A preliminary investigation into the use of biomarkers and a fish community index to assess estuarine health in selected Eastern Cape estuaries

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By

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Abstract

The aims of this study were to determine the potential use of biomarkers at multiple levels of biological organisation together with a fish community bioindicator to assess the estuarine health status of three temporarily open/closed estuaries. The estuaries investigated were the East Kleinemonde (EK), Old Woman's (OW) and Mtana (MTN), all of which are situated in the Eastern Cape Province. Three biomarkers, the acetylcholinesterase (AChE) assay, lipid peroxidation (LPx) assay, liver histopathology and a condition factor were used to determine sub-organism health and one bioindicator, the Estuarine Fish Community Index (EFCI), was used as a bioindicator of community health. The estuarine-dependent marine species *Rhabdosargus holubi* was selected as an indicator species for the sub-organism level analyses.

The results from the community analyses indicated that the EK and OW estuaries were in 'good' condition, while the MTN was found to be in 'moderate' condition. Histological analyses revealed that *R. holubi* from all three estuaries showed signs of pathological changes to the liver, with the fish from the MTN eliciting the highest occurrence of these changes. The LPx assay found that *R. holubi* from both the OW and MTN showed signs of oxidative damage in the liver tissue, but those from the EK did not appear to be affected. The AChE assay showed that only the fish from the OW had been affected by anticholinesterase compounds.

A laboratory study was undertaken using *R. holubi* as a positive control for the AChE and LPx assay. The fish were exposed to 3 µg/L chlorpyrifos, a known cholinesterase inhibitor, for six hours and their tissues were examined for changes to LPx levels and AChE activities. AChE activity was significantly inhibited (Mann Whitney U test, $z = 3.65$, $n = 38$, $P < 0.001$) by the exposure, but LPx levels were not significantly affected.

A composite index incorporating the biomarkers at different biological levels of organisation was developed. The index was designed to assist managers and scientists to determine whether the ichthyofauna of a system was being affected by

environmental stressors and what management interventions could be undertaken to ameliorate the water quality in an estuary. The index was applied to the three estuaries investigated during the present study and both the OW and MTN were assessed to be in need of immediate management intervention.

The fish in the OW were found to be stressed at all the sub-organism levels measured and the reason for this was hypothesised to be as a result of golf course activities in this adjacent estuary. A number of management actions are proposed to reduce the sub-organism stress observed in the fish from the OW. The livers of fish from the MTN were shown to be under stress; however the causative agent of this stress was unknown because there is no formal development in the MTN catchment. However, a possible contaminant source is proposed and management interventions to alleviate the stress on the biota of the MTN are suggested. The EK does not require immediate management intervention, however, continuous routine monitoring is recommended to ensure that conditions do not deteriorate. Shortcomings of the index were outlined and a number of suggestions were made in terms of other measures of biological health which could be incorporated into the index.

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List of Abbreviations

ACh: Acetylcholine

AChE: Acetylcholinesterase

AHH: Aryl Hydrocarbon Hydroxylase

ALT: Alanine Aminotransferase

BChE: Butyrylcholinesterase

BHI: Biological Health Index

CAT: Catalase

CDI: Community Degradation Index

CF: Condition Factor

Cs: Carbamates

EC50: Effect Concentration: dosage at which the desired effect is present in 50% of the population being tested

EFCI: Estuarine Fish Community Index

EK: East Kleinemonde Estuary

EROD: 7-ethoxyresorufin O-deethylase

FAC: Fluorescent Aromatic Compounds

FRI: Fish Recruitment Index

GPx: Glutathione peroxidase

GSI: Gonad Somatic Index

H&E: Haemotoxylin & Eosin

HSI: Hepatosomatic Index

LPx: Lipid peroxidation

MAR: Mean Annual Run-off

MDA: Malondialdehyde

MFO: Mixed Funciton Oxidase

MMCs: Melanomacrophage Centres

MNLs: Mono-nuclear Leucocytes

MT: Metallothionein

MTN: Mtana Estuary

NHLS: National Health Laboratory Services

OP: Organophosphorous insecticide

OPP: Organophosphorous Pollutants

OW: Old Woman's Estuary

PAH: Polycyclic Aromatic Hydrocarbons

PCA: Principle Component Analysis

PCB: Polychlorobiphenyls

PSU: Practical Salinity Units

PUFAs: Polyunsaturated Fatty Acids

ROS: Reactive Oxygen Species

SL: Standard Length

SODs: Superoxide Dimutases

TBARS: Thiobarbituric Acid Reactive Substances

TOCEs: Temporarily Open/Closed Estuaries

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Chapter 1

General introduction

1.1 Introduction

Estuaries are highly productive systems that provide shelter for fish on an otherwise exposed South African coastline (Harrison *et al.* 2000). These systems are key environments for many fish species as they serve as nursery areas for juveniles. Unfortunately, estuaries are vulnerable to anthropogenic development on several fronts. Firstly, ever increasing numbers of people are using estuaries for recreational purposes which results in growing pressure on these systems (Lamberth & Turpie 2003). Secondly, anthropogenic developments along the banks of estuaries include urban, agricultural and industrial developments, all of which can have different impacts on estuaries (Niemi *et al.* 2004). Thirdly, estuaries may be impacted by upstream catchment activities due to agricultural runoff and industrial effluents entering these systems from inflowing river water (Niemi *et al.* 2004).

The dichotomy of needs between anthropogenic development and conservation of these ecologically important systems has resulted in government policies which aim to develop the coastline taking into consideration both needs (Integrated Coastal Management Bill 2002). Monitoring protocols which appropriately describe the health of estuaries are essential in order to determine the effectiveness of these policies and to measure the impact of various kinds of development on estuaries.

There are several ways to monitor estuarine health, e.g. using water chemistry data, sediment contamination data etc. However these measures provide limited insight into the state of the biota of a system (Vasseur & Cossu-Leguille 2003), e.g. chemical and sediment analyses are restricted in terms of the number of chemicals which can be tested for, and also give little to no insight into the effect these chemicals have on the biota of a system (Stentiford *et al.* 2003). The results of these analyses also provide no insight into the impact of habitat change, such as flow alterations, changes in food availability and predator prey relationships on the estuarine biota (Adams 2005).

Biomonitoring using sentinel species is increasingly becoming recognised as a more appropriate method for monitoring and assessing estuarine health. Biomonitoring techniques indirectly measure parameters such as water and sediment quality (these being the major vectors through which the biota of a system are exposed to xenobiotics) while at the same time providing insight into the biological condition of the estuary. The biomonitoring approach used during the present study investigated adverse biological effects which have been caused by xenobiotics and have had an impact on the fitness of the affected individual. These effects at the individual level provided an indirect measure of estuarine health.

Biomonitoring techniques used in the field are direct measures of the impact of xenobiotics on the biota of a system. Anthropogenic activities in and around estuaries tend to result in both point and non-point source pollution (Adams *et al.* 2000). The impact of these multiple stressors on the biota of an estuary are difficult to determine using traditional laboratory based toxicological techniques because these techniques lack ecological realism in terms of the various fluctuating parameters which naturally occur in the estuarine environment on both a short (e.g. temperature and salinity changes) and long term (e.g. sediment characteristic changes) basis (Adams *et al.* 2000). Another problem with the traditional laboratory based approach is that links between biological organisation (for example from cellular to organ level biomarkers) are seldom investigated (Adams *et al.* 2000) and therefore establishing the sequence of events that follow a pollution event is difficult because there is a lack of data from this field.

Biomonitoring can give a good indication of the general category of pollutant (e.g. pesticides versus polynuclear aromatic hydrocarbons) which is likely to be producing the effects observed, despite the fact that these techniques cannot directly identify the specific xenobiotics which are causing the disturbance. Using the findings of the biomonitoring study, the chemical or sedimentary analyses required to identify the pollutant that is causing the observed effect can be narrowed down, thereby saving time and being more cost effective. Biomonitoring studies also provide a weight-of-evidence approach to proposing cause and effect relationships between pollutant effects at lower levels of biological organisation to effects observed at higher levels (Collier & Adams 2003).

Adams & Greely (2000) provide two examples of the successful use of biomonitoring techniques using multiple levels of biological organisation. In the first example, a stream was investigated over a period of 10 years of remedial action instituted to lower the contaminant loading within the stream. The system had a distinct decreasing downstream gradient of pollution which changed over temporal and spatial scales through the duration of the study as remedial measures were put into place. The authors studied a suite of biomarker responses in fish, including cellular, physiological, histopathological, individual, population and community measures. Over this period, improvements in biomarkers responses were recorded at the same time as improvements in sedimentary and water quality parameters. Improvements were first seen at the cellular level and were then tracked across higher levels of biological organisation over time. All of these changes could be related to management actions undertaken to ameliorate the water quality and sediment contamination. Biomarkers of exposure, such as the mixed function oxidases (MFO) enzymes, decreased in a downstream direction along the decreasing contamination gradient. Individual level biomarkers, such as steroid concentration, also improved in a downstream direction. Community analyses also showed an increase in the distribution and abundance of sensitive species (which were excluded from certain sites prior to the remedial action due to high levels of toxicants in the water and sediments) over time. These species moved further upstream as the conditions in locations nearer to the source of contamination improved over time (Adams & Greeley 2000).

In the second study the Pigeon River was monitored over the period of nine years following remedial action to ameliorate the quality of discharged effluent from a paper mill (Adams & Greeley 2000). The authors used the same biomarkers as those used in the in the aforementioned study and could also trace spatial and temporal changes in all the biomarkers in conjunction with an improvement in water and sediment contamination criteria (Adams & Greeley 2000). They concluded that these types of studies demonstrate that spatial and temporal changes in contaminant levels in the sediment and water were reflected in terms of all the biological responses measured.

Thus, biomonitoring using multiple levels of biological organisation has been successfully used to monitor riverine recovery, thereby providing an indication of aquatic ecosystem health. Biomonitoring was used to measure the effect of remedial management actions and enabled causal relationships to be established between biomarker responses recorded in the field and contaminant loadings at those sites using a weight-of-evidence approach (Adams & Greeley 2000). A similar approach was adopted during the present study using fish community analyses from three temporarily open/closed estuaries (TOCEs) in the Eastern Cape and at the same time collecting a number of biomarker samples for analysis in the laboratory. During this study, estuarine health was determined using the ichthyofaunal component of each system and assessed according to the fitness (from the cellular level to the community level) of fish from each estuary as recommended by Elliott (2003). A reduction in fitness beyond a certain level was considered to be indicative of an unhealthy system (for more information on this level see Chapter 7).

A community analysis using the fish component of the estuarine biota was chosen as a bioindicator of estuarine health for the present study. The fish community structure of an estuary was used to indicate the departure from reference conditions of a community and was used to describe detrimental changes to an estuary. Sub-organism biomarkers were used to determine the health status of the fish from each estuary. Biomarkers are defined as:

"A change in a biological response (ranging from the molecular through cellular and physiological responses to behavioural changes) which can be related to exposure to or toxic effects of environmental chemicals" (Van der Oost *et al.* 2003 p59).

The biomarker approaches used in this study were the acetylcholinesterase assay (AChE assay), the lipid peroxidation assay (LPx assay), condition factor and histological examination of the liver. These biomarkers, along with the community analysis results, were then combined to determine the health of the three estuaries investigated. A brief overview of why these particular techniques were chosen, as well as an outline of the selected study sites, is given below. There is also a brief explanation of why the estuarine-dependent marine species *Rhabdosargus holubi* was chosen as an indicator species and finally the aims and objectives of this study are outlined.

1.2 The use of fish community indices to measure estuarine health

Several fish community indices have been used internationally to determine aquatic health. Adams *et al.* (2005) measured the recovery of a polluted stream in America over 15 years using both fish and invertebrate communities as indicators of improvement to the water quality. The fish community component of the above study looked at species richness and presence of sensitive species, and concluded that in terms of the attributes studied, fish communities were sensitive indicators of water and sediment contamination. Adams *et al.* (2005) also showed that as contaminant loading decreased over the recovery period (15 years), an improvement in species diversity and an increase in sensitive species was recorded. The authors highlighted, however, that in order to be able to detect and demonstrate causal relationships between changes in an environment and the responses exhibited by the biota, several levels of biological organisation (such as organ level indicators of health, biochemical indicators etc) need to be included (Adams *et al.* 2005).

Elliott *et al.* (1988) used a fish community functioning and carrying capacity index in order to determine the effect of management actions undertaken in the Fourth estuary in Scotland. These management actions were to lower contaminant levels in the water to environmental quality standard levels. The community integrity index was calculated as a ratio of secondary producer production to fish consumer production (Elliott *et al.* 1988). The authors found that the ecological efficiency calculation (ratio of fish to secondary producers) was a useful indicator of ecosystem health but highlighted that this index was highly susceptible to changes in habitat quality through, for example, loss of highly productive mud-flats (which may not be a direct result of water quality issues).

O'Connor *et al.* (2000) investigated the use of multiple taxonomic groups to determine ecosystem health in 19 lakes in New England, USA. Using a range of indices, they assessed the condition of assemblages of diatoms, zoobenthos, zooplankton, fish and birds in terms of anthropogenic disturbance (e.g. zooplankton communities were examined for size ratios, trophic links and species richness). The percentage of intolerant, omnivores and piscivorous species were measured in fish communities, as well as the native species richness and the total species richness. The

results of the above study suggested that each taxonomic group was individually sensitive to different types of disturbances. Unfortunately the sample sizes were too small to obtain significant correlations between the effects of various anthropogenic impacts (such as pH and nutrient changes) and the community being studied (e.g. diatoms). The only strong correlation reported was fish distribution and disturbance of riparian-littoral condition in the lakes (O'Connor *et al.* 2000), thus indicating that adult fish were sensitive to changes in ecosystem health.

Fish larvae have also been used as indicators of ecosystem health. Quinn *et al.* (1999) developed a Fish Recruitment Index (FRI) to assess the fresh water flow requirements of South African estuaries to ensure successful recruitment of larval and juvenile fish into estuaries. This index was developed taking into consideration optimal recruitment periods for a number of taxa (generally spring and summer) as well as the degree of estuarine dependence of the species. In addition to this, it was recognised that the largest amount of recruitment occurred during periods of moderate to high fresh water flow which coincided with a steep salinity gradient along the estuary. Thus periods of open mouth conditions, together with a steep salinity gradient along the estuary during the spring/summer, resulted in maximum fish recruitment (Quinn *et al.* 1999). The FRI was based on several assumptions and the authors cautioned against using it as an absolute representation of the actual recruitment into estuaries. It did, however, provide managers with a clearer idea of the impact of freshwater flow alterations on the recruitment success into estuaries for a number of marine species. The FRI also indicated that when freshwater flow decreased by more than 50% there was a dramatic decrease in fish recruitment success for particular estuaries (Quinn *et al.* 1999). Water abstraction is a major concern for the health of South African estuaries (Grange *et al.* 2000, Scharler & Baird 2003), and a decrease in larval recruitment could have important consequences for the overall fitness of estuarine-dependent marine species.

A more recent study by Strydom & Neira (2006) also found that larvae of *Glossogobius callidus* and *Redigobius dewaali* have the potential to be used as indicators of estuarine health. The authors surveyed seven open estuaries and five intermittently open estuaries in the warm temperate region of South Africa and determined that the distribution of the above species was primarily determined by

estuary type and freshwater input into each estuary (Strydom & Neira 2006). It was shown that freshwater was a major driving factor in determining the distribution of these two species because river pulses trigger spawning in the adults (Strydom & Whitfield 2000) and also because estuaries receiving more fresh water inflow have higher productivity in terms of primary and secondary producers (Grange *et al.* 2000). Neira & Sporcic (2002) also assessed the use of ichthyoplankton as indicators of ecosystem health. The authors compared two data sets collected during 1983/1984 and 1995/1996 from Port Philip Bay, Australia. An interesting finding was that the larvae of *Neoodax balteatus* was linked to the occurrence of the distribution of the exotic polychaete worm *Sabella spallanzanii* (Neira & Sporcic 2002). Biological 'pollution' has been listed as an increasingly important new pollutant source in many estuaries around the world (Elliott 2003). This type of pollution can have direct effects on the fitness of fishes, and therefore the results from the Port Philip Bay study are significant because they demonstrate that fish larvae are useful indicators of ecosystem change.

Fish community analyses have been used to determine estuarine health in South Africa for a number of years. Ramm (1990) developed the Estuarine Community Degradation Index (CDI) and successfully applied it to 62 estuaries in KwaZulu Natal. The index worked on an observed (field collected data) versus expected (reference conditions) approach to the presence and absence of species in the estuaries. The CDI looked at dissimilarity between the expected and observed values. The index highlighted the fact that the estuaries under investigation had to be assigned hydrological categories (for example using characteristics such as catchment size, catchment soil characteristics etc) before they could be analysed using this method (Ramm 1990).

Cooper *et al.* (1994) further developed the CDI into the estuarine Biological Health Index (BHI). This index still used presence/absence data but the reference conditions were defined by the least impacted representative estuarine community in the area. The BHI looked at similarity rather than dissimilarity between an estuary and reference conditions, and also incorporated an importance rating (in terms of the ichthyofaunal composition) for each estuary in a given area. This community index also recognised the importance of delineating estuary groupings using hydrology (Cooper *et al.* 1994).

Harrison *et al.* (2000) developed the Estuarine Fish Health Index which was the first index to have both qualitative and quantitative comparisons of communities to reference communities in South African estuaries. The reference conditions in this index were based on the most frequently occurring taxa in a particular type of estuary, and excluded any exotic or introduced species because these were considered to be a result of human interference. This index also incorporated other estuarine health aspects such aesthetic alteration (in terms of anthropogenic development around the estuary) and water quality (in terms of dissolved oxygen, oxygen absorbed, ammonia nitrogen, faecal coliforms, nitrate nitrogen and ortho-phosphate) (Harrison *et al.* 2000).

The EFCI (Estuarine Fish Community Index) used during the present study is a more focused index that includes aspects such as trophic integrity, which were not present in previous South African estuarine health assessments. The EFCI includes important factors such as defining estuaries in terms of zoogeography and hydrology, both of which have been shown to be the principle driving factors in terms of community assemblages within South African estuaries (Ramm 1990, Cooper *et al.* 1994, Harrison *et al.* 2000). The index primarily uses the composition, diversity, nursery function and trophic integrity of an estuarine fish community and this is compared to reference conditions established for that type of estuary in a particular biogeographic region (Harrison & Whitfield 2004, Harrison & Whitfield 2006). Once the scores for all the metrics are added up, the final estuarine health rating can then be determined and an estuary can be classified as critical, very poor, poor, moderate, good or very good (for more information on this index refer to Chapter 2).

The reason why the EFCI index was chosen for this study was because it was the most detailed and appropriate of all the indices described above. Using an index that was designed specifically for the South African context, taking into account differences in terms of zoogeography, hydrology and estuary size, was considered the most appropriate way of evaluating the integrity of the fish community in the three study estuaries.

1.3 Histological biomarker techniques

Community indices have been criticised because they only measure a stress after it has occurred and resulted in a change in the community structure and do not provide any indication of the type of stressor which caused the change (Adams *et al.* 2005). Biomarkers have been proposed as a method of detecting sub-organism level stress before it becomes apparent at the population or community level (Adams *et al.* 2005). Biomarkers are used on individual organisms and examine processes at lower biological organisation scales than monitoring tools such as the EFCI. Biomarkers can therefore act as 'early warning systems' for managers to intervene and reverse or prohibit a stressor before effects of that stressor are measured at the population level. Biomarkers measure the fitness of an individual within a population at the sub-organism level, and negative effects on the fitness at this level can be related to the overall health of the estuary.

Liver histopathology enables researchers to detect different types of stressors on the liver and can be used to narrow down the likely pollutants which are causing damage at the organ level. In this way pollutant induced stress can be detected before any damage becomes apparent at the population or community level (Stentiford *et al.* 2003).

Liver histology was chosen as the sub-organism indicator of health, because the liver is of key importance to the overall homeostasis of the body in terms of nutrition, defence against toxicants and reproductive development (Hinton & Lauren 1990). It is the primary site of detoxification and therefore greater exposure to xenobiotics results in more liver damage. The liver was considered to most appropriately reflect the extent to which an organism has been exposed to toxicants. The degradation of each liver analysed was undertaken according to methods described in Van Dyk *et al.* (2007) where the liver is assigned to one of five categories, with zero being the lowest and four being the highest sign of degradation (see Chapter 3 for more information on this method).

Estuarine monitoring using fish liver histopathology has been successfully applied in several international studies. For example, Stentiford *et al.* (2003) successfully used histopathological biomarkers in the liver to determine estuarine health in four

estuaries in the UK. The authors identified several lesions in three species of fish (*Platichthys flesus, Pomatoschistus minutus* and *Zoarces viviparous*) which had been shown by laboratory and mesocosm studies to be directly related to exposure to xenobiotics. Their study showed that histopathological studies were powerful tools in estuarine health assessments.

Malins *et al.* (1998) conducted a five year study of the development of hepatic (liver) neoplasm and other diseases in the English sole (*Parophrys vetulus*) from the Puget Sound, United States. The authors reported significant correlations between the number of lesions (for example neoplasms, foci of cellular alterations, megalocytic hepatosis[1]) and the concentration of aromatic compounds such as benzo(*a*)pyrene in the bile (which is produced in the liver). These chemicals were also detected in the sediments at the sampling sites and the results suggest that contaminated sediments result in the occurrence of hepatic neoplasia in flatfish (Malins *et al.* 1988). Stehr *et al.* (2003) conducted a similar study and found that bile metabolites containing traces of xenobiotics, as well as sediment contamination, were correlated with the occurrence of certain pathological lesions in *P. vetulus* in Canada.

Myers *et al.* (1998) undertook a study on sub-adult English sole (*P. vetulus*) in the Puget Sound and were able to quantify a number of preneoplastic lesions in the liver which are possible precursors to neoplasms that are a result of exposure to certain xenobiotics. The authors successfully showed that biochemical responses (in biomarkers) could be used to indicate the susceptibility to the development of preneoplastic lesions, indicating that certain biomarkers are early warning systems for organ level changes as a result of exposure to xenobiotics.

Thus histopathological studies have successfully been applied in biomonitoring programs in estuaries as well as in the marine environment. This is a well established technique which uses data generated from laboratory and mesocosm experiments, as well as field based observations to determine causative effects of contaminants at the organ level.

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[1] For more information on these lesions please see Appendix 1.

Damage in the liver, which has several important functions in terms of overall fish homeostasis, was considered to be a good biomarker to determine fish health and could then be used to infer estuarine health. This approach provides a definite biological end point of exposure and can be used to determine the type of stressor which is causing the damage observed (Stentiford *et al.* 2003). Estuarine health was measured from the cellular to the community level during this study, and although these different levels do not provide the same level of information in terms of determining the overall health of the system, damage to the liver has the potential to affect fish populations and therefore the estuarine community.

Condition factor is a coarse measure of the nutritional status of an organism (Kirby *et al.* 2000). It is based on a length-weight relationship which can give an indication of the feeding level of an organism. This is because fish weight and not length changes according to feeding frequency and can therefore provide a measure of the condition of a fish (Blaber 1975). Fish condition may be used to give an indirect indication of the condition of an estuary, because estuaries are considered to be food rich environments which provide refuge areas for juvenile fish (Paterson & Whitfield 2000). Thus if the condition of a fish is poor, the estuarine environment is not fulfilling its role as a food rich environment and can also be considered to be poor.

1.4 Cellular biomarker techniques

Cellular level biomarkers are a measure of recent exposure to xenobiotics and can be indicative of a narrow range of pollutants. Recent pollution occurrences, particularly brief low dose events, may not be measured at the other levels of biological organisation, but are important components of estuarine health assessments because cellular biomarkers are a measure of the fitness of a sub-sample of the biological component of an estuary.

All animals contain a suite of enzymes that allow them to biotransform xenobiotics (Livingstone 1998). They convert hydrophobic lipid-soluble xenobiotics into water-soluble excretable products, generally in a two step process involving phase I and II metabolism. The enzymes involved in phase I metabolism undertake reduction and oxidation reactions as well as hydrolysing and hydrating the xenobiotics. This is

achieved by adding hydrophilic functional groups to the xenobiotics (Livingstone 1998). The phase II metabolism enzymes add larger polar molecules such as glutathione to the xenobiotic, thus enabling the new modified xenobiotic to be excreted from the cell (Livingstone 1998). Each xenobiotic reacts differently with phase I and II enzymes. The secondary and tertiary compounds resulting from interactions between the enzymes and the xenobiotics can be used to test for exposure to certain contaminants. Generally, the products being tested for in the biomarker assays are there as a result of damage or impairment to cellular functioning.

Cellular level biomarkers have been defined as "sub-lethal biological measures of the response to, and effects of, pollutants in living organisms" (Napierska & Podolska 2005 p759). These biomarkers are advantageous because they are used on individual organisms and examine processes at lower biological organisation scales than monitoring tools such as the EFCI and histology. A range of biomarkers are now commonly used, varying from the biomolecular/biochemical level (for example stress proteins, DNA integrity, endocrine disruptors) to the individual physiological level (total body lipids, condition factors etc) (Adams & Greeley 2000).

The two cellular level biomarkers selected for this study are generalised expressions of a response to stress; the acetylcholinesterase activity assay (AChE assay) and the lipid peroxidation assay (LPx assay). The AChE assay is an indicator of exposure to a range of xenobiotics including pesticides, insecticides, metals, organochlorines and surfactants. The acetylcholinesterase enzyme binds with the xenobiotic and is unable to degrade the neurotransmitter acetylcholine (Pan & Dutta 1998). The AChE assay measures the extent of enzyme inhibition caused by exposure to xenobiotics (for more information on this assay see Chapter 5). Acetylcholinesterase inhibition can have direct impacts in the fitness of an individual because acetylcholinesterase inhibition decreases the motor abilities of the affected individual. The ability of a fish to avoid predators and forage become compromised and therefore it becomes more vulnerable to predation, starvation and disease (Pan & Dutta 1998). This is an important component that contributes to the overall assessment of estuarine health.

The lipid peroxidation (LPx) assay measures the amount of oxidative damage an organ has undergone from oxidative stress. The measured oxidative damage is a result of damaged lipids in cellular membranes, which can impair the cells ability to continue normal functions. Oxidative damage occurs as a result of exposure to different types of pollutants, that include quinones, certain dyes, bipyridyl herbicides, certain transition metals, aromatic nitro compounds and some pesticides (Kelly *et al.* 1998, Dorval *et al.* 2005, Valavanidis *et al.* 2006) (for more information on this assay see Chapter 4).

These above two assays were chosen for this study because they are relatively generalised and can be used to detect different types of pollutants. The AChE assay was chosen to detect pesticides, particularly because one of the sites investigated had suspected pesticide pollution. The LPx assay was chosen because it related to the liver histological study. Both the histology and LPx level biomarkers use liver tissue, and it was hypothesised that effects at a higher level (tissue) should be measurable at a lower level (cellular). In addition, if damage is measured at the lower cellular level but no damage is recorded at the organ level, this may indicate that the stress has been of limited duration, or of limited concentration, because effects cannot be seen at the organ level as yet. This type of information is important because it can give a more quantitative measure of the type of stressor that is affecting the liver.

1.5 Selection of an indicator species for the biomarker studies

Borotone *et al.* (2005) suggest that a number of factors should be considered when choosing an indicator species. These include the life stage of the organism which will be used (e.g. larvae, juveniles or adults), the level of biological organisation which will be considered (cellular, organ level, population and community), the trophic status of the organism (primary, secondary or tertiary consumer), the estuarine dependency of the species chosen (estuarine resident, estuarine-dependent marine species, catadromous species, marine species which sometimes occur in estuaries or freshwater species which sometimes occur in the estuarine environment), sensitivity of the species, and prior information regarding the species (Bortone *et al.* 2005).

The Cape stumpnose (*Rhabdosargus holubi*) was selected as an indicator species for the present study for several reasons:

1. This species occurs in estuaries during the juvenile stage and negative effects of xenobiotics at this stage of development (for example damage to the liver) could affect the future ability of this species to combat other toxicant insults. Xenobiotics may also affect future fecundity and thus result in population effects.
2. Several levels of biological organisation were being investigated (cellular, organ level and community); therefore it was necessary to select a species which occurs in large numbers and is relatively easy to catch. This is the case with *R. holubi* (De Kock & Lord 1988).
3. *R. holubi* is a primary and secondary consumer, mainly feeding on aquatic macrophytes, filamentous algae, crustaceans, bivalves and polychaetes (Whitfield 1998). A generalist consumer can give a good indication of the condition of an estuary because it is exposed to a variety of food chains and therefore a variety of potential toxicants.
4. *R. holubi* is an estuarine-dependent marine species and, as such, is representative of several other estuary-associated marine fishes. Many of these species are important to both subsistence and commercial fisheries in South Africa and therefore any detrimental effects which are occurring during their development within estuaries can have important management consequences for marine fish stocks (Lamberth & Turpie 2003).
5. No toxicity testing has been conducted using *R. holubi* except for a study which investigated the kinetics of uptake and elimination of certain polychlorinated biphenyls (De Kock & Lord 1988).
6. Although little is known about the sensitivity of *R. holubi* to xenobiotics, the biology and ecology of the species is well understood (Whitfield 1984, Cyrus & Blaber 1987a, Cyrus & Blaber 1987b, Cowley *et al.* 2001, Lukey *et al.* 2006).

Thus it can be seen that *R. holubi* complies with most of the criteria listed by Bortone *et al.* (2005) for an indicator species and it was therefore chosen as an indicator species for this project.

1.6 Study sites

The East Kleinemonde, Old Woman's and Mtana estuaries were the three sites chosen for this study. All three estuaries are situated on the Eastern Cape coast and are similar in size and geomorphology. They are moderate to large systems (surface area of between 2 and 150 ha) and are classified as temporarily open/closed estuaries (TOCEs) or type 1B estuaries (Harrison *et al.* 2000). The three systems obtained the same score when assessed using the Biological Health Index, Estuarine Water Quality Index and the Aesthetic Health Index (Harrison *et al.* 2000).

The estuaries were chosen because they are representative of the medium-sized TOCEs in the Eastern Cape. These types of systems typically have urban or recreational developments around the perimeter of the estuary but no heavy industry is present. The selected sites are in close proximity to each other and in the same biogeographical area (warm temperate zone). They are also similar in terms of hydrology and mouth morphology, which is an important factor to consider in terms of comparability of estuaries. Mouth state is a primary determinant in terms of species diversity and composition in these types of systems (Harrison & Whitfield 1995, Cowley & Whitfield 2001a , Vorwerk *et al.* 2001).

The East Kleinemonde estuary (Figure 1.6.1) has a surface area of approximately 35 ha, a catchment size estimated at 46.3 km^2 and mean annual runoff of approximately 2 x 10^6 m^3 (Cowley & Whitfield 2001a) (Table 1.6.1). The catchment area is used primarily for cattle grazing and the river and estuarine slopes are covered by valley bushveld vegetation (Cowley & Whitfield 2001b). Higher up in the catchment there are pineapple farms which probably apply an array of pesticides and herbicides on the crops. The East Kleinemonde also has a small residential community which lives in houses surrounding the estuary. The town is a popular holiday destination during school vacations when the estuary becomes the focus of recreational activities. The East Kleinemonde estuary has been the focus of a large amount of research on many aspects (e.g. juvenile fish recruitment (Bell *et al.* 2001), fish community composition (Vorwerk *et al.* 2001), invertebrate community composition (Teske & Wooldridge 2003).

Figure 1.6.1: Aerial photograph of the East Kleinemonde estuary (upper estuary in the photograph) showing the residential development around the mouth of estuary.

The Old Woman's (Figure 1.6.2) estuary is separated from the sea by a low, approximately 100 m wide sand berm. Sea water only enters the system during storm overtopping events or when the sand bar breaches following river flooding. Salinities in the mouth region are approximately 35 psu (Harrison *et al.* 1997) and the estuary has a surface area of approximately 25.1 ha and a mean annual runoff of $1.16 \times 10^6 m^3$ from the catchment (Table 1.6.1). During a 1997 survey, the overall water quality was described as 'good' (apart from some *Escherichia coli* detected in the water column) and had an estuarine health score of 8 out of 9 (Harrison *et al.* 1997). The estuary is situated within the Fish River Sun holiday complex and is bordered by a golf course. It also has two road bridges and one foot bridge which cross it in the upper reaches. Due to the presence of the golf course it was hypothesised that the fishes in this system would be under more physiological stress than the fishes in other systems because of the possible contamination by the pesticides (such as Chlorpyrifos, Hydramethylnon, Cyfluthrin, Trifluralin, Chlorothanonil) used on the golf course.

Figure 1.6.2: Aerial photograph of the Old Woman's estuary showing the golf course area along the middle and upper reaches of the system.

The Mtana estuary (Figure 1.6.3) is a poorly studied system that is infrequently open to the sea and separated by a 150-200 m wide sand berm between it and the sea. The catchment of this estuary is undeveloped, with local rural communities using the area to graze their cattle. The estuary has a surface area of approximately 15.7 ha and a mean annual runoff of 2.29 x 10^6 m^3 from the catchment (Table 1.6.1). The water quality index obtained during a 1997 survey was rated as 'good' but was moderately impaired due to the presence of *E. coli* in the water column (Harrison *et al.* 1997). The overall score allocated to the system during the 1997 survey was also 8 out of 9 which reflects the virtually undeveloped estuary surrounds and catchment.

Table 1.6.1: Overall physical characteristics of each estuary

Estuary	Size (ha)	Mean Annual Runoff	Catchment activities
East Kleinemonde	35	2 x 10^6 m^3	Cattle grazing, pineapple farming, residential development
Old Woman's	21.5	1.16 x 10^6 m^3	Golf course
Mtana	15.7	2.29 x 10^6 m^3	No anthropogenic development, some cattle grazing

Figure 1.6.3: Aerial photograph of the Mtana estuary.

1.7 Aims and objectives

This study was a pilot project aimed at determining the appropriateness of various biomonitoring tools for assessing the health of Eastern Cape TOCEs. The tools used measured responses to stressors across a range of biological organisation levels (from the biochemical level to the community level) in order to assess cumulative, synergistic and/or antagonistic effects in the environment (Adams *et al.* 2000). This approach provides information on past and present stressors and enables researchers to detect response integration across biological levels of organisation. Causal relationships can be elucidated between pollutants and their impacts on estuarine communities at different levels of biological organisation (Adams *et al.* 2000). Thus, in comparison to the other methods mentioned, the biomarker approach uses the early

warning capacity of the biomarker techniques as well as community indices to generate an overall picture of the health of an estuary.

A primary purpose of this study was to compare three approaches to biomonitoring estuaries; a community based index, a histological (organ level) indicator and two cellular level biomarkers. The condition factor of each fish was also calculated as a coarse measure of the individual level health of each fish. These measures were then compared and the feasibility of extrapolating results from lower levels of biological organisation to the community level as a measure of estuarine health was investigated. This study highlights the positive and negative aspects of each assessment method and the sensitivity of each method is discussed.

The principal aims of this project were:

1. To determine the health of the East Kleinemonde, Old Woman's and Mtana estuaries in terms of the selected parameters.
2. To assess the applicability of the biomarker approach in determining the estuarine health of three estuaries and to evaluate the different methods tested.
3. To investigate differences in terms of community structure, liver histology of individual fish, acetylcholinesterase activity and lipid peroxidation levels in fish between the estuaries.
4. To propose a tiered biomonitoring approach using different levels of biological organisation to provide an assessment of estuarine health.

Chapter 2 of the thesis deals with the results from the community analysis, while Chapter 3 is focused on the histology analyses. Chapter 4 describes the LPx assay results as well as the condition factor results. Chapter 5 describes the AChE assay results. Chapter 6 details the laboratory exposure work undertaken using *R. holubi* and Chapter 7 integrates the results from Chapters 2-6 to provide an overall indication of the health of each estuary. Chapter 7 also proposes a tiered approach for an integrated biomarker and community level health assessment index that can be applied to South African estuaries.

Chapter 2

Biomonitoring using community indices

2.1 Introduction

Estuaries are traditionally described and assessed using physical, chemical and biological parameters. Physico-chemical measures give limited insight into the biological health of an estuary which is an important component of the overall condition of the estuary (Vasseur & Cossu-Leguille 2003). Biological measures of estuarine condition incorporate a range of biotic and abiotic factors and are therefore the most promising measures of estuarine health and integrity (Vasseur & Cossu-Leguille 2003). The use of biological communities is justified in Harrison & Whitfield (2004), with the principal reasons being that the biota are sensitive indicators of a wide range of environmental stressors, and portray the cumulative result of all these stressors on the community being studied. Fish community analyses are particularly promising in terms of their potential as indicators of estuarine health (Whitfield & Elliott 2002, Bortone *et al.* 2005). The advantages proposed by Harrison & Whitfield (2004) of using fish as biological indicators are that;

- Fish are found in most aquatic habitats except severely polluted ones;
- Fish are easy to identify and there is substantial information on their biology and ecology;
- Fish are relatively long lived and can provide a long-term record of exposure to stressors;
- Fish are generally found to exhibit morphological and physiological responses to stress;
- Presence and absence patterns can be interpreted in terms of pollution concentration gradients;
- Fish communities are generally composed of several trophic levels which have the potential of indicating bioaccumulative effects of pollutants;
- People are more likely to relate to fish than other biological communities such as invertebrates (Harrison & Whitfield 2004);

- Changes to or loss of habitat will directly affect the fish community and can therefore be measured;
- Changes to water quality in terms of, for example, turbidity will also directly affect certain species and can be measured using fish.

There are also a number of disadvantages associated with using fish which have been outlined in Harrison & Whitfield (2004);

- Gear selectivity may eliminate certain species from a sample due to their size;
- Certain substances may be harmful to some species but may not affect fish;
- Fish move around and this may introduce sampling error due to the uncertainty regarding the duration of stay at the site of capture (unless the fish have been acoustically tagged);
- Even in severely impacted sites, the composition of a fish community may still be diverse and therefore may not reflect the extent to which a site has been altered (Harrison & Whitfield 2004).

Despite these disadvantages, fish communities have been successfully used to determine the health status of aquatic environments (Ramm 1990, Niemi *et al.* 2004, Adams 2005, Harrison & Whitfield 2006).

The community index which was used as a bioindicator during the present study was the Estuarine Fish Community Index (EFCI) (Harrison & Whitfield 2004). It has been specifically designed for South African estuaries and enables scientists to communicate large amounts of data on a particular estuary or a group of estuaries to managers in a clear and simple manner. The EFCI is a community index based on 14 metrics which together give an in-depth analysis of the ichthyofaunal community structure of an estuary. It analyses abundance data of species composition as well as presence/absence data and compares this to a set of zoogeographically and hydrologically specific reference conditions. The scores for each individual metric are then combined and a final score between 16 (poor) and 68 (very good) is obtained. The EFCI has been validated through a long term study of the previously highly polluted Sezela Estuary, KwaZulu Natal, which demonstrated the sensitivity of the EFCI as well as its reproducibility (Harrison & Whitfield 2004). It is important to note that this is a rapid assessment method that can produce either a single 'snap shot'

or a series of 'snap shots' on the changing state of an estuary. This data can be used to determine whether further study or attention to a particular region or estuary is required.

The EFCI is calculated using four basic fish community characteristics; species community and diversity, species composition, nursery function and trophic integrity (Harrison & Whitfield 2004). A total of 14 metrics (Table 2.1.1) based on these four characteristics are used to assess the state of an estuary. Each metric has the same weighting. Fish collection methods are described later in the methods section.

Table 2.1.1: An outline of the 14 metrics in the EFCI (Harrison & Whitfield 2004).

Category	Metric No.	Description
Species diversity and composition	1	The total number of taxa
	2	The presence or absence of rare or threatened species
	3	The presence of exotic or introduced species
	4	The similarity of the species composition of an estuary to a reference community
Species proportions	5	The proportion of species in an estuary in relation to a reference community
	6	The number of taxa required to make up 90% of the total abundance (as a measure of dominance)
Nursery function	7	The number of estuarine resident taxa
	8	The number of estuarine-dependent marine taxa
	9	The relative abundance of estuarine resident taxa
	10	The relative abundance of estuarine-dependent marine taxa
Trophic integrity	11	The number of benthic invertebrate feeding fish species
	12	The number of piscivorous taxa
	13	The percent abundance of benthic invertebrate feeding fish species
	14	The percent abundance of piscivorous fish species

The species diversity and composition metrics indicate the nature of the community. The number of taxa (metric 1) is a direct measure of diversity. The presence of rare or threatened species (metric 2) is often indicative of an undisturbed environment because these taxa are often more specialised than the rest of the species and tend to be the first to be forced out of a system under stressful conditions (Harrison & Whitfield 2004). Metric 3 gives an indication of artificial introductions into the estuary and the extent to which the fish assemblage has deviated from the natural species composition. Metrics 4, 5 and 6 provide a measure of the degree of departure

from the pristine fish assemblage composition within an estuary. Metric 4 allows the investigator to assess the amount of similarity between the reference and study communities, whilst metric 5 gives a quantitative assessment of metric 4. Metric 6 focuses on the dominant taxa in an estuary and can also be used to identify which species are missing from this group when compared to reference conditions. The nursery function of estuaries is imperative for both estuarine-dependent marine species and estuarine resident species. The measure of how many species from these two groups are present (metrics 7 & 8) permits an assessment of the importance of a particular estuary to both groups of fish. The relative abundance of both the groups (metrics 9 & 10) reflects the amount by which one group dominates over another. The trophic integrity metrics (metrics 11-14) are an indirect measure of the zoobenthic and ichthyofaunal productivity of an estuary.

All of the above metrics are calculated by comparing field data to reference conditions that were developed by taking into consideration zoogeographical and hydrological differences between South African estuaries. Three zoogeographical regions were identified, namely cool temperate, warm temperate and subtropical regions (Harrison 2002). Within these regions, three primary estuarine morphologies were identified, namely permanently open estuaries, moderate to large closed estuaries and small closed estuaries (Harrison & Whitfield 2004). Reference conditions were established for all nine of these categories. These reference conditions were developed from a data set collected during a 'State of South African estuaries' study (Harrison *et al.* 2000) which sampled most South African estuaries. Using this dataset, the highest or 'best' metric values from all the estuaries within a particular category were selected as 'best' reference conditions. The thresholds for each metric were then calculated as good (score of 5, similar to reference conditions), moderate (score of 3, different from reference conditions) or poor (score of 1, considerably different from reference conditions) (Harrison & Whitfield 2004).

The three selected estuaries fall within the warm temperate zoogeographic area and are all classified as moderate to large closed estuaries (for more information on these study sites please refer to Chapter 1).

2.2 Methods

Each estuary was sampled during the day in April 2005 using a 30 m x 1.7 m x 15 mm bar mesh seine net fitted with a 5 mm bar mesh cod end. A fleet of gill nets (each gill net was 10 m x 2 m, with three equal length panels of 45 mm, 75 mm and 100 mm stretch mesh) were deployed overnight and the fish collected the following morning. Fish were identified according to Smith & Heemstra (1986). The number of seine net hauls and the number of gill nets used in each system depended on the size of the estuary and the availability of appropriate sites. Seine netting was conducted from the mouth to the upper reaches of each estuary until no new species were caught. Gill nets were placed between the lower and upper reaches. Ten seine hauls were conducted in the East Kleinemonde, and five gill nets were deployed overnight. Five seine net hauls were conducted in the Old Woman's estuary, and three gill nets were used in this estuary. Finally, seven seine net hauls were conducted in the Mtana estuary and four gill nets were deployed overnight.

Fish caught using the seine net were counted and identified to species level before being released back alive into the estuary. Fish caught using the gill nets were measured and identified to species level. The location and number of seine net hauls and gill nets used during the present study were the same as those used during the State of South African Estuaries study conducted by Harrison *et al.* (2000) to ensure comparability of data.

2.3 Results

A total of 3728 fish from 18 taxa were caught in the East Kleinemonde Estuary (EK), with the numerically dominant taxa being *Atherina breviceps, Gilchristella aestuaria* and *Rhabdosargus holubi* (Table 2.3.1). In the Old woman's Estuary (OW), a total of 903 fish from 15 taxa were recorded, with the numerically dominant taxa being *A. breviceps, Glossogobius callidus* and *R. holubi* (Table 2.3.1). Finally, 5010 fish were sampled in the Mtana Estuary (MTN) from 20 species. The catches were dominated by large numbers of *G. aestuaria* (4073 individuals were caught during the survey), with other numerically important taxa being *G. callidus* and *R. holubi* (Table 2.3.1). The MTN estuary had the lowest EFCI score of the three estuaries investigated, with a total

score of 44, which represents an estuary in moderate condition. Despite scoring quite differently in terms of individual metrics, the EK and OW both had overall scores of 48, which is indicative of estuaries that are in good condition. Table 2.3.2 contains the individual values and consequent EFCI scores for each estuary for each metric.

The highest score for the category *species diversity and composition* (metrics 1-4) was found in the MTN estuary (total score of 16 for this section). This is followed by the EK which scored a total of 14, and the OW which scored 12 for this section. The main differences in this category occurred in metric 1 where the MTN had the highest value, and in metric 4 where the OW scored lower than the other two estuaries. The MTN contained the largest number of taxa, and was very similar to the reference conditions in terms of this category. The EK also had a high score for this category, however only 14 species were recorded in this estuary, resulting in a lower score than the MTN for metric 1 (total number of taxa). The EK was also found to be very similar to reference conditions. The OW had the same number of species as the EK, however it was less similar to the reference conditions than the MTN and the OW.

In terms of the *species proportions* category (metrics 5 and 6), the OW had the highest overall score (8), followed by the EK (6) and the MTN (4). The MTN and EK had lower scores for Metric 5 than the OW. The MTN was the least similar to reference conditions in terms of metric 6, with the other two estuaries being very similar to the reference conditions. Species representation (metrics 5 & 6) was dissimilar between the three estuaries, with the OW scoring highest overall for this category despite the low overall abundance of fishes within this system. This high score is mainly due to the close similarity in the relative proportions of the catch data to reference conditions. The MTN had the highest fish abundance of the three estuaries but catches were overwhelmed by the large numbers of *G. aestuaria* recorded in this system. The EK also had relatively few species comprising 90% of the catch (metric 5), but the relative abundance of the remaining taxa was more similar to reference conditions than in the MTN.

For the *nursery function* category (metrics 7-10) of the EFCI, the EK scored the highest (18 overall score) and the OW and MTN both scored 16. The MTN had the highest number of estuarine resident and estuarine-dependent marine species (metrics

7 and 8), followed by the EK. The OW had the lowest number, scoring three for both these metrics. However in terms of relative abundance, the OW and EK were closer to the reference conditions than the MTN (metrics 9 and 10). The OW and MTN estuaries scored inversely for metrics 7 & 8 and 9 & 10 which can, in part, be attributed to the dominance of the estuarine resident species *G. aestuaria* in the MTN (resulting in lower scores for metric 9). The OW had four estuarine resident species and 10 estuarine-dependent marine species (lowering the scores for metrics 7 and 8), but the relative abundance of the overall community was similar to the reference community (increasing the scores for metrics 9 and 10).

Table 2.3.1: Number of individuals by species caught in each estuary using gill nets and seine nets in the East Kleinemonde, Old Woman's and Mtana estuaries.

Species	East Kleinemonde	Old Woman's	Mtana
Argyrosomus japonicus	5	0	0
Atherina breviceps	870	332	71
Caffrogobius gilchristi	0	0	1
Diplodus sargus	0	1	0
Elops machnata	1	0	5
Galeichthys feliceps	0	1	0
Gilchristella aestuaria	1493	34	4073
Glossogobius callidus	55	156	225
Heteromycteris capensis	2	1	4
Lichia amia	0	2	0
Lithognathus lithognathus	9	10	1
Liza alata	0	0	1
Liza dumerili	32	32	8
Liza macrolepis	0	0	6
Liza richardsonii	21	57	3
Liza tricuspidens	7	42	14
Monodactylus falciformis	5	9	74
Mugil cephalus	4	0	8
Myxus capensis	24	18	18
Oreochromis mossambicus	5	2	64
Pomadasys commersonnii	1	0	1
Psammogobius knysnaensis	53	0	1
Rhabdosargus globiceps	14	0	15
Rhabdosargus holubi	1127	206	417
Total	3728	903	5010

Table 2.3.2: Individual EFCI metric results for each estuary. EK = East Kleinemonde, OW = Old Woman's, MTN = Mtana estuaries.

Category	Metric no.	Description	EK	EK EFCI score	OW	OW EFCI score	MTN	MTN EFCI score
Species diversity and composition	1	Total number of taxa	18	3	15	3	20	5
	2	Presence of rare/threatened species	0	3	0	3	0	3
	3	Presence of exotic/introduced species	0	3	0	3	0	3
	4	Species composition (% similarity to reference conditions)	80	5	75.7	3	80.9	5
Species proportions	5	Number of species that make up 90% of the community composition	3	1	6	3	3	1
	6	Species relative abundance (% similarity to reference conditions)	75.4	5	60.7	5	49.3	3
Nursery function	7	Number of estuarine resident species	4	3	3	3	5	5
	8	Number of estuarine-dependent marine species	13	5	11	3	14	5
	9	Relative abundance of estuarine resident species	66.3	5	57.8	5	87.3	3
	10	Relative abundance of estuarine-dependent marine species	33.6	5	42.0	5	11.5	3
Trophic integrity	11	Number benthic invertebrate feeding species	5	3	4	3	6	3
	12	Number of piscivorous species	2	5	1	3	1	3
	13	Relative abundance of benthic invertebrate feeding species	3.2	1	18.6	5	4.7	1
	14	Relative abundance of piscivorous species	0.16	1	0.22	1	0.10	1
Total				**48**		**48**		**44**
EFCI rating				**Good**		**Good**		**Moderate**

The score for the *trophic integrity* category (metrics 11-14) was highest in the OW (overall score for this section was 12). This was followed by the EK which had an overall score of ten and the MTN which scored eight overall. The EK and MTN scored higher than the OW for metric 11, however the EK and OW scored higher than the MTN for metric 12. The OW was closest to reference conditions in terms of the relative abundance of benthic invertebrate feeding species (metric 13) and all the estuaries scored the lowest possible score for the metric 14 (relative abundance of piscivorous species).

2.4 Discussion

Species diversity and composition

All three estuaries are known to have opened approximately 4 months prior to sampling, and mouth opening events are crucial to marine fish recruitment into these types of estuaries (Cowley & Whitfield 2001a, Vorwerk *et al.* 2001). The fact that the MTN had more species than the other two estuaries could be indicative of a longer open mouth phase that would have allowed more opportunities for marine fish recruitment. Whitfield (1999) highlighted several factors which determine estuarine ichthyofaunal assemblages. These were; salinity, water temperature, river flow, turbidity, mouth phase, dissolved oxygen content, habitat variability, catchment and estuary size, larval linkages and estuary type. Overriding these factors when it comes to temporarily open/closed estuaries (TOCEs) is the frequency, seasonality and duration of mouth opening events (Cowley & Whitfield 2001b).

Harrison & Whitfield (1995) investigated the ichthyofaunal composition of three TOCEs in KwaZulu-Natal over two years. One of these estuaries (Zotsha) was open throughout the spring/summer seasons and had the highest species diversity. The Damba estuary was closed for most of the study and had the lowest species diversity of the three systems. The Mhlanga estuary was open for longer periods than the Damba, but not as long as the Zotsha and had intermediary species composition (Harrison & Whitfield 1995). The results of their study indicate that the species composition in TOCEs is strongly affected by mouth condition.

Another important factor in determining fish species composition is estuary and catchment size. Whitfield (1999) proposed that a larger catchment size results in increased runoff and therefore a higher organic and nutrient input into a system. Due to this, estuaries with larger catchments have greater primary and secondary production, resulting in higher fish biomass within these systems (Whitfield 1999). The higher diversity and biomass seen in the MTN during the present study may be a result of higher runoff into the MTN than in the other two estuaries. The MTN has a smaller estuarine surface area compared to the OW (MTN = 15.7 ha, OW = 25.1ha) but the former system has a greater mean annual run-off (MAR) (MTN MAR = 2.29 $x10^6m^3$, OW MAR = 1.16 $x10^6m^3$). The higher MAR into the MTN may bring more nutrients into this system and result in a longer open phase, thus resulting in an increased fish diversity and biomass when compared to the OW. In addition, there are differences in estuary size following breaching events. When the EK breaches, it empties substantially, resulting in a decrease in habitat availability due to the fringing macrophyte beds and mud flats no longer being covered in water. The MTN and the OW on the other hand retain much of their water surface area following breaching.

Species proportions

Dominance by a few species in an estuary may be indicative of a stressor that removes the more vulnerable species (Harrison & Whitfield 2004), resulting in less diverse ichthyofaunal assemblages and less complex trophic relationships. During this study, the majority of hauls dominated by *G. aestuaria* were recorded in the upper reaches of the MTN which is similar to the findings of Vorwerk *et al.* (2001) in other temporarily open/closed estuaries of the region. The reason for the occurrence of high numbers of *G. aestuaria* in this system may be related to the higher MAR entering the MTN estuary. *G. aestuaria* have been reported to pulse spawn following fresh water flow events into estuaries (Strydom *et al.* 2002). As mentioned in the previous section, the EK empties substantially when it breaches, which may result in many *G. aestuaria* eggs and larvae being flushed out of the estuary. The MTN and OW also breach, but these systems do not empty to the same extent as the EK. The MTN has a higher MAR than the OW which may have triggered more extensive pulse spawning in the resident *G. aestuaria* population. The differences in MAR between the OW and MTN may also explain the inverse abundances recorded in these two estuaries (Table 2.3.1).

Vorwerk *et al* (2003) found that mouth condition and estuary size were the most important factors in determining community structure in a range of Eastern Cape estuaries. These authors found that although permanently open systems contained more species, TOCEs had a higher relative abundance of species. Vorwerk *et al.* (2003) reported that one of the major differences between permanently open and temporarily open/closed systems were the relative abundance of *G. aestuaria* and *A. breviceps*, with *G. aestuaria* being more dominant in permanently open systems than in temporarily open/closed estuaries. This is in contradiction with the findings from the MTN and EK during the current study where *G. aestuaria* was more abundant than *A. breviceps*.

R. holubi was a frequently caught species during the present study. Cowley & Whitfield (2001a) hypothesised that TOCEs have variable fish populations due to unpredictable mouth opening and overwash events, linked primarily to irregular rainfall and unpredictably rough seas. Specifically, the authors attributed an increase in *R. holubi* numbers between two mark recapture surveys in the EK to the ability of *R. holubi* to take advantage of both mouth opening and overwash events. Further studies have shown that *R. holubi* and mugilid 0+ juveniles enter TOCEs through overwash events (Bell *et al.* 2001, Kemp & Froneman 2006). This is in contrast to species such as *Lithognathus lithognathus*, which have a restricted spawning season and are dependent on mouth opening events occurring during the limited larval and juvenile recruitment 'window' (Cowley & Whitfield 2001a). Therefore, it is proposed that one of the major reasons for the biomass dominance of *R. holubi* in all three estuaries is their extended spawning season and versatile recruitment strategy from the marine environment into estuaries. In addition, differences in species abundance between the estuaries during the current study may also be a result of differing mouth opening magnitudes, durations and frequencies between the three systems.

Another finding from the Cowley & Whitfield (2001b) study was that there appeared to be distinct trends in distribution patterns of *G. callidus* and *P. knysnaensis*, with the former species occurring more frequently in muddier substrata and the latter more frequently in sandier substrata. The same trend was observed in the Kariega Estuary using sediment composition and *G. callidus* distribution (Richardson *et al.* 2006). These results are interesting because during the present study, the MTN and OW catch data contained almost no *P. knysnaensis* but high numbers of *G. callidus*. The EK on the

other hand had lower numbers of *G. callidus* in the catch data and many more *P. knysnaensis*. Although the MTN and OW contain sandy sediments in the estuary mouth region, the extent of sandy substrata is much greater in the EK. This is probably the principle reason for the observed *G. callidus* and *P. knysnaensis* proportions in the present study.

Nursery function

Estuaries are well documented as being nursery areas for juvenile fishes and the *nursery function* category is a measure of the extent to which an estuary is fulfilling this role. The higher number of estuarine resident and estuarine-dependent marine species in the EK and MTN may be a result of the more extensive littoral areas in these two estuaries when compared to the OW. Shallow water habitats have been shown to provide juvenile fish with optimum nursery areas in South African estuaries (Paterson & Whitfield 2000) and the smaller size of the OW results in less suitable shallow water habitat than the other two estuaