (94.9%) remaining specimens total plasma protein concentration determined fell between 3 and 6 g/100 ml, and scored 30 on the NFHAI scoring system.

Calculated NFHAI ranged from 50 to 90 between individual specimens. An average of 73.08 ± 15.46 was determined for the control group (Table 6.9). The bile of all fish showed differences in colour, ranging from straw yellow to a dark green, and it also ranged from being empty to being full. The percentage of cecum covered by mesenteric fat ranged from no mesenteric fat present to more than 5% cecum coverage.

6.3.2 Cadmium exposure groups

10% LC₅₀ cadmium exposure

After an external investigation was performed on each specimen it was found that macroscopically all specimens were in a state of good health in terms of the condition of the fins, eyes, mouth, scales and behaviour, as well as their general external appearance. No structural abnormalities were recorded regarding the condition of the major visceral organs of any of the specimens, and no inflammation of the hindgut was noted. The group was free of external or internal parasitic infections. No mortalities occurred within the specified exposure times.

Of the 20 specimens, 14 (70%) specimens showed a deviation from the normal reddish to coffee brown colour accepted as normal by the NFHAI. These livers were classified as discoloured and scored 30 on the NFHAI scoring system.
Of the 20 haematocrit readings obtained for the 10% LC$_{50}$ cadmium exposure group, 7 (35.0%) specimens haematocrit fell below 18% and scored a 30 on the NFHAI score. A further 12 (60.0%) specimens haematocrit readings fell between 19% and 29% and scored a 20 on the NFHAI score. Only 1 specimen’s haematocrit fell within the normal specified range of 30% to 45%. All 20 of the 20 (100%) leucocrit readings determined for the control group fell within the normal accepted ranges. Total plasma protein concentration analysis revealed that 14 (70.0%) specimens total plasma protein concentration fell between 3 and 6 g/100 ml. These 14 specimens therefore scored 30 on the NFHAI scoring system. The remaining 6 (30%) specimens total plasma protein concentration fell within the normal acceptable range.

Bile colour ranged from straw coloured bile to dark green, and showed variant degrees of fullness. The percentage of the cecum covered by mesenteric fat also showed great variance from no fat coverage to less than 50% fat coverage. Calculated NFHAI varied from 50 to 90 between individual specimens. An average of 64.5 ± 13.6 was determined for the control group (Table 6.9).

**20% LC$_{50}$ cadmium exposure**

After an external investigation of all 19 specimens was executed it was found that all specimens were in a state of good health in terms of the macroscopic condition of the fins, eyes, mouth, scales and behaviour, as well as their general external appearance. No structural abnormalities were recorded regarding the condition of the major visceral organs of the specimens. No external or internal parasitic infections were observed, and no mortalities occurred within the specified exposure times.
All of the examined fish from this exposure showed a deviation from the normal reddish to coffee brown colour. All the examined livers were therefore classified as discoloured and scored a 30 on the NFHAI scale.

Blood for haematocrit analysis could not be collected from 1 specimen. For this exposure group, 10 (52.6%) specimens haematocrit fell below 18% and scored a 30 on the NFHAI score. The remaining 8 (42.1%) specimens haematocrit readings fell between 19% and 29% and scored a 20 on the NFHAI score. All 20 of the 20 (100%) leucocrit readings determined for the control group fell within the normal accepted ranges. All 19 (100%) specimens from this exposure group total plasma protein concentration fell between 3 and 6 g/100 ml, and therefore scored a 30 on the NFHAI scale.

Bile colour showed great deviation between the specimens from straw coloured bile to dark green bile. Specimens showed great deviation in the amount of mesenteric fat present. Calculated NFHAI varied from 50 to 90 between individual specimens. An average of 85.6 ± 5.1 was determined for the control group (Table 6.9).

### 6.3.3 Chromium exposure groups

**10% LC50 chromium exposure group**

An external investigation of each specimen was executed and it was found that all specimens were in a state of good health in terms of the condition of the fins, eyes, mouth, scales, opercula and behaviour, as well as their general external appearance. No structural abnormalities were recorded regarding the condition of the major visceral organs of the specimens. The group was also free of any external or internal parasitic infections. No mortalities occurred within the specified exposure times.
All of the examined livers from this exposure group deviated from the reddish to coffee brown colour specified to be normal by NFHAI. These livers were classified as discoloured according to the NFHAI criteria, and scored a 30 on the scoring system.

No blood was collected from 2 specimens. Of the 16 haematocrit readings obtained for this exposure group, 3 (18.8%) specimens haematocrit fell below 18% and scored a 30 on the NFHAI score. A further 10 (62.5%) specimens haematocrit readings fell between 19% and 29% and scored a 20 on the NFHAI score. Only 1 specimen’s haematocrit reading fell below 45% and can therefore be considered to be normal. All leucocrit readings fell within the normal accepted ranges. Total plasma protein analysis done on the 16 specimens. The total plasma protein concentration of 11 (68.8%) specimens fell between 3 and 6 g/100 ml, and scored 30 on the NFHAI scoring system. The remaining 2 (12.5%) specimens total plasma protein fell within the normal accepted range and scored 0 on the NFHAI scoring system.

Bile colour ranged from straw coloured bile to dark green, and showed variant degrees of fullness. The percentage of the cecum covered by mesenteric fat also showed great variance, from no fat coverage to less than 50% fat coverage. An average NFHAI of 71.0 ± 14.0 was determined for this exposure group (Table 6.9).

**LC\textsubscript{50} chromium 20% exposure group**

After an external investigation of each specimen was it found that all specimens were in a state of good health in terms of the macroscopic condition of the fins, eyes, mouth, scales, opercula and behaviour, as well as their general external appearance. No structural abnormalities were recorded regarding the condition of the major visceral organs of the specimens. This exposure group was also free of external or internal...
parasitic infections. No mortalities occurred within the specified exposure times.

Of the 20 liver examined for this exposure group, 19 (95.0%) of the livers deviation from the normal reddish to coffee brown colour specified by NFHAI. These livers were classified as discoloured according to the NFHAI criteria, and scored a 30 on the scoring system.

No blood was collected from 1 specimen. The remaining 19 (10%) specimens total plasma protein concentration all fell between 3 and 6 g/100 ml, and scored 30 on the NFHAI scoring system. Of the 19 haematocrit readings obtained for this exposure group, 8 (52.6%) specimens haematocrit fell below 18% and scored a 30 on the NFHAI score. A further 10 (62.5%) specimens haematocrit readings fell between 19% and 29% and scored a 20 on the NFHAI score, and 1 specimens haematocrit was normal. All leucocrit readings fell within the normal accepted ranges.

Bile colour showed great deviation between the specimens ranging from straw coloured bile to dark green bile. Specimens showed great deviation in the amount of mesenteric fat present. An average NFHAI of 81.7 ± 9.3 was determined for this exposure group (Table 6.9).

6.3.4 Nickel exposure groups

LC\textsubscript{50} nickel 10% exposure group

After an external investigation of all 19 specimens was executed, all specimens were found to be in a state of good health in terms of the condition of the fins, eyes, mouth, scales and behaviour, as well as their general external appearance. No structural abnormalities were recorded regarding the condition of the major visceral organs of any of the specimens.
The group was also free of any external or internal parasitic infections. No mortalities occurred within the specified exposure times.

All 19 of the 19 livers examined for this exposure group, deviated from the normal reddish to coffee brown colour specified by NFHAI. All the livers for this exposure group were classified as discoloured according to the NFHAI criteria, and scored a 30 on the scoring system.

Of the 19 haematocrit readings obtained for this exposure group, 5 (26.3%) specimens haematocrit fell below 18% and scored a 30 on the NFHAI score. A further 10 (52.6%) specimens haematocrit readings fell between 19% and 29% and scored a 20 on the NFHAI score, and 4 (21.1%) specimens haematocrit readings fell below 45% and scored a 0 on the NFHAI score. All 19 (100%) leucocrit readings fell within the normal accepted ranges. The total plasma protein concentration of 16 (84.2%) specimens fell between 3 and 6 g/100 ml, and scored 30 on the NFHAI scoring system, 2 (10.5%) specimens total plasma protein fell within the normal acceptable range. A total plasma protein concentration below 3 g/100 ml was recorded for 1 specimen, which resulted in a score value of 10 according to the NFHAI criteria.

Bile colour ranged from straw coloured bile to dark green, and showed variant degrees of fullness. The percentage of the cecum covered by mesenteric fat also showed great variance, from no fat coverage to less than 50% fat coverage. An average NFHAI of 71.0 ± 14.0 was determined for this exposure group (Table 6.9).

**LC\(_{50}\) nickel 20% exposure group**

After an external investigation of all 19 specimens was executed, it was found that all specimens were in a state of good health in terms of the condition of the fins, eyes, mouth, scales and behaviour, as well as their
general external appearance. No structural abnormalities were recorded regarding the condition of the major visceral organs of any of the specimens. The group was also free of any external or internal parasitic infections. No mortalities occurred within the specified exposure times.

All of the 19 livers examined for this exposure group, deviated from the normal reddish to coffee brown colour specified by NFHAI. All the livers for this exposure group were classified as discoloured according to the NFHAI criteria, and scored a 30 on the scoring system.

Of the 19 haematocrit readings obtained for this exposure group, 7 (36.8%) specimens haematocrit fell below 18% and scored a 30 on the NFHAI score. A further 10 specimens (52.6%) haematocrit readings fell between 19% and 29% and scored a 20 on the NFHAI score, and 2 (10.5%) specimens haematocrit readings fell below 45% and scored a 0 on the NFHAI score. All leucocrit readings fell within the normal accepted ranges. The total plasma protein concentration of 10 (52.6%) specimens fell between 3 and 6 g/100 mℓ, and scored 30 on the NFHAI scoring system, 5 (26.3%) specimens total plasma protein fell within the normal acceptable range. A total plasma protein concentration below 3 g/100 mℓ was recorded for 4 (21.1%) specimens, and thus scored a 10 according to the NFHAI criteria.

Bile colour ranged from straw coloured bile to dark green, and showed variant degrees of fullness. The percentage of the cecum covered by mesenteric fat also showed great variance, from no fat coverage to less than 50% fat coverage. An average NFHAI of 71.0 ± 14.0 was determined for this exposure group (Table 6.9).
Table 6.9: Average NFHAI scores for the different variables examined during the necropsy (NFHAI - necropsy based fish health assessment index; n - number of specimens; SD - standard deviation; Hct - haematocrit; L - leucocrit; [TP] - total plasma protein concentration).

<table>
<thead>
<tr>
<th>NFHAI variables</th>
<th>Control</th>
<th>Cadmium</th>
<th>Chromium</th>
<th>Nickel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10% LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>20% LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>10% LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>20% LC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>n</td>
<td>39</td>
<td>20</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Eyes ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Skin ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Gills ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Opercula ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Liver ± SD</td>
<td>26.0 ± 11.0</td>
<td>21.0 ± 14.1</td>
<td>30.0 ± 0.0</td>
<td>30.0 ± 0.0</td>
</tr>
<tr>
<td>Spleen ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Kidney ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Hindgut ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Parasites ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Hct ± SD</td>
<td>23.0 ± 8.1</td>
<td>23.0 ± 7.2</td>
<td>26.0 ± 5.1</td>
<td>18.8 ± 8.9</td>
</tr>
<tr>
<td>L ± SD</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>[TP] ± SD</td>
<td>26.0 ± 10.3</td>
<td>21.0 ± 14.1</td>
<td>30.0 ± 0.0</td>
<td>19.0 ± 14.8</td>
</tr>
<tr>
<td>NFHAI ± SD</td>
<td>73.1 ± 15.5</td>
<td>64.5 ± 13.6</td>
<td>85.6 ± 5.1</td>
<td>71.0 ± 14.0</td>
</tr>
</tbody>
</table>
6.3.5 **Statistical analysis**

**Non-parametric statistical analysis**

The Kruskal-Wallis test was preformed on the data obtained from all the exposure groups. All data sets were ranked according to the means of the different groups. The Chi-square test was preformed on the ranked data. Table 6.10 summarizes the results obtained from the Chi-square test.

*Table 6.10*: Results obtained from the Chi-square test preformed data obtained from the NFHAI of the different exposure groups.

<table>
<thead>
<tr>
<th><strong>Chi-square (x^2)</strong></th>
<th><strong>NFHAI</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28.38</td>
</tr>
<tr>
<td><strong>Degrees of freedom (df)</strong></td>
<td>6</td>
</tr>
<tr>
<td><strong>Significant value (p)</strong></td>
<td>0.000</td>
</tr>
</tbody>
</table>

According to the results obtained from the Chi-square test, there are significant differences \( p \leq 0.05 \) between the calculated NFHAI values of the different exposure groups.

**Parametric statistical tests**

The data set for the NFHAI violated the test of homogeneity of variances (Levene statistic). The data set were then subjected to the robust quality of means (Brown-Forsythe) test, which hinted at a difference between the data sets. The Brown-Forsythe test was then followed by the Dunnet T3 test to determine which exposure groups are significantly different from the control group. 20% LC\(_{50}\) cadmium exposure was found to be significantly different from the control group.
Figure 6.6: Median, quartiles, and extreme values of the calculated necropsy based fish health assessment index (NFHAI) of *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (× - not significantly different; ✓ - significantly different).
6.4 Somatic indices

As discussed in section 5.5.2 the organo-somatic indices calculated for this study includes the hepato-somatic index (HSI), spleno-somatic index (SSI), gonadal somatic index (GSI) as well as the fat-tissue index (FTI). Table 6.11 summarizes the average values of the organo-somatic indices as calculated for the exposure groups.

6.4.1 Control specimens

For the 39 fish from the control group, an average liver weight of 0.67 ± 0.26 g and an average spleen weight of 0.09 ± 0.05 g were recorded. An average HSI of 1.34 ± 0.36% and an SSI of 0.19 ± 0.08% was calculated. For the 19 females from this group, an average body weight of 42.56 ± 11.46 g were recorded, and an average GSI of 2.76 ± 1.70% was calculated. The average weight recorded for the 21 males were 50.06 ± 14.75 g and an average GSI of 0.51 ± 0.27% was calculated (Table 6.11).

6.4.2 Cadmium specimens

For the 10% LC₅₀ cadmium exposure group an average liver weight and spleen weight of 0.46 ± 0.12 g and 0.11 ± 0.03 g, was recorded. An average HSI of 0.87 ± 0.22% and SSI of 0.20 ± 0.06% was calculated for this exposure group. An average body weight of 49.61 ± 9.34 g was recorded for the 5 females from this group, and an average body weight of 54.80 ± 8.69 g was recorded for the 15 males. An average GSI of 1.95 ± 1.35% was calculated for the females, and 0.43 ± 0.19% was calculated for the males (Table 6.11).

For the 20% LC₅₀ cadmium exposure group, an average liver weight of 0.42 ± 0.10 g and an average spleen weight of 0.10 ± 0.14 g were recorded. An
average HSI of 1.05 ± 0.21% and an SSI of 0.25 ± 0.36% was calculated. As summarized in Table 6.11, an average body weight of 38.77 ± 10.63 g were recorded, and an average GSI of 0.71 ± 0.26% was calculated for the 4 females. The average weight recorded for the 15 males were 41.43 ± 6.39 g and an average GSI of 0.80 ± 0.91% was calculated (Table 6.11).

6.4.3 Chromium specimens

As summarized in Table 6.11, an average liver and spleen weight of 0.83 ± 0.21 g and 0.11 ± 0.05 g, was respectively recorded for the 10% chromium exposure group. An average HSI of 1.51 ± 0.38% and SSI of 0.20 ± 0.05% was calculated for this exposure group. An average body weight of 52.02 ± 16.64 g was recorded for the 8 females from this group, and an average body weight of 61.11 ± 15.40 g was recorded for the 10 males. An average GSI of 3.39 ± 1.61% was calculated for the females, and 0.76 ± 0.53% was calculated for the males.

For the 20% LC$_{50}$ chromium exposure group, an average liver weight of 0.47 ± 0.13 g and an average spleen weight of 0.08 ± 0.03 g were recorded. An average HSI of 1.24 ± 0.24% and an SSI of 0.21 ± 0.07% was calculated. For the 10 females an average body weight of 36.65 ± 4.31 g were recorded, and an average GSI of 3.85 ± 1.34% was calculated. The average weight recorded for the 10 males were 38.88 ± 6.07 g and an average GSI of 0.77 ± 0.23 was calculated (Table 6.11).

6.4.4 Nickel specimens

For the 10% LC$_{50}$ nickel exposure group an average liver weight and spleen weight of 1.01 ± 0.35 % and 0.11 ± 0.04%, was recorded. An average HSI of 1.88 ± 0.54% and SSI of 0.21 ± 0.07% was calculated for this exposure group. An average body weight of 51.43 ± 10.70 g was recorded for the 7 females from this group, and an average body weight of 56.61 ± 14.41 g
was recorded for the 12 males. An average GSI of $1.28 \pm 1.10\%$ was calculated for the females, and $0.39 \pm 0.32\%$ was calculated for the males (Table 6.11).

For the 20% LC$_{50}$ nickel exposure group, an average liver weight of $52.11 \pm 14.35$ g and an average spleen weight of $0.11 \pm 0.04$ g were recorded. An average HSI of $1.69 \pm 0.46\%$ and an SSI of $0.22 \pm 0.07\%$ was calculated. An average body weight of $55.06 \pm 16.46$ was recorded for the 9 females from this group, and an average body weight of $49.45 \pm 12.42$ was recorded for the 10 males. An average GSI of $0.81 \pm 0.84\%$ was calculated for the females, and $0.34 \pm 0.13\%$ was calculated for the males (Table 6.11).
### Table 6.11: Average organo-somatic indices calculated for the exposure groups (HSI - hepato-somatic index; SSI - spleno-somatic index, GSI - gonadal somatic index; FTI - fat-tissue index).

<table>
<thead>
<tr>
<th>Exposure groups</th>
<th>Sex</th>
<th>n</th>
<th>Total weight ± SD (g)</th>
<th>Liver weight ± SD (g)</th>
<th>HSI ± SD</th>
<th>Spleen weight ± SD (g)</th>
<th>SSI ± SD</th>
<th>Gonad weight ± SD (g)</th>
<th>GSI ± SD</th>
<th>Fat ± SD (g)</th>
<th>FTI ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>F</td>
<td>18</td>
<td>42.56 ± 11.46</td>
<td>0.66 ± 0.25</td>
<td>1.52 ± 0.40</td>
<td>0.07 ± 0.03</td>
<td>0.17 ± 0.06</td>
<td>0.58 ± 0.39</td>
<td>2.76 ± 1.70</td>
<td>0.31 ± 0.51</td>
<td>0.60 ± 0.92</td>
</tr>
<tr>
<td>M</td>
<td>21</td>
<td></td>
<td>50.06 ± 14.57</td>
<td>0.68 ± 0.27</td>
<td>1.35 ± 0.32</td>
<td>0.10 ± 0.06</td>
<td>0.20 ± 0.09</td>
<td>0.13 ± 0.08</td>
<td>0.51 ± 0.27</td>
<td>0.41 ± 0.55</td>
<td>0.71 ± 0.77</td>
</tr>
<tr>
<td>T</td>
<td>39</td>
<td></td>
<td>46.60 ± 13.60</td>
<td>0.67 ± 0.26</td>
<td>1.43 ± 0.36</td>
<td>0.09 ± 0.05</td>
<td>0.19 ± 0.08</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

| **Cadmium**     | F   | 5  | 49.61 ± 9.34          | 0.45 ± 0.12           | 0.91 ± 0.11 | 0.11 ± 0.03            | 0.21 ± 0.02  | 0.45 ± 0.28          | 1.95 ± 1.35  | 0.13 ± 0.11  | 0.24 ± 0.18 |
| M               | 15  |    | 54.80 ± 8.69          | 0.46 ± 0.13           | 0.85 ± 0.24 | 0.11 ± 0.04            | 0.20 ± 0.07  | 0.11 ± 0.06          | 0.43 ± 0.19  | 0.14 ± 0.15  | 0.26 ± 0.29 |
| T               | 20  |    | 53.5 ± 8.91           | 0.46 ± 0.12           | 0.87 ± 0.22 | 0.11 ± 0.03            | 0.20 ± 0.06  |                     | -         | -            | -       |

| **Chromium**    | F   | 8  | 52.02 ± 16.64         | 0.81 ± 0.17           | 1.61 ± 0.35 | 0.09 ± 0.03            | 0.18 ± 0.05  | 0.78 ± 0.32          | 3.39 ± 1.61  | 0.38 ± 0.52  | 0.74 ± 1.13 |
| M               | 10  |    | 61.11 ± 15.40         | 0.84 ± 0.25           | 1.43 ± 0.41 | 0.13 ± 0.05            | 0.21 ± 0.05  | 0.71 ± 0.26          | 3.77 ± 1.53  | 0.06 ± 0.00  | 0.20 ± 0.00 |
| T               | 18  |    | 57.07 ± 16.16         | 0.83 ± 0.21           | 1.51 ± 0.38 | 0.11 ± 0.05            | 0.20 ± 0.05  |                     | -         | -            | -       |

| **Nickel**      | F   | 7  | 51.43 ± 10.70         | 1.03 ± 0.24           | 2.01 ± 0.26 | 0.09 ± 0.03            | 0.19 ± 0.06  | 0.31 ± 0.25          | 1.28 ± 1.10  | 0.85 ± 0.53  | 1.67 ± 0.95 |
| M               | 12  |    | 56.61 ± 14.41         | 1.01 ± 0.41           | 1.80 ± 0.65 | 0.13 ± 0.04            | 0.23 ± 0.07  | 0.12 ± 0.12          | 0.39 ± 0.32  | 0.61 ± 0.50  | 1.07 ± 0.75 |
| T               | 19  |    | 54.70 ± 13.10         | 1.01 ± 0.35           | 1.88 ± 0.54 | 0.11 ± 0.04            | 0.21 ± 0.07  |                     | -         | -            | -       |

**Results**
6.4.5 Statistical analysis

Figures 6.7, 6.8, 6.9, 6.10 and 6.11 graphically summarizes the median, quartiles, and extreme values calculated for the HSI, SSI, GSI (male and female), and the FTI respectively. Matrixes denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests.

Non-parametric statistical analysis

The Kruskal-Wallis test was preformed on the data obtained from all the exposure groups. All data sets were ranked according to the means of the different groups. The Chi-square test was preformed on the ranked data. Table 6.12 summarizes the results obtained from the Chi-square test.

Table 6.12: Results obtained from the Chi-square test preformed on the organosomatic indices determined for the different exposure groups (HSI - hepato-somatic index; SSI - spleno-somatic index; GSI - gonadal-somatic index; F - female; M - male; FTI - fat tissue index).

<table>
<thead>
<tr>
<th></th>
<th>HSI</th>
<th>SSI</th>
<th>GSI F</th>
<th>GSI M</th>
<th>FTI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-square ($x^2$)</td>
<td>68.103</td>
<td>13.686</td>
<td>20.437</td>
<td>26.953</td>
<td>28.569</td>
</tr>
<tr>
<td>Degrees of freedom (df)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Significant value (p)</td>
<td>0.000</td>
<td>0.033</td>
<td>0.002</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

According to the p value obtained from the Chi-square test, there are significant differences ($p \leq 0.05$) between the all the exposure groups (including the control group) for all the somatic indices used during this study.
Parametric statistical analysis

- **Hepato-somatic index (HSI)**
  The data set for the HSI violated the test of homogeneity of variances (Levene statistic). The data set was then subjected to the robust quality of means (Brown-Forsythe) test, which hinted at a difference between the data sets. The Brown-Forsythe test was then followed by the Dunnet T3 test to determine which exposure groups are significantly different from the control group. A statistical significant difference was observed between the control group and the 10% LC$_{50}$ cadmium exposure as well as the control group and the 20% LC$_{50}$ cadmium exposure group (Figure 6.7).

- **Spleno-somatic index (SSI)**
  The data set for the SSI violated the test of homogeneity of variances (Levene statistic). The data set also failed the robust quality of means (Brown-Forsythe) test. There were no significant difference between the control group and the different exposure groups (Figure 6.8).

- **Gonadal-somatic index - Female (GSI F)**
  The data sets passed the test of homogeneity of variances (Levene statistic). The One-way ANOVA pointed to a significant difference between the data sets ($f(6, 54) = 4.009; p = 0.002$). The Scheffe test of multiple comparisons was then performed to determine which exposure groups are significantly different from the control group. No significant differences were however found between the control group and the exposure groups (Figure 6.9).

- **Gonadal-somatic index - Male (GSI M)**
  The data set for the HSI violated the test of homogeneity of variances (Levene statistic). The data set was then subjected to the robust quality of means (Brown-Forsythe) test, which hinted at a difference between the data sets. The Brown-Forsythe test was then followed by the Dunnet T3 test to determine which exposure groups are significantly different from the control
group. No significant differences were found between the control group and the exposure groups (Figure 6.10).

Figure 6.7: Median, quartiles, and extreme values of the calculated hepato-somatic index (HSI) of *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (× - not significantly different; ✓ - significantly different).
Figure 6.8: Median, quartiles, and extreme values of the calculated total spleno-somatic index (SSI) of *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (× - not significantly different; ✓ - significantly different).
Figure 6.9: Median, quartiles, and extreme values of the calculated gonadal-somatic index (GSI) of female *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (× - not significantly different; ✓ - significantly different).
Figure 6.10: Median, quartiles, and extreme values of the calculated gonadal-somatic index (GSI) of male *O. mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences as determined by non-parametric as well as parametric statistical tests. (× - not significantly different; ✓ - significantly different).
Fat tissue index (FTI)

The data set for the FTI violated the test of homogeneity of variances (Levene statistic). The data set was then subjected to the robust quality of means (Brown-Forsythe) test, which hinted at a difference between the data sets. The Brown-Forsythe test was then followed by the Dunnet T3 test to determine which exposure groups are significantly different from the control group. No significant differences were found between the control group and the exposure groups.
Figure 6.11: Median, quartiles, and extreme values of the calculated fat tissue index (FTI) of *Oreochromis mossambicus* after short-term exposure to various metals. Matrices denote exposure groups with statistically significant differences. (× - not significantly different; ✓ - significantly different).
6.5 Histological assessment

The following section presents both qualitative and quantitative histological results. Representative micro slides of the histological changes found in all the exposure groups, are listed in section 6.5.6. Section 6.5.7 presents the results obtained from the gonad maturity index.

6.5.1 Control group

6.5.1.1 Qualitative assessment

During the histological analysis of the 39 livers of the control fish, the following was observed:

The liver histology of the 39 control specimens appeared relatively normal and was evaluated in terms of the criteria stipulated in histopathological assessment protocol (section 5.6.1). However, the normal structure of the liver was not clearly visible in 9 (23.1%) specimens, and mild congestion of blood vessels and sinusoids was visible in 2 (5.2%) of the control specimens. Cell membranes of individual cells were clearly noticeable. Granular degradation of the hepatocytes was identified in 37 (94.9%) specimens, and fat accumulation was visible in 11 (28.2%) specimens. Intercellular deposits were found in 19 (48.7%) specimens. Varying degrees of nuclear alterations to the hepatocytes nuclei were identified in 29 (74.36%) specimens. No alteration to the interstitial tissues was identified. An increase in connective tissue around blood vessels was however identified for 1 specimen. In general all the examined bile ducts were in a good condition. Necrosis of 1 bile duct was recorded, and 3 (7.7%) specimens showed nuclear alterations. An increase in the amount of connective tissue around blood vessels was identified in 11 (28.2%) specimens.
During the histological analysis of the 39 gills of the control fish, the following was observed:

No structural alterations were of the primary or secondary lamella was found in any of the 39 specimens of the control group. No congestion of blood vessels was identified. The epithelium of the primary lamella of 31 (79.5%) specimens was vacuolated, and inter cellular deposits were identified in 32 (82.1%) specimens. Mild hyperplasia of the secondary gill epithelia was identified in 37 (94.9%) specimens.

During the histological analysis of the 18 ovaries of the control fish, the following was observed:

Oogenesis was not inhibited and all stages of ova development could be identified in all 18 specimens. No structural alterations to the ovaries, oogonia and oocytes were identified. Deposits in the interstitial tissue were recorded for 12 (66.7%) specimens.

During the histological analysis of the 21 testis of the control fish, the following was observed:

The basal membrane of all 21 specimens was intact, and all specimens retained lobular organization. No signs of inhibition of spermatogenesis were detected in any of the examined specimens; all stages of spermatogenesis were represented. No histological changes were noted to the interstitial tissue, spermatids or spermatozoa. Vacuolation of the spermatogonia of 17 (81.0%) specimens were identified, as well as hypertrophy of spermatocytes of all 21 (100%) specimens. No inflammation or tumours were identified.

The control specimen’s histological characteristics were considered as normal.
6.5.1.2 Quantitative assessment

Table 6.13 summarizes the quantitative results obtained from the quantitative histopathological assessment tool (discussed in detail in section 5.6.1.2). The reaction index \( I_{\text{org } rp} \) gives an indication of the quality of the lesions in the specific organ, and the organ index \( I_{\text{org}} \) gives an indication to the degree of damage. The results obtained from the quantitative analysis of the liver signify circulatory disturbances (CD), regressive changes (RC), progressive changes (PC) as well as inflammation (I). An organ index of 15.2 was calculated for the liver. Only regressive (RC) and progressive changes (PC) were identified in the gills, resulting in a final organ index value of 11.2. Only regressive changes (RC) were recorded for the ovaries from the control group, which resulted in an organ index of 1.6. Regressive and progressive changes were found within the testis of the control group. Organ index of 13.8 was calculated for the testis. A total organ index of 41.8 was calculated for the control group.

**Table 6.13:** Summary of the reaction index \( I_{\text{org } rp} \), organ index \( I_{\text{org}} \), total reaction index \( I_{rp} \) and the total index \( \text{Tot-I} \) of the selected organs as calculated for the control group (CD-circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour).

<table>
<thead>
<tr>
<th>Rp</th>
<th>Gills</th>
<th>Liver</th>
<th>Ovary</th>
<th>Testis</th>
<th>( I_{rp} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( I_{\text{org } rp} )</td>
<td>( I_{\text{org } rp} )</td>
<td>( I_{\text{org } rp} )</td>
<td>( I_{\text{org } rp} )</td>
<td></td>
</tr>
<tr>
<td>CD</td>
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<td>0.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.4</td>
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<td>RC</td>
<td>4.8</td>
<td>13.5</td>
<td>1.6</td>
<td>10.5</td>
<td>30.4</td>
</tr>
<tr>
<td>PC</td>
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<td>1.2</td>
<td>0.0</td>
<td>3.3</td>
<td>11.0</td>
</tr>
<tr>
<td>I</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>T</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>( I_{\text{org}} )</td>
<td>11.2</td>
<td>15.2</td>
<td>1.6</td>
<td>13.8</td>
<td>( \text{Tot-I} ) 41.8</td>
</tr>
</tbody>
</table>
6.5.2 Cadmium exposure

6.5.2.1 Qualitative assessment

During the histological analysis of the 20 livers from the 10% LC\textsubscript{50} cadmium exposure group, the following was observed:
The normal structure of the liver was lost in 10 (50.0%), and congestion of blood vessels and sinusoids was visible in 5 (25.0%) specimens. Granular degradation of the hepatocytes was identified in 19 (95.0%) specimens, and fat accumulation was visible in 3 (15.0%) specimens. Intercellular deposits were found in 2 (10.0%) specimens. An increase in macrophage centres (MC) was apparent in 9 (45%) specimens. Varying degrees of nuclear alterations to the hepatocyte nuclei were identified in 11 (55%) specimens. Hyperplasia of the hepatocytes was identified in 3 (15%) specimens, and only 1 specimen showed signs of necrosis. Infiltration by was identified in 2 (10%) specimens. No alteration to the interstitial tissues or bile ducts was identified.

During the histological analysis of the 19 livers from the 20% LC\textsubscript{50} cadmium exposure group, the following was observed:
The normal structure of the liver was lost in 8 (42.1%). No congestion of blood vessels and sinusoids was visible. Granular degradation of the hepatocytes was identified in all 19 (100%) specimens, and fat accumulation was visible in 5 (26.3%) specimens. Intercellular deposits were found in 7 (36.8%) specimens. Hepatocytes showed varying degrees of nuclear alterations in 12 (63.2%) specimens. Nuclear alterations were also identified in the biliary epithelium of 3 (15.8%) specimens. Hypertrophy of the hepatocytes was identified in 7 (36.8%) specimens. Mild inflammation was identified in 2 (10.5%) specimens.
During the histological analysis of the 20 gills from the 10% LC$_{50}$ cadmium exposure group, the following was observed:
No congestion of blood vessels was identified. The general structure of the gill lamellae was lost in 8 (40.0%) specimens. Vacuolation of the primary lamellar epithelium was identified in 12 (60.0%) specimens. Inter cellular deposits were identified in 18 (90.0%) specimens. Hyperplasia of the primary and secondary gill epithelia was identified in all the specimens. Enlargement of mucus cells and increase in mucus secretion was seen in all the specimens.

During the histological analysis of the 19 gills from the 20% LC$_{50}$ cadmium exposure group, the following was observed:
No congestion of blood vessels was identified. The general structure of the gill lamellae was lost in 6 (31.6%) specimens. The primary lamellar epithelium of 18 (94.7%) specimens was vacuolated. Inter cellular deposits were identified in all the specimens examined. Hyperplasia of the primary gill epithelia was identified in all the specimens. Enlargement of mucus cells and increase in mucus secretion was seen in all the specimens.

During the histological analysis of the 5 ovaries from the 10% LC$_{50}$ cadmium exposure group, the following was observed:
No inhibition of oogenesis was noted, all stages of ova development could be identified in all 18 specimens. No structural alterations to the ovaries, oogonia and oocytes were identified. Deposits in the interstitial tissue were recorded in all the specimens.

During the histological analysis of the 4 ovaries from the 20% LC$_{50}$ cadmium exposure group, the following was observed:
Oogenesis was not inhibited and all stages of ova development could be identified in all the specimens. No structural alterations to the ovaries, oogonia and oocytes were identified. Deposits in the interstitial tissue were recorded for all specimens.
During the histological analysis of the 15 testis from the 10% LC₅₀ cadmium exposure group, the following was observed:
The basal membrane of all specimens was intact. The lobules of 4 (26.7%) specimens were disorganized. No signs of inhibition of spermatogenesis were detected in any of the specimens; all stages of spermatogenesis were represented. No histological changes were noted to the interstitial tissue, spermatids or spermatozoa. Vacuolation of the spermatogonia was identified in 1 specimen. Spermatocytes of 15 (100%) specimens stained hyperchromatic because of plasma alterations. Vacuolated spermatocytes were found in 9 (60.0%) specimens. Hypertrophy of interstitial tissue was identified in only 1 specimen. No signs of inflammation identified. A tumour was found in one specimen.

During the histological analysis of the 15 testis from the 20% LC₅₀ cadmium exposure group, the following was observed:
The basal membrane of all 15 specimens was intact. Lobular organization was lost in 4 (26.7%) specimens. No signs of inhibition of spermatogenesis were detected in any of the examined specimens; all stages of spermatogenesis were represented. No histological changes were noted to the interstitial tissue, spermatogonia, spermatids or spermatozoa. Vacuolation of the spermacytes were identified in 12 (80.0%) specimens. Hypertrophy of the spermatocytes was identified in all the specimens. No inflammation or tumours were identified.

6.5.2.2 Qualitative assessment

10% LC₅₀ cadmium exposure group
Table 6.14 summarizes the quantitative results obtained from the 10% LC₅₀ cadmium exposure group for the quantitative histopathological assessment tool (discussed in detail in 5.6.1.2). The reaction index \((I_{org\ rp})\) gives an indication of the quality of the lesions in the specific organ, and the organ index \((I_{org})\) gives an indication to the degree of damage. Circulatory
disturbances (CD), regressive changes (RC), progressive changes (PC) as well as inflammation (I) was recorded for the liver from the 10% LC$_{50}$ cadmium exposure. An organ index of 17.8 was calculated for the liver. Only regressive (RC) and progressive changes (PC) were identified in the gills, resulting in a final organ index value of 18.2. Only regressive changes (RC) were recorded for the ovaries, which resulted in an organ index of 3.6. Regressive changes (RC), progressive changes (PC) and a tumour (T) were found within the testis. Organ index of 12.5 was calculated for the testis.

Table 6.14: Summary of the reaction index ($I_{org\ rp}$), organ index ($I_{org}$) and the total organ index (Tot-I) of the selected organs as calculated for the 10% LC$_{50}$ cadmium exposure group (CD-circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour).

<table>
<thead>
<tr>
<th>Rp</th>
<th>Gills</th>
<th>Liver</th>
<th>Ovary</th>
<th>Testis</th>
<th>$I_{rp}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td></td>
</tr>
<tr>
<td>CD</td>
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<td>1.2</td>
<td>0.0</td>
<td>0.0</td>
<td>1.2</td>
</tr>
<tr>
<td>RC</td>
<td>6.2</td>
<td>14.9</td>
<td>3.6</td>
<td>9.1</td>
<td>33.8</td>
</tr>
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<td>PC</td>
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<td>I</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>T</td>
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<td>0.0</td>
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<td>0.8</td>
</tr>
<tr>
<td>$I_{org}$</td>
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<td>17.8</td>
<td>3.6</td>
<td>12.6</td>
<td>Tot-I 52.2</td>
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</table>

20% LC$_{50}$ cadmium exposure group

Table 6.15 summarizes the quantitative results obtained form the quantitative histopathological assessment tool (discussed in detail in 5.6.1.2). The results obtained from the quantitative analysis of the livers from the 20% LC$_{50}$ cadmium exposure group, signify circulatory disturbances (CD), regressive changes (RC), progressive changes (PC) as well as inflammation (I). An organ index of 14.5 was calculated for the liver. Only
regressive (RC) and progressive changes (PC) were identified in the gills, resulting in a final organ index value of 14.2. Only regressive changes (RC) were recorded for the ovaries, which resulted in an organ index of 2.0. Regressive and progressive changes were found within the testis of the control group. Organ index of 14.0 was calculated for the testis.

Table 6.15: Summary of the reaction index ($I_{org\ rp}$), organ index ($I_{org}$) and the total organ index ($Tot-I$) of the selected organs as calculated for the 20% LC$_{50}$ cadmium exposure group (CD-circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour).

<table>
<thead>
<tr>
<th>Rp</th>
<th>Gills</th>
<th>Liver</th>
<th>Ovary</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
</tr>
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<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RC</td>
<td>6.6</td>
<td>12.7</td>
<td>2.0</td>
<td>10.0</td>
</tr>
<tr>
<td>PC</td>
<td>7.6</td>
<td>1.2</td>
<td>0.0</td>
<td>3.6</td>
</tr>
<tr>
<td>I</td>
<td>0.0</td>
<td>0.4</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>T</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>$I_{org}$</td>
<td>14.2</td>
<td>14.5</td>
<td>2.0</td>
<td>14.0</td>
</tr>
</tbody>
</table>

6.5.3 Chromium exposure group

6.5.2.1 Qualitative assessment

During the histological analysis of 18 livers of fish from the 10% LC$_{50}$ chromium exposure group, the following was observed:
Clear structural hepatic cords were distinguishable in all specimens, with minor congestion of blood vessels visible in 2 (11.1%) of the specimens. Granular degradation of the hepatocytes was identified in 17 (94.4%) specimens, and fat accumulation was visible in 9 (50.0%) specimens. Intercellular deposits were found in 17 (94.4%) specimens. An increase in
the amount of macrophage centres present were noted for 1 specimen. Hepatocytes showed varying degrees of nuclear alterations in 14 (77.8%) specimens. Necrosis of the liver tissue was recorded for 1 specimen, and hepatocyte hypertrophy was identified in 5 (27.8%) specimens.

**During the histological analysis of 20 livers of fish from the 20% LC₅₀ chromium exposure group, the following was observed:**

No congestion of blood sinusoids, portal or central veins was evident in any of the liver samples. Clear structural hepatic cords were not distinguishable in 5 (25.0%) specimens. Granular degradation of the hepatocytes was identified in all specimens, and fat accumulation was visible in 12 (60.0%) specimens. Intercellular deposits were found in 15 (75.0%) specimens. Nuclear alterations of the biliary epithelium were found in 1 specimen. An increase in the amount of connective tissue around blood vessels (wall proliferation) was found in only 1 specimen.

**During the histological analysis of the 18 gills from the 10% LC₅₀ chromium exposure group, the following was observed:**

No congestion of blood vessels was identified. The general structure of the primary and secondary gill lamellae was retained in all but 1 specimen. Vacuolation of the primary lamellar epithelium was identified in 16 (88.9%) specimens. Inter cellular deposits were identified in 17 (94.4%) specimens. Hyperplasia of the primary gill epithelia was identified in all (100%) the specimens.

**During the histological analysis of the 20 gills of the 20% LC₅₀ chromium exposure group, the following was observed:**

The general structure of the gill lamellae was retained, and no congestion of blood vessels was identified. The primary lamellar epithelium of 19 (95.0%) specimens was vacuolated, and inter cellular deposits were identified in all (100%) the specimens. Hyperplasia of the primary gill epithelia was identified in all (100%) the specimens.
During the histological analysis of the 8 ovaries from the 10% LC50 chromium exposure group, the following was observed:
No inhibition of oogenesis was noted, all stages of ova development could be identified in all 8 (100%) specimens. No structural alterations to the ovaries, oogonia and oocytes were identified. Deposits in the interstitial tissue were recorded in 5 (62.5%) of the specimens.

During the histological analysis of the 10 ovaries from the 20% LC50 chromium exposure group, the following was observed:
Oogenesis was not inhibited and all stages of ova development could be identified in all the specimens. No structural alterations to the ovaries, oogonia and oocytes were identified. Deposits in the interstitial tissue were recorded for 5 (50.0%) specimens.

During the histological analysis of the 10 testis from the 10% LC50 chromium exposure group, the following was observed:
The basal membrane of all 10 specimens was intact, and all specimens retained lobular organization. No signs of inhibition of spermatogenesis were detected in any of the specimens; all stages of spermatogenesis were represented. No histological changes were noted to the interstitial tissue, spermatids or spermatozoa. Vacuolation of the spermatogonia was identified in 9 (90.0%) specimens, and nuclear alterations of the spermatogonia were identified in 7 (70.0%) specimens. Hypertrophy of the spermatocytes was identified in 8 (80.0%) specimens.

During the histological analysis of the 10 testis from the 20% LC50 chromium exposure group, the following was observed:
The basal membrane of all the specimens was intact. Lobular organization was lost in only 1 specimen. No signs of inhibition of spermatogenesis were detected in any of the examined specimens; all stages of spermatogenesis were represented. No histological changes were noted to the interstitial tissue, spermatogonia, spermatids or spermatozoa. Vacuolation of the
spermatocytes were identified in 8 (80.0%) specimens. Spermatocytes of all 10 (100%) specimens stained hyperchromatic. Hypertrophy of the spermatocytes was identified in 7 (70.0%) specimens. No inflammation or tumours were identified.

6.5.3.2 Qualitative assessment

Table 6.16 and Table 6.17 summarize the quantitative histopathological results obtained from the 10% and 20% LC\textsubscript{50} chromium exposure groups respectively.

10% LC\textsubscript{50} chromium exposure groups
Circulatory disturbances (CD), regressive changes (RC), progressive changes (PC) as well as inflammation (I) was recorded for the liver from the 10% LC\textsubscript{50} chromium exposure, and an organ index of 15.7 was calculated for the liver. Only regressive (RC) and progressive changes (PC) were identified in the gills, resulting in an organ index value of 12.2. Only regressive changes (RC) were recorded for the ovaries from the control group. An organ index of 1.3 was calculated for the ovaries. Regressive changes (RC), progressive changes (PC) and inflammation (I) were found within the testis. Organ index of 10.6 was calculated for the testis (Table 6.16).
Table 6.16: Summary of the reaction index ($I_{org \ rp}$), organ index ($I_{org}$) and the total organ index (Tot-$I$) of the selected organs as calculated for the 10% LC$_{50}$ chromium exposure group (CD-circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour).

<table>
<thead>
<tr>
<th>Rp</th>
<th>Gills</th>
<th>Liver</th>
<th>Ovary</th>
<th>Testis</th>
<th>$I_{rp}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
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</tr>
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<tr>
<td>T</td>
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<td>0.0</td>
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<td>0.0</td>
</tr>
<tr>
<td>$I_{org}$</td>
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<td>1.3</td>
<td>10.6</td>
<td>Tot-$I$ 39.8</td>
</tr>
</tbody>
</table>

10% LC$_{50}$ chromium exposure groups
Circulatory disturbances (CD), regressive changes (RC), progressive changes (PC) as well as inflammation (I) was recorded for the liver from the 20% LC$_{50}$ chromium exposure, and an organ index of 14.9 was calculated for the liver. Regressive (RC) and progressive changes (PC) were identified in the gills, resulting in an organ index value of 13.3. Only regressive changes (RC) were recorded for the ovaries, and an organ index of 1.0 was calculated for the ovaries. Regressive changes (RC) and progressive changes (PC) were identified within the testis. Organ index of 13 was calculated for the testis (Table 6.17).
Table 6.17: Summary of the reaction index ($I_{org\ rp}$), organ index ($I_{org}$) and the total organ index (Tot-I) of the selected organs as calculated for the 20% LC$_{50}$ chromium exposure group (CD - circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour).

<table>
<thead>
<tr>
<th>Rp</th>
<th>Gills</th>
<th>Liver</th>
<th>Ovary</th>
<th>Testis</th>
<th>$I_{org\ rp}$</th>
<th>$I_{org\ rp}$</th>
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<tr>
<td>CD</td>
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</tr>
<tr>
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<td>0.0</td>
</tr>
<tr>
<td>T</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
<tr>
<td>$I_{org}$</td>
<td>13.3</td>
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<td>13.0</td>
<td><strong>Tot-I</strong></td>
<td>42.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.5.4 Nickel exposure group

6.5.4.1 Qualitative assessment

During the histological analysis of 19 livers of fish from the 10% LC$_{50}$ nickel exposure group, the following was observed:

No congestion of blood sinusoids, portal or central veins was evident in any of the liver samples examined from this exposure group. The normal liver structure was lost in 2 (10.5%) specimens. Granular degradation of the hepatocytes was identified in 17 (89.5%) specimens, and fat accumulation was visible in 8 (42.1%) specimens. Intercellular deposits were found in all specimens, and 1 specimen showed an increase in the amount of macrophage centres present. Varying degrees of hepatocyte nuclear alterations were identified in 10 (52.6%) specimens. Hepatocyte hyperplasia was recorded for only 1 specimen, and hypertrophy was recorded for 7 (36.8%) specimens.
During the histological analysis of 19 livers of fish from the 20% LC$_{50}$ nickel exposure group, the following was observed:

The normal hepatic structure was lost in only one specimen. No congestion of blood sinusoids, portal or central veins was evident in any of the liver samples. Granular degradation of the hepatocytes was identified in 18 (94.7%) specimens, and fat accumulation was visible in 8 (42.1%) specimens. Intercellular deposits were found in all specimens. Nuclear alterations to the hepatocytes were identified in 13 (68.4%) specimens. Hypertrophy of the hepatocytes was recorded for 5 (26.3%) specimens, and 1 specimen showed signs of infection.

During the histological analysis of the 19 gills of the 10% LC$_{50}$ nickel exposure group, the following was observed:

No congestion of blood vessels was identified, structural alterations to the gill lamella were however found in 1 specimen. The epithelium of the primary lamella of 11 (57.9%) specimens was vacuolated, and inter cellular deposits were identified in 15 (78.9%) specimens. Hyperplasia of the secondary gill epithelia was identified in all 19 (100%) specimens.

During the histological analysis of the 19 gills of the 20% LC$_{50}$ nickel exposure group, the following was observed:

No structural alterations were of the primary or secondary lamella and no congestion of blood vessels was identified. The epithelium of the primary lamella of 13 (68.4%) specimens was vacuolated, and inter cellular deposits were identified in 17 (89.5%) specimens. Hyperplasia of the secondary gill epithelia was identified in 18 (94.7%) specimens.

During the histological analysis of the 7 ovaries of the 10% LC$_{50}$ nickel exposure group, the following was observed:

Oogenesis was not inhibited and all stages of ova development could be identified in all 18 specimens. No structural alterations to the ovaries, oogonia and oocytes were identified. Deposits in the interstitial tissue were
recorded for 5 (71.4%) specimens, and inflammation was identified in 1 specimen.

**During the histological analysis of the 9 ovaries of the 20% LC$_{50}$ nickel exposure group, the following was observed:**
Oogenesis was not inhibited and all stages of ova development could be identified in all 9 (100%) specimens. No structural alterations to the ovaries, oogonia and oocytes were identified. Deposits in the interstitial tissue were recorded for 3 (33.3%) specimens.

**During the histological analysis of the 12 testis of the 10% LC$_{50}$ nickel exposure group, the following was observed:**
The basal membrane of all 9 specimens was intact. Lobular organization was retained in all specimens. No signs of inhibition of spermatogenesis were detected in any of the examined specimens; all stages of spermatogenesis were represented. No histological changes were noted to the interstitial tissue, spermatids or spermatozoa. Vacuolation of the spermatogonia of 2 (16.7%) specimens were identified, as well as nuclear alterations in all 12 (100%) specimens. Hypertrophied spermatocytes were present in all 12 (100%) specimens. A tumour was identified in 1 specimen.

**During the histological analysis of the 10 testis of the 20% LC$_{50}$ nickel exposure group, the following was observed:**
The basal membrane of all 10 specimens was intact. Lobular organization was lost in 9 (90.0%) specimens. No signs of inhibition of spermatogenesis were detected in any of the examined specimens; all stages of spermatogenesis were represented. No histological changes were noted to the interstitial tissue, spermatids or spermatozoa. Vacuolation of the spermatogonia of 4 (40.0%) specimens were identified, as well as hypertrophy of spermatocytes of all 10 (100%) specimens. No inflammation or tumours were identified.
6.5.4.2 Qualitative assessment

Table 6.16 and Table 6.17 summarize the quantitative histopathological results obtained from the 10% and 20% LC$_{50}$ nickel exposure groups respectively.

10% LC$_{50}$ nickel exposure group

Circulatory disturbances (CD), regressive changes (RC), progressive changes (PC) as well as inflammation (I) was recorded for the liver from the 10% LC$_{50}$ nickel exposure, and an organ index of 16.2 was calculated for the liver. Only regressive (RC) and progressive changes (PC) were identified in the gills, resulting in an organ index value of 12.2. Only regressive changes (RC) were recorded for the ovaries, which resulted in an organ index of 2.0. Regressive changes (RC), progressive changes (PC), inflammation (I) and a tumour (T) were found within the testis. Organ index of 12.6 was calculated for the testis (Table 6.18).

Table 6.18: Summary of the reaction index ($I_{org\ rp}$), organ index ($I_{org}$) and the total organ index (Tot-I) of the selected organs as calculated for the 10% LC$_{50}$ nickel exposure group (CD - circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour).

<table>
<thead>
<tr>
<th>Rp</th>
<th>Gills</th>
<th>Liver</th>
<th>Ovary</th>
<th>Testis</th>
<th>$I_{org\ rp}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
<td>$I_{org\ rp}$</td>
</tr>
<tr>
<td>CD</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>RC</td>
<td>4.6</td>
<td>14.0</td>
<td>1.4</td>
<td>9.3</td>
<td>29.3</td>
</tr>
<tr>
<td>PC</td>
<td>7.6</td>
<td>1.9</td>
<td>0.0</td>
<td>2.5</td>
<td>12.0</td>
</tr>
<tr>
<td>I</td>
<td>0.0</td>
<td>0.0</td>
<td>0.6</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>T</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>$I_{org}$</td>
<td>12.2</td>
<td>16.2</td>
<td>2.0</td>
<td>12.6</td>
<td>43.0 Tot-I</td>
</tr>
</tbody>
</table>
20% LC$_{50}$ nickel exposure group

Circulatory disturbances (CD), regressive changes (RC), progressive changes (PC) as well as inflammation (I) was recorded for the liver from the 20% LC$_{50}$ nickel exposure, and an organ index of 17.6 was calculated for the liver. Only regressive (RC) and progressive changes (PC) were identified in the gills, resulting in an organ index value of 12.1. Only regressive changes (RC) were recorded for the ovaries, which resulted in an organ index of 1.3. Regressive changes (RC), progressive changes (PC), inflammation (I) and a tumour (T) were found within the testis. Organ index of 14.5 was calculated for the testis (Table 6.19).

**Table 6.19:** Summary of the reaction index ($I_{org\, rp}$), organ index ($I_{org}$) and the total organ index (Tot-I) of the selected organs as calculated for the 20% LC$_{50}$ nickel exposure group (CD-circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour).

<table>
<thead>
<tr>
<th>Rp</th>
<th>Gills</th>
<th>Liver</th>
<th>Ovary</th>
<th>Testis</th>
<th>$I_{org, rp}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>RC</td>
<td>5.4</td>
<td>16.1</td>
<td>1.3</td>
<td>11.3</td>
<td>34.1</td>
</tr>
<tr>
<td>PC</td>
<td>6.7</td>
<td>1.1</td>
<td>0.0</td>
<td>3.2</td>
<td>11.0</td>
</tr>
<tr>
<td>I</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>T</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>$I_{org}$</td>
<td>12.1</td>
<td>17.6</td>
<td>1.3</td>
<td>14.5</td>
<td>Tot-I 45.5</td>
</tr>
</tbody>
</table>

6.5.5 Statistical analysis

Statistical analysis was done on the organ index ($I_{org}$) calculated for all the organs from all the exposure groups. Figure 6.12, 6.13, 6.14, and 6.15 graphically summarizes mean values of the reaction index ($I_{org\, rp}$) and the organ index ($I_{org}$) as calculated for the liver, gills, ovary and testes respectively. Matrixes denote
exposure groups with statistically significant differences with regard to the organ index ($I_{org}$). 

**Non-parametric statistical analysis**

The Kruskal-Wallis test was preformed on the data obtained from all the exposure groups. All data sets were ranked according to the means of the different groups. The Chi-square test was preformed on the ranked data. Table 6.20 summarizes the results obtained from the Chi-square test.

Table 6.20: Results obtained from the Chi-square test preformed on the organ index ($I_{org}$) as calculated for the different exposure groups.

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Gills</th>
<th>Ovary</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-square ($x^2$)</td>
<td>4.559</td>
<td>61.738</td>
<td>13.508</td>
<td>3.770</td>
</tr>
<tr>
<td>Degrees of freedom (df)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Significant value (p)</td>
<td>0.602</td>
<td>0.000</td>
<td>0.036</td>
<td>0.708</td>
</tr>
</tbody>
</table>

According to the results obtained from the Chi-square test, there are significant differences ($p \leq 0.05$) between the calculated $I_{org}$ of the gills and ovaries from the exposure groups.

**Parametric statistical test**

- **Liver**
  The data sets passed the test of homogeneity of variances (Levene statistic). The results obtained from the one-way ANOVA were not significant. No significant differences could therefore be found between the control group and the exposure groups.

- **Gills**
  The data set violated the test of homogeneity of variances (Levene statistic). The data set was then subjected to the robust quality of means (Brown-Forsythe) test, which hinted at a difference between the data sets. The
Brown-Forsythe test was then followed by the Dunnet T3 test to determine which exposure groups are significantly different from the control group. Significant differences were found between the control group and Cd10, Cd20 and Cr20 exposure groups.

- **Ovary**
The data set failed the test of homogeneity of variances (Levene statistic). The data set violated the robust quality of means (Brown-Forsythe) test (one group has 0 variances). No significant differences could therefore be found between the control group and the exposure groups.

- **Testis**
The data set failed the test of homogeneity of variances (Levene statistic), and the robust quality of means (Brown-Forsythe) test. No significant differences could therefore be found between the control group and the exposure groups.
Figure 6.12: Quantitative histopathological assessment of the liver of *O. mossambicus* after short-term exposure to various metals. Mean values of the reaction index ($I_{org \, rp}$): CD - circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour. The sum of the 5 reaction index indicates the organ index ($I_{org}$). Matrices denote exposure groups with statistically significant differences in the $I_{org}$ as determined by non-parametric as well as parametric statistical tests. (C - control exposure, Cd10 - 10% LC$_{50}$ Cd exposure; Cd20 - 20% LC$_{50}$ Cd exposure; Cr10 - 10% LC$_{50}$ Cr exposure, Cr20 - 20% LC$_{50}$ Cr exposure, Ni10 - 10% LC$_{50}$ Ni exposure, Ni20 - 20% LC$_{50}$ Ni exposure). (× - not significantly different; ✔ - significantly different).
Figure 6.13: Quantitative histopathological assessment of the gill of *O. mossambicus* after short-term exposure to various metals. Mean values of the reaction index ($I_{org\ rp}$): CD - circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour. The sum of the 5 reaction index indicates the organ index ($I_{org}$). Matrixes denote exposure groups with statistically significant differences in the $I_{org}$ as determined by non-parametric as well as parametric statistical tests. (C - control exposure, Cd10 - 10% LC$_{50}$ Cd exposure; Cd20 - 20% LC$_{50}$ Cd exposure; Cr10 - 10% LC$_{50}$ Cr exposure, Cr20 - 20% LC$_{50}$ Cr exposure, Ni10 - 10% LC$_{50}$ Ni exposure, Ni20 - 20% LC$_{50}$ Ni exposure). (× - not significantly different; ✓ - significantly different).
Figure 6.14: Quantitative histopathological assessment of the ovary of *O. mossambicus* after short-term exposure to various metals. Mean values of the reaction index ($I_{org rp}$): CD - circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour. The sum of the 5 reaction index indicates the organ index ($I_{org}$). Matrixes denote exposure groups with statistically significant differences in the $I_{org}$ as determined by non-parametric as well as parametric statistical tests. (C - control exposure, Cd10 - 10% LC50 Cd exposure; Cd20 - 20% LC50 Cd exposure; Cr10 - 10% LC50 Cr exposure, Cr20 - 20% LC50 Cr exposure, Ni10 - 10% LC50 Ni exposure, Ni20 - 20% LC50 Ni exposure). (× - not significantly different; ✓ - significantly different).
Figure 6.15: Quantitative histopathological assessment of the testis of *O. mossambicus* after short-term exposure to various metals. Mean values of the reaction index ($I_{org \; rp}$): CD - circulatory disturbance, RC - regressive changes, PC - progressive changes, I - inflammation, T - tumour. The sum of the 5 reaction index indicates the organ index ($I_{org}$). Matrixes denote exposure groups with statistically significant differences in the $I_{org}$ as determined by non-parametric as well as parametric statistical tests. (C - control exposure, Cd10 - 10% LC$_{50}$ Cd exposure; Cd20 - 20% LC$_{50}$ Cd exposure; Cr10 - 10% LC$_{50}$ Cr exposure, Cr20 - 20% LC$_{50}$ Cr exposure, Ni10 - 10% LC$_{50}$ Ni exposure, Ni20 - 20% LC$_{50}$ Ni exposure). (× - not significantly different; ✓ - significantly different).
6.5.6 Micro slides

Similar histological changes, although variant in degree, were observed in all the examined fish. Representative micro slides which best displays these histological alterations are listed below.
Figure 6.16: Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 10% LC50 Ni exposure.

- **Congestion (C)** of sinusoids (S) of blood vessels (BV) and the central vein (CV).
- **Intercellular deposits (I)** are scattered throughout the section, but are concentrated around the central vein.
- Fatty degradation identified by **fatty vacuoles (F)** are scattered throughout the section.

(Stained with H & E; 40 X magnification.)

Figure 6.17: Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 10% LC50 Ni exposure.

- The general disruption of cord structure caused by severe fat accumulation (*) with in hepatocytes throughout the section.
- Nuclear activity, such as **pyknosis (P, encircled in black)** and **karyorrhexis (K, encircled in white)**, can also be identified on this section.

(Stained with H & E; 100 X magnification.)
Figure 6.18: Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 20% LC₅₀ Ni exposure.

Degeneration is the state in which the physiological substances present in tissues increase or appear in other places. Both fatty degeneration (6.13 A) and granular degradation (6.13 B) are clearly identifiable in this section.
(Stained with H & E; 40X magnification)

A) Fatty degradation

Fat is stored in the cytoplasm in the form of triglyceride-rich droplets (*) are stored in the hepatocytes which appear as big vacuoles throughout this section. The hepatocytes lost their hexagonal shape and the general structure of liver tissue is disrupted. Fatty degeneration is accompanied by pyknosis (encircled in black on insert).

(Insert: Stained with H & E; 100 X magnification.)

B) Granular degeneration

Proteinous granules are visible in the cytoplasm of the hepatocytes throughout this section. Cytoplasm appears to clumped. Hepatocytes appear swollen and lost their hexagonal shape. Pyknosis of the nucleus is observed (encircled in white).

(Insert: Stained with H & E; 100 X magnification.)
**Figure 6.19:** Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 20% LC$_{50}$ Ni exposure.

- **Intercellular deposits (I)** were found around the central vein (CV).
- **Mono macrophage centres (MMC)** are located between the intercellular deposits.
- **Infiltration of mono nuclear leucocytes (MNL)** was also found around the central vein.

(Stained with H & E; 40X magnification).

**Figure 6.20:** Representative histopathological characteristics observed in the liver of *O. mossambicus* after exposure to 10% LC$_{50}$ Cr exposure.

- Hepatocytes show varied degrees of **hypertrophy (Ht)** throughout the section.
- **Granular degradation (G)** of the cytoplasm of the hepatocytes can be seen in most hepatocytes on this section.
- Fragmentation of the nucleus, **karhorrhexis (K)**, was found throughout the section.

(Stained with H & E; 40X magnification)
Figure 6.21: Representative histopathological characteristics observed in the gill of *O. mossambicus* after acute exposure to 10% LC₅₀ chromium exposure.

- The epithelial cells on the primary lamellae are **vacuolated (V)**.
- **Deposits (De)** are found within the epithelial cells.

(Stained with H & E; 100X magnification)

Figure 6.22: Representative histopathological characteristics observed in the gill of *O. mossambicus* after acute exposure to 10% LC₅₀ nickel exposure.

- **Hyperplasia (Hp)** of the primary gill epithelium is noted.
- **Deposits (De)** are found within the epithelial cells.

(Stained with H & E; 100X magnification)
Figure 6.23: Representative histopathological characteristics observed in the gill of *O. mossambicus* after acute exposure to 10% LC$_{50}$ cadmium exposure.

- **Deposits (De)** are found within the epithelial cells throughout the section.
- **Hyperplasia (Hp)** of both the secondary and the primary gill epithelia are demonstrated in this section.
- **Epithelia lifting (EL)**
- A slight increase in **mucus cells (MC)** was noted.

(Stained with H & E; 40X magnification)

---

Figure 6.24: Representative histopathological characteristics observed in the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel exposure.

- The presence of ovum (O) in the testis (intersex).
- All developmental stages were identified in this section.
- All developmental stages were vacuolated (V)
- Primary and secondary spermatocytes stained hyperchromatic (Hc).

(Stained with H & E; 100X magnification)
**Figure 6.25:** Section thought the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel. 
- General lobular organization is lost.
- Primary spermatocytes are **vacuolated (V)** and stain **hyperchromatic (Hc)**.  
  (Stained with H & E; 40X magnification)

**Figure 6.26:** Section through the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel. 
- General lobular organization is retained.
- Not all developmental stages identified in this section (only primary and secondary spermatocytes identified)
- Primary spermatocytes are **vacuolated (V)**.  
- Spermatocytes stain **hyperchromatic (Hc)**.  
  (Stained with H & E; 100X magnification)
Figure 6.27: Section through the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ nickel.

A) General lobular structure is retained, and all developmental stages are represented.
(Stained with H & E; 400 X magnification.)

B) Spermatocytes stained hyperchromatic (Hc) and are valuolated (V).
(Stained with H & E; 100 X magnification.)
Figure 6.28: Section through the testis of *O. mossambicus* after acute exposure to 10% LC$_{50}$ cadmium.

- General lobular structure is lost.
- No spermatids or spermatozoa identified.
- Primary and secondary spermatocytes appear vacuolated (V) and stain hyperchromatic (Hc).
6.5.7  Gonad maturity index

The maturity of the individual fish was determined histological by applying the maturity of gonads index as described in section 5.6.3.

Of the 61 females examined, 4 were classified to be in stage 2 development. The majority of developing follicles are late vitellogenic. Oocytes were smaller in diameter (600-1000 µm), and eosinophilic yolk globules were distributed throughout the cytoplasm. A conspicuous chorion was present. The remaining 57 females were all classified to be in stage 3 development, with the majority of late vitellogenic follicles are. Table 6.21 lists the frequencies of the developmental stages of the females in each exposure group.

Table 6.21: Frequencies of the developmental stages identified for the ovaries.

<table>
<thead>
<tr>
<th>Females</th>
<th>n</th>
<th>Developmental stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>Cd10</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Cd20</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Cr10</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Cr20</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Ni10</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Ni20</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>∑ Females</td>
<td>61</td>
<td>-</td>
</tr>
</tbody>
</table>

Of the 93 males examined, 12 were classified to be mid-spermatogenic (stage 2 development), with an equal mix of spermatocytes, spermatids and spermatozoa present. The remaining 81 specimens were classified as late spermatogenic. All developmental stages were identified, but spermatozoa
predominate. Table 6.22 lists the frequencies of the developmental stages of the males in each exposure group.

**Table 6.22:** Frequencies of the developmental stages identified for the testis.

<table>
<thead>
<tr>
<th>Male</th>
<th>n</th>
<th>Developmental stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Cd10</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Cd20</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Cr10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cr20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Ni10</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Ni20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Σ Males</strong></td>
<td><strong>93</strong></td>
<td><strong>12</strong></td>
</tr>
</tbody>
</table>

**6.6 Summary of results**

Table 6.23 summarizes the parametric and non-parametric statistical results obtained from all the biomarkers applied during this study.

Chapter 8 will discuss and compare the results obtained during this study with results from previous exposure studies. A final conclusion will be formulated and future recommendations will be made.
**Table 6.23:** Summary of the statistical results obtained after exposure to cadmium, chromium and nickel. (Non-parametric test compare all values from all the exposure groups with each other; parametric statistical test compare the control group against the different exposure groups.) (× - not significantly different; ✓ - significantly different; outside the normal range; inside the normal range).

<table>
<thead>
<tr>
<th>Applied biomarkers</th>
<th>Statistical analysis</th>
<th>Parametric: Control exposure group vs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-parametric: All exposure groups*</td>
<td>Cd10</td>
</tr>
<tr>
<td>CF F</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>CF M</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>H</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>L</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>[TP]</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>NFHAI</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>HSI</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>SSI</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>GSI F</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>GSI M</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>FTI</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
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<td>x</td>
<td>x</td>
</tr>
<tr>
<td>I&lt;sub&gt;org&lt;/sub&gt; Gills</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>I&lt;sub&gt;org&lt;/sub&gt; Ovary</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>I&lt;sub&gt;org&lt;/sub&gt; Testis</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

(* Control, Cd10; Cd20; Cr10, Cr20, Ni10, Ni20).
7.1 Test organism

During the 6 weeks acclimation period prior to the onset of the exposure, fish were kept at ± 23°C and fed commercial trout pellets on a daily basis. All the fish used during this study were from same breeding stock and therefore similar in age. All the fish used during this study should therefore be of similar health, condition and developmental stage prior to exposure. The genetic purity of the *O. mossambicus* strain used in this exposure study was confirmed (5.1; Moralee et al., 2000).

7.2 Water quality

7.2.1 Physical water quality parameters

The physical water quality variables measured during the exposures (Table 6.1, 6.2 and 6.3) were within the target water quality guidelines for aquatic ecosystems (DWAF, 1996), and fell within the environmental requirements for optimum growth and survival of the Tilapia species (as listed in table 5.1). All water quality variables were kept constant and there were no fluctuations in temperature, abnormally low or high pH levels, or low dissolved oxygen levels that may have had a negative effect on the test organisms. The metals added to the exposure groups were therefore the only stressor which may elicit a physiological response by the test organism.
7.2.2 Metal speciation

The determination of the available percentage of free metal ions (Cd\(^{2+}\), CrO\(_4^{2-}\) and Ni\(^{2+}\)) in each of the separate exposure systems provides an estimated amount of the specific metal ion species that will be bioavailable through the aquatic environment, and therefore elicit a reaction by the target cells, resulting in a biomarker response. The available percentage of free metal ions was determined by taking the daily physical water quality variables into consideration. From the results obtained it was concluded that >97% of the added metals was available during the 96 hours of exposure (Table 6.4).

7.3 Necropsy

In order to simplify the discussion of the results obtained during this study, the control group will also be considered as a separate exposure group.

All fish used in the exposure were adults, and did not show any sign of discomfort or annoyance. An increase in mucus production on the gills and the skin was noted for all the exposure groups compared to the control groups, a sign the fish were experiencing stress. The same observation was made by Nussey (1994; 1996), where juvenile *O. mossambicus* were exposed to high concentrations of copper.

Macroscopically all the fish from all the exposure groups seemed to be in similar health and condition when considering the external organs and structures. Macroscopic deviation in the colour of the liver was noted in all the exposure groups. These macroscopic changes in liver colour showed no correlation to the histology of these specimens. Robinson (1996) also recorded similar results regarding the discolouration of the liver of *O. mossambicus* and speculates that this discolouration might be considered as normal for *O. mossambicus*. The variations in bile colour and mesenteric fat
accumulation may be an indication of the different feeding patterns of individual fish prior to feeding termination, before commencement of the exposure period.

### 7.3.1 Condition factor (CF)

The average calculated condition factors of all the groups fell within normal acceptable range of ± 1 (Adams et al., 1993; discussed in section 3.2.1). No significant difference in the condition factor was noted between the exposure groups and the control group. Studies done by Miller et al. (1992) and Kiceniuk and Khan (1987) on different metal mixtures all showed a decrease in the condition factor. However, both these studies were chronic exposures. The present study was an acute exposure of only 96 hours to the selected metals. Because the condition factor is considered as an organism response (Goede and Barton, 1990), it might be that an acute exposure does not allow for enough time to result in a significant change in the gross weight of the exposed specimens.

During this study specimens were exposed to low concentrations of the selected metals (10% and 20% of the LC$_{50}$). It is therefore possible that the organism adapted biochemical and physiological processes to cope with the insult, and that the concentration of the added metals were therefore not high enough to elicit an effect. The liver may therefore still able to detoxify the metals. According to Goede and Barton (1990) a decrease in weight due to loss of energy stores can also be masked by an increase in body water.

According to the results obtained from to CF it might be concluded that the fish from all the exposure groups as well as the control group are in similar overall health and nutritional status (Adams et al., 1993).
7.3.2 Blood variables

According previous studies, haematocrit values increase during situations of acute stress (Barton et al., 1985; Casillas and Smith, 1977; Douellou and Guillaume, 1986; Soivio and Oikari, 1976), and when fish are exposed to metals for longer than 24 hours (Cyriac et al., 1989). This was not found during this study after 96 hours exposure.

There was a decrease in the haematocrit of the 10% and 20% cadmium exposure in relation to the control group. However, this decrease proofed not to be significant. The change in haematocrit values may illustrate a decrease in erythrocytes under stress conditions (Van Vuren, 1986). Previous studies done by Gill and Plant (1985) reported erythrocyte abnormalities and anaemia in *Puntius conchonius* after a 12 week exposure to cadmium.

Schiffman and Fromm, (1959) reported higher haematocrit readings for rainbow trout from water contaminated with chromium salts. This was not found during this study. Although the haematocrit of the chromium 10% exposure group showed a slight increase from the control group, this change was not significant. The chromium 20% exposure group showed a smaller deviation from the control group.

The average haematocrit readings obtained from the nickel exposure groups was slightly higher than that of the control group. However, this increase was not significantly higher than control group. Ghazaly (1992) reported that *Tilapia nilotica* exposed to 32.0 and 51.0 mg/l nickel for 96 hours resulted in an elevated erythrocyte number. Again it can be argued that the concentration of the metals used during the present study was too low to elicit an effect at this level. The 20% nickel exposure group showed a smaller deviation from the control group.
The effects on the haematocrit are less pronounced in the 20% chromium and nickel exposure groups compared to the effect on the haematocrit of the 10% chromium and nickel exposure groups. This can be explained by a possible increase in metallothioneins (MT) induction. Metallothioneins have been known to bind with a variety of metals possibly exporting the metal from the liver to the kidneys for excretion.

The calculated leucocrit values of all the exposure groups fell within the normal range (<4%). The cadmium 20% exposure group and the chromium 10% exposure group had a slightly higher average leucocrit than control group. This increase proofed not to be significant. From these results it can be deduced that the fish health were not compromised by an infection. The cadmium 10% exposure group and the nickel 10% exposure group showed a decrease in the average leucocrit when compared to the control group, although not statistically significant. According to Donaldson (1981) and Schreck (1981), circulating lymphocytes are suppressed by elevated corticosteroid. Under conditions of acute stress, the hypothalamus-pituitary-interrenal axis (HPI-axis) are activated, resulting in a increase release of the hormones the such as glucocorticoids, mineralocorticoids, and androgens from adrenal cortex (Pelgrom, 1995). Studies done by Fu et al. (1989) have shown an increase in cortisol (a glucocorticosteroid) in exposed fish during sub-lethal exposure to cadmium. At a higher concentration of cadmium, this effect may be more pronounced.

The total plasma protein concentration of the cadmium 20% exposure group was significantly lower than that of the control group. A low concentration of total plasma proteins is indicative of a haemodilution caused by kidney and liver damage, depletion of energy stores, and impaired water balance (Cunjak, 1988; Lockhart and Metner, 1984; Wedemeyer and Yasukake, 1977). Degenerative changes have been reported by Forlin et al. (1986) in the kidney of Salmo gairdneri exposed to cadmium for 4 weeks, as well as glycogen depletion of the liver. Atrophy and necrosis of hepatocytes have
been reported in the liver of *Cyprinus carpio* exposed to ambient cadmium (Morsey and Protasowicki, 1990). The gills are the primary site of damage during water born cadmium exposure (Pelgrom, 1995; Williams and Giesy, 1978). According to studies done by Pratap and Wendelaar Bonga (1993) ambient cadmium reduce $\text{Na}^+/\text{K}^+-\text{ATPase}$ activity, which may in turn lead to impaired water balance. The increase in total plasma protein of the 20% cadmium exposure group may thus be as a result of any of the above mentioned causes. Further histological investigation is needed to confirm the cause (discussed in section 7.4.2 under Gills).

### 7.3.3 Necropsy based fish health assessment index (NFHAI)

The average NFHAI value as calculated for the 20% cadmium exposure group are significantly higher than the value calculated for the control group. It can therefore be concluded that the general health of the specimens from the 20% cadmium exposure group are compromised. The increase in the NFHAI is attributed to discoloured liver, low total plasma protein concentration, increase in liver discoulouration and the low haematocrit of this exposure group (Table 6.9).

### 7.3.4 Organo-somatic indices

Except for cadmium 10% exposure group, all the hepato-somatic indices (HSI) determined for all the exposure groups ranged between the normal 1-2% (Gingerich, 1982). The HSI determined for both the cadmium 10% and cadmium 20% exposure groups are significantly lower than the average HSI determined for the control group. Larson *et al.* (1984) also reported a decrease in the hepato-somatic index of *Perca fluviatilis* exposed to a mixture of metals. This decrease in the hepato-somatic index might be the result of atrophy or necrosis of the hepatocytes (Busacker *et al.*, 1990). However, Van Dyk *et al.* (2007) did not find any significant differences in the HSI of fish exposed to a low concentration (5% and 10% of the LC$_{50}$ concentration)
of cadmium and zinc. According to Gingerich (1982) fish liver may store blood during periods of quiescence. This suggests that the activity of the fish immediately prior to capture, and the protocol used to procure the liver, may affect relative liver size. To address this issue, all methods were standardized and care was taken to minimize stress and handling of fish before sampling. Further histological investigation is needed to confirm the cause of the decrease in the HSI.

The cadmium 20% exposure group shows a slight decrease in the calculated spleno-somatic index (SSI), however this decrease proved not to be statistically significant. Decrease in the spleno-somatic index might point to a decrease in haematopoietic function of the spleen or necrosis (Schmitt et al., 2000). When comparing the results of the SSI with the lower haematocrit (also not significantly lower than the control) calculated for this group, it may be argued that there was indeed a reduction in the haematopoietic function of the spleen. Previous study by Pulsford et al. (1995), showed a decrease in the SSI of Gadus morrhuvus after chronic exposure to selected metals. The effect of cadmium might be more pronounced after chronic exposure.

Previous studies by Pieterse (2004) and Friedman et al. (1996) showed that exposure to Cu and Hg can result in gonadal alterations such as a decreased gonadal-somatic index (GSI). No significant difference in the GSI was however found between the control group and exposure groups during this study. From these results it can be concluded that all the specimens used during the exposures are in similar reproductive maturity. The reproductive maturity was confirmed when the histology of the gonads were investigated (Section 6.3.6)

As with the GSI, no significant differences were found in the fat tissue index (FTI) between the exposure groups. Both these indices are organism level responses. Lesions observed at the organism level of organization suggests that the adaptive immunological, physiological and biochemical mechanisms
have been overwhelmed. The exposed specimens might have adapted to the insult caused by the metals. Examples of such adaptation is metallothionein induction as well as heat shock protein production (HSP) upon metal exposure. It can also be argued that acute exposure does not allow for enough time to result in a significant change in the gross weight of these organs.

7.4 Histological assessment

7.4.1 Qualitative assessment

Liver
The following changes were identified in the liver:

- Congestion
  Congestion of blood vessels and sinusoids were found in 5.13% of the control fish. A definite increase in the level of congestion was observed in the cadmium 10% exposure group and the chromium 10% (25% and 10% of the examined samples, respectively). Van Dyk et al. (2007) also reported congestion of blood vessels (especially portal veins) in fish exposed to low concentrations of cadmium and zinc. An increase in the intensity of the congestion was seen with an increase in concentration of the metal exposure.

- Structural alterations
  Structural alterations to the hepatic cord were identified in individual fish from the control group. An increase in cord disarray was demonstrated in fish from the 10% LC<sub>50</sub> cadmium exposure, and the 20% LC<sub>50</sub> cadmium exposure when compared to the control group. Similar changes in cord structure were recorded by Van Dyk et al. (2007) after 96 hour exposure to low concentrations of cadmium and zinc exposure.
• **Plasma alterations**

**Granular degradation**
For the purpose of this study, granular degradation includes calcareous degeneration, amyloid degeneration and colloid degeneration, which all results in the formation proteinious cytoplasmic clump (discussed in section 3.4.1.2). Almost all of the 150 fish examined from all the exposure groups, including the control group, demonstrated granular degradation. No correlation could be established between the degree of granular degradation and metal exposure.

**Fatty degeneration (or fatty change)**
Fatty change of hepatocytes was observed in both the control and exposed specimens. However, fatty change of hepatocytes is a common response associated with exposure of fish to a variety of different agents (Meyers and Hendricks, 1985). This histological change could signify various biochemical lesions: (1) Inhibition of protein synthesis; (2) energy depletion; (3) disaggregation of microtubules; (4) shifts in substance utilization.

The large vacuole in the cell forces the nuclei to the periphery of the hepatocytes and this fatty change is usually accompanied by nuclear atrophy (Damjanov, 1996; Hibiya, 1982). Similar results were obtained for chromium and nickel exposure groups. The same degree of vacuolation was visible in livers exposed to both concentrations; no real difference could be concluded regarding this aspect. Van Dyk *et al.* (2007) reported similar changes in the liver of *O. mossambicus* after acute exposure (96 hour) to cadmium. This was however not confirmed for the cadmium exposure groups regarding the present study.

• **Intercellular deposits**
Intercellular deposits are common biomarkers of metal exposure in mammals but have not been commonly reported in fish (Hinton and Laurén, 1990).
Scattered intercellular deposits were found in all 150 fish examined during this study. A noted increase of intercellular deposits, were recorded for the chromium and nickel exposures. Intercellular deposits were found scattered throughout the liver tissue, but were slightly more concentrated around the portal veins. No change in the severity of the deposits was found between the different concentrations of the metals.

- **Increase in macrophage aggregates**
  Macrophage aggregates (MA) have been recognized as normal structures of fish liver (as discussed in section 4.2.2). An increase in the number or size of the MA’s has proven to be an indicator of contaminant exposure in fish tissue (Schmitt et al., 2000, discussed in Section 4.2.4.1).

  MA’s were identified in all the specimens examined during this study, including some control specimens. No pattern in terms of the exposure or metal concentration and the MA’s could be established during this study. Irregular variations in amount did, however, occur in individual specimens from the cadmium 10% exposure group. This increase in amount could have been caused by the metal exposure (Van Dyk et al., 2007).

- **Mono-nuclear leukocyte infiltration**
  Irregular variations in amount of mono-nuclear leucocyte (MNL) infiltration were observed in selected specimens from the cadmium exposure groups and the 20% LC₅₀ nickel exposure group. This infiltration was of low intensity and did not reflect any pattern in terms of the exposure period or metal concentration. This increase in amount of MNL could have been caused by the metal exposure. No final conclusion could be made.

- **Nuclear alterations**
  Irregular variations in the degree of nuclear alterations were observed in selected specimens from all the exposure groups, including the control group.
Nuclear alterations identified include pyknosis, karyolysis and karyorrhexis (described in Section 3.4.1.2). No pattern in terms of the exposure period or metal concentration could be found, and no final conclusion could be made.

- **Hypertrophy**

Hypertrophy of hepatocytes is an adaptive change after contaminant exposure (Damjanov, 1996). According to Hinton and Laurén (1990), hypertrophy of cells is the result of an increase in organelles, such as the endoplasmic reticulum. Studies done by Klauning et al. (1979) and Schoor and Couch (1979) revealed a correlation between amount of endoplasmic reticulum and cytochrome p-450 in the hepatocytes of fish exposed to Aroclor 1254. Cytochrome P-450 is a mixed function oxidase responsible for detoxification of xenobiotic substances such as metals (previously discussed in Section 4.2.3.1). The correlation between the increase in hypertrophy and the increase in Cytochrome P-450 will provide a useful link in future studies regarding metal exposures.

A moderate increase in hepatocytes hypertrophy was identified in the 20% LC$_{50}$ cadmium exposure group, the 10% LC$_{50}$ chromium exposure group, and both the nickel exposure groups. These results confirm that even though the HSI of the fish from these exposure groups are in the normal range, fish are under stress and adapting to cope with the chemical insult. No atrophy were identified to confirm the low HSI obtained for the 10% LC$_{50}$ cadmium exposure group, and therefore no conclusion could be made in this regard.

**Gills**

The following changes were identified in the gills:

- **Structural alterations**

Variant degrees of epithelial lifting were record for both of the cadmium and chromium exposure groups and the 10% LC$_{50}$ nickel exposure group. A higher degree of damage was recorded for the 10% and 20% LC$_{50}$ cadmium
exposure groups. This was not unexpected, because uptake of cadmium occurs mainly through the gills (Williams and Giesy, 1978).

- **Plasma alterations**
  Vacuolation of the epithelial cells were identified in all the specimens examined during this study, including some control specimens. An increase in the severity of the vacuolation was seen in the exposure groups. No pattern in terms of the metal or metal concentration and epithelial vacuolation could be established during this study. No final conclusion could be made.

- **Intercellular deposits**
  Eosinophilic intercellular deposits were identified on the primary lamellae of all the specimens examined during this study. A slight increase in the severity of the deposits was seen in the exposure groups. No pattern could be established in terms of the metal or metal concentration and the deposits during this study. No final conclusion could be made.

- **Hyperplasia**
  According to Hinton and Laurén (1990) hyperplasia of the undifferentiated cells on the primary lamella is a non-specific response to a wide variety of chemical exposures. Some of the undifferentiated cells might differentiate into chloride cells to replace the degeneration chloride cells.

Several studies have shown that fish adapting to sub-lethal ion losses exhibit a compensatory increase in chloride cells. Ambient cadmium reduce the movement of $\text{Ca}^{2+}$ into the gill epithelia (Verbost *et al.*, 1988), and inhibit $\text{Na}^{+}/\text{K}^{+}$-ATPase activity (Pratap and Wendelaar Bonga, 1993) (discussed in Section 2.5.4), therefore disrupting the internal ion balance of the exposed fish. As a result, undifferentiated epithelial cells undergo hyperplasia to compensate for the function loss of the existing chloride cells. Gardner and Yevich (1970) reported hyperplasia of gill epithelia of *Fundulus heteroclitus*
after acute exposure to cadmium. Similar results were obtained during the present study from the cadmium exposure groups.

According to studies done by Farag et al. (2006) there is an increase in gill epithelium and apoptosis of chloride cells of *Oncorhynchus tshawytscha* after chronic exposure to chromium. Results from the chromium exposure from the current study, follow the trend of increasing undifferentiated epithelial cells. After a longer exposure period the damage to the chloride cells may become more apparent.

Although an increase in mucus on gills of the exposed specimens was observed during the necropsy health assessment index (macroscopic investigation), no hyperplasia of mucus cells were found during histological analysis. Mucus has the ability to bind metals from solution, thereby protecting the gills from severe damage (discussed in Section 4.3.3).

- **Tumour**
  A tumour was identified in only 1 specimen from the 20% LC$_{50}$ cadmium exposure group. An acute exposure does not allow enough time for the formation of a tumour, and was therefore considered not to be a change caused by the metal exposure.

- **Ovaries**
  Oocyte atresia, or resorption of unfertilized eggs by the ovaries, is a normal physiological event in all fish, but it has become the only histopathological reproductive biomarker of contaminant exposure (Hinton and Laurén, 1990). Oocyte atresia was identified in *Colisa fasciata* after acute exposure (96 h) to 64.0 mg/l nickel (Nath and Kumar, 1989). No oocyte atresia was identified in any of the examined samples. Chronic exposure to cadmium caused a delay in oogenesis in *Salmo trutta* (Brown et al., 1994). No signs of inhibition of oogenesis were identified in any of the examined specimens. The following changes were found in the ovaries:
• **Plasma alterations: intercellular deposits**
The intercellular deposits observed in the interstitial tissue of all the specimens from all the exposure groups (including the control group), were of low intensity and did not reflect any pattern in terms of the exposure period or metal concentration. No final conclusion could be made.

• **Mono-nuclear leukocyte infiltration**
MNL infiltration was observed in 1 specimen from the 20% LC$_{50}$ nickel exposure group. This infiltration was of low intensity and was therefore not considered to be a significant change.

**Testis**
The following changes were found in the testis:

• **Disorganization of lobules**
The loss of lobular structure observed in the 10% and 20% LC$_{50}$ cadmium exposure, as well as the 20% LC$_{50}$ nickel exposure group might be as a result of endocrine disruption. Testicular degeneration was also identified in *Colisa fasciata* after acute exposed to nickel (Nath and Kumar, 1989). According to studies done by Sangalang and O'Halloran (1972), endocrine disruption in *Salvelinus fontinalis* is noticeable after 24 hours of exposure to cadmium.

• **Plasma alterations: vacuolation**
Vacuolation of spermatocytes were identified in all the specimens examined during this study, including some control specimens. No pattern in terms of the metal or metal concentration and the degree vacuolation could be established during this study. No conclusion could be made.

• **Nuclear alterations**
Hyperchromatic staining of spermatocytes cells were identified in all the specimens examined during this study. No pattern in terms of the metal or
metal concentration and epithelial vacuolation could be established during this study. No conclusion could be made.

- **Hyperplasia**
  A low degree of hyperplasia of interstitial tissue was observed in 1 specimen from the 10% LC$_{50}$ cadmium exposure group. This change was regarded as insignificant.

- **Hypertrophy**
  Hypertrophy of spermatocytes was identified in all the specimens examined during this study, including the control specimens. No pattern in terms of the metal or metal concentration and the degree of hypertrophy could be established during this study. No final conclusion could be made.

- **Tumours**
  A tumour was identified in 1 fish from the 10% LC$_{50}$ cadmium exposure group, and 1 fish from the 10% LC$_{50}$ nickel exposure. As mentioned before, an acute exposure does not allow enough time for the formation of a tumour. The formation of these tumours was therefore not considered as a change caused by the metal exposure.

- **Inter sex**
  The presence of ova in the testis was found in all the exposure groups, including the control groups. The presence of intersex was therefore not considered to be as a result of metal exposure during this study, but as a result of prior exposure to contaminants. Intersex was not included in the quantitative histological assessment.

### 7.4.2 Quantitative assessment

Similar histological changes, similar in degree, were identified in all the exposure groups including the control group for the liver, testis and ovaries. The quantitative histological assessment tool allows for a comparison of the
exposure groups to the control group which then elucidates toxic histological changes as a result of the metal exposures.

**Liver**

After statistical analysis of the quantitative results obtained from the histological investigation of the liver, no significant changes were found between the livers from the different exposure groups (Figure 6.12). The main function of the liver is to detoxify xenobiotic substances such as metals (discussed in Section 4.2.3). It was therefore concluded that the cytochrome-P450 enzyme system were still able to cope with the insult.

Histopathological studies done by Van Dyk et al. (2007) showed a decrease in pathological changes found in the liver of specimens exposed to 5% and 10% LC$_{50}$ cadmium and zinc after 96 hours. This decrease might be related to metallothionein induction during the exposure time. According to the results obtained by Basson (2006) there is a statistical significant increase in hepatic HSP70 production after 24 hours exposure to 20% LC$_{50}$ cadmium. Similar results were obtained for HSP74 and HSP76 of the 20% LC$_{50}$ cadmium exposure group, however the increase was not statistical significant. After exposure to 10% and 20% chromium and nickel, respectively, an increase in HSP74 and HSP76 was noted after 72 hours of exposure. If can therefore be argued that the damage caused by the metals happens before 96 hours exposure and allows for adaptation of the fish to the insult.

Pathological changes were more pronounced in the 10% LC$_{50}$ exposure groups compared to the 20% LC$_{50}$ exposure groups. This effect might be due to an increase in metallothioneins induction or induction of HSP to cope with the increase in metal concentration. Results obtained by Basson (2006) shows that there is a higher induction of HSP at the 20% LC$_{50}$ exposure groups which points to the ability of the fish to adapt quicker to a higher concentration of metal exposure. It was concluded that acute exposure to
low concentrations of metals did not overpower the liver's ability to detoxify these xenobiotic substances.

- **Gills**

  According to Pelgrom *et al.* (1995) gills are the primary uptake site of toxicologically effective concentrations of waterborne metals and are a crucial organ during induction of compensatory responses. Significant differences were found between the control group and 10% and 20% LC₅₀ cadmium exposure groups, and the 20% LC₅₀ exposure group respectively. The 10% LC₅₀ cadmium exposure group was also significantly higher than the 20% LC₅₀ cadmium exposure group, and the 20% LC₅₀ chromium exposure group.

  As mentioned before in section 7.3.2., the [TP] of the cadmium 20% exposure group was significantly lower than that of the control group. From the preceding results, it can be deduced that the low [TP] might in part be due to impaired water balance.

  The pathological effect was again more pronounced in the 10% LC₅₀ of both the cadmium and nickel exposure groups (similar effect was found in the liver). This effect was attributed to an increase in stress protein induction, such as metallothioneins and HSP, to cope with the increase in metal concentration, therefore protecting the gills from severe damage.

- **Ovaries**

  No significant differences in the organ index were found between the control group and the exposure groups. According to results obtained from Nath and Kumar (1989), the ovary might prove to be an effective biomarker for at a higher concentration of metal exposures. Further studies are however required in this regard.
Testis
Cadmium is a known endocrine disruptor (Richard et al., 1998; Sangalang and O'Halloran, 1972). No significant differences in the organ index were found between the control group and the exposure groups. Acute exposure to low concentrations of metals did not result in noticeable pathological changes to the testis.

7.4.3 Maturity of gonads

Biomarkers such as the condition factor (CF) and gonadal-somatic index (GSI) vary seasonally with the reproductive cycle. The histopathological assessment tool assesses all the developmental stages present, and will therefore also be affected by the maturity of the examined fish. All the specimens from all the exposure groups were in the same stage of development.

7.5 Conclusion

After 96 hours of exposure to 10% and 20% LC$_{50}$ chromium and nickel, respectively, no changes were found with regard to behaviour, CF, haematological parameters, NFHAI or the organo-somatic indices of the exposed fish. From these results it could be concluded that all the exposed fish were of similar health and condition as the control group fish.

Statistical significant difference was found between the control group and the 20% LC$_{50}$ cadmium exposure group with regard to the [TP] and NFHAI value. The [TP] calculated for the 20% LC$_{50}$ cadmium exposure was statistically lower was the calculated [TP] for the control group, which also resulted in a higher calculated NFHAI value. Histology analysis of the liver and the gills suggests that this is as a result of liver damage and impaired water balance.
Statistical significantly differences were found between the HSI calculated for the control group and the HSI calculated for the 10% and 20% LC$_{50}$ cadmium exposure groups. Although these values are significantly different from the values obtained for the control group, all values still falls within the range accepted as normal by the HSI. Qualitative histological analysis of the liver however did not show atrophy of the hepatocytes to confirm the low HSI obtained for the 10% and 20% LC$_{50}$ cadmium exposure group.

From the above mentioned results it can be concluded that general health of fish are not affected after acute exposure to a relative low concentration (10% and 20% of the LC$_{50}$) of Cd, Cr and Ni. The levels of the metals are not high enough to impair the physiological functioning of the organs. Test organisms are allowed to efficiently adapt to the insult. At higher concentrations of the metals, a more pronounce effect might be seen.

Qualitative histological analysis of the liver showed a moderate increase in hepatocytes hypertrophy in the 20% LC$_{50}$ cadmium exposure group, the 10% LC$_{50}$ chromium exposure group, and both the nickel exposure groups. These results confirm that even though the HSI of the fish from these exposure groups are in the normal range, fish are under stress and adapting to cope with the insult. Qualitative histological analysis is therefore a sensitive parameter for identifying physiological changes at cellular level, before a change in the organo-somatic indices can be identified.

Quantitative histological analysis of the gills showed the gill to be the primary site for damages after water born exposure to 10% and 20% LC$_{50}$ cadmium and 20% LC$_{50}$ chromium exposure. Similar histological, changes variant in degree, were identified in the gills from the cadmium exposure group and the chromium exposure group. This indicated that these histological changes are not metal specific.
The metal concentrations to which the test organisms were exposed are relatively low (10% and 20% of the LC$_{50}$ of the specific metals). From all the results obtained from this study it can concluded that after 96 hours of exposure to relatively low concentrations of cadmium, chromium and nickel the general health of the fish are not adversely affected. The exposed fish are able to adapt and cope with the insult. A toxic response was identified in the livers of the exposed specimens, but the liver retained the ability to detoxify. Histology of the gill proved to be a sensitive biomarker for low concentrations of metal exposure. At a higher concentration of metal exposure or after a longer exposure period, these results found during this study might be amplified.

**Final remarks**

The histopathological assessment tool allows for systematic analysis of the specific target organs. By applying this tool, it enables the researcher to statistically compare histological changes to target organs from different groups with each other, but still retains the ability to describe the different alterations found. The application of the histopathological assessment tool was useful in reaching the aim of this study: qualitatively and qualitative assess the histology of the selected organs after acute exposure to cadmium, chromium and nickel.

### 7.6 References


of Chinook salmon (*Oncorhynchus tshawytscha*). *Aquat. Toxicol.*, 76(3-4): 246-257.


## Addendum A
### Water quality

#### 10% Cadmium exposure

**Date of exposure:** 14 September 2004 to 13 October 2004

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<tr>
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Obtained from Rand water:  [http://www.reservoir.co.za](http://www.reservoir.co.za) (17/09/2005)

These values are a qualitative measure only analyzed at outlets of the Water Purification Works.
## 20% Cadmium exposure

**Date of exposure:** 10 November 2004 to 28 November 2004

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<tr>
<th>Parameter</th>
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<th>Mean</th>
<th>Median</th>
<th>Mean + 1SD</th>
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<td>µg/t</td>
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Obtained from Rand water: [http://www.reservoir.co.za](http://www.reservoir.co.za) (17/09/2005)

These values are a qualitative measure only analyzed at outlets of the Water Purification Works.
### 10% Chromium exposure

*Date of exposure: 16 March 2005 to 14 April 2005*

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<th>Parameter</th>
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<th>Mean - 1SD</th>
<th>Mean</th>
<th>Median</th>
<th>Mean + 1SD</th>
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These values are a qualitative measure only analyzed at outlets of the Water Purification Works.
# 20% Chromium exposure

**Date of exposure:** 18 May 2005 to 16 April 2005

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<th>Median</th>
<th>Mean + 1SD</th>
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<td>3.3</td>
<td>5.1</td>
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<td>6.8</td>
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<td>mg/t as Mn</td>
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<tr>
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<tr>
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<td>7.4</td>
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</tr>
<tr>
<td>Sulphate</td>
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</tbody>
</table>

Obtained from Rand water:  [http://www.reservoir.co.za](http://www.reservoir.co.za) (17/09/2005)

These values are a qualitative measure only analyzed at outlets of the Water Purification Works.
### 10% Nickel exposure

**Date of exposure:** 1 January 2005 to 3 February 2005

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units of measure</th>
<th>No of Samples</th>
<th>Mean - 1SD</th>
<th>Mean</th>
<th>Median</th>
<th>Mean + 1SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Trihalomethanes</td>
<td>µg/t</td>
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<td>21.9</td>
<td>30.9</td>
<td>31.9</td>
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<tr>
<td>Phenils C₆H₅OH</td>
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<td>2.6</td>
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<tr>
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<td>µg/t</td>
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<td>2.6</td>
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<td>3.2</td>
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<tr>
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<td>µg/t as Sb</td>
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<td>2.50</td>
<td>2.50</td>
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<tr>
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<td>µg/t as As</td>
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<tr>
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<tr>
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<tr>
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Obtained from Rand water: http://www.reservoir.co.za (17/09/2005)

These values are a qualitative measure only analyzed at outlets of the Water Purification Works.
### 20% Nickel exposure

**Date of exposure:** 10 February 2005 to 10 March 2005

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<tr>
<th>Parameter</th>
<th>Units of measure</th>
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<th>Mean</th>
<th>Median</th>
<th>Mean + 1SD</th>
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<tr>
<td>Dis.Organic Carbon</td>
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</tr>
<tr>
<td>Fluoride</td>
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<tr>
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<td>0.0</td>
</tr>
<tr>
<td>Nitrate</td>
<td>mg/t as N</td>
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<td>0.4</td>
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</tr>
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<td>Potassium</td>
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<td>Sulphate</td>
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<td>17.0</td>
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<td>0.0</td>
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<td>0.1</td>
</tr>
</tbody>
</table>

Obtained from Rand water: [http://www.reservoir.co.za](http://www.reservoir.co.za) (17/09/2005)

These values are a qualitative measure only analyzed at outlets of the Water Purification Works.
Addendum B

Calculations

Cadmium

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td><strong>Cd LC₅₀ (Cyprinus carpio)</strong></td>
<td>0.24 mg/l</td>
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<tr>
<td><strong>Cd Molecular mass (M)</strong></td>
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<td><strong>Cd mol mass (n)</strong></td>
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10% Cd exposure

10% of LC₅₀

Converted to g/l

Mass Cd in 1g CdCl₂

X CdCl₂ needed to yield 10% of LC₅₀

90 ℓ

900 ℓ

20% Cd exposure

20% of LC₅₀

Converted to g/l

Mass Cd in 1g CdCl₂

X CdCl₂ needed to yield 20% of LC₅₀

90 ℓ

900 ℓ
Chromium

<table>
<thead>
<tr>
<th>Chromium</th>
<th>0.108 mg/l</th>
</tr>
</thead>
</table>

Cr LC$_{50}$ (*Cyprinus carpio*)

Cr Molecular mass (M)  52
Cr mol mass (n)  294.19

10% Cr exposure

10% of LC$_{50}$  0.0108 mg/l
Converted to g/l  1.08E-05 g/l

Mass Cr in 1g K$_2$Cr$_2$O$_7$  0.1768 g
17.6757%

X K$_2$Cr$_2$O$_7$ needed to yield 10% of LC$_{50}$  6.11E-05 g

90 l  0.005499 g
900 l  0.054991 g

20% Cr exposure

20% of LC$_{50}$  0.0216 mg/l
Converted to g/l  2.16E-05 g/l

Mass Cr in 1g K$_2$Cr$_2$O$_7$  0.1768 g
17.6757%

X K$_2$Cr$_2$O$_7$ needed to yield 20% of LC$_{50}$  0.000122 g

90 l  0.010998 g
900 l  0.109982 g
Nickel

**Ni LC$_{50}$** (*Cyprinus carpio*) 10.6 mg/ℓ
**Ni Molecular mass (M)** 58.71
**Ni mol mass (n)** 237.7

**10% Ni exposure**

10% of LC$_{50}$ 1.06 mg/ℓ
Converted to g/ℓ 0.00106 g/ℓ

Mass Ni in 1g NiCl$_2$ 0.2470 g
24.6992%

X NiCl$_2$ needed to yield 10% of LC$_{50}$ 0.004292 g

90 ℓ 0.386247 g
900 ℓ 3.862473 g

**20% Ni exposure**

20% of LC$_{50}$ 2.12 mg/ℓ
Converted to g/ℓ 0.00212 g/ℓ

Mass Ni in 1g 0.2470 g
24.6992%

X NiCl$_2$ needed to yield 20% of LC$_{50}$ 0.008583 g

90 ℓ 0.772495 g
900 ℓ 7.724946 g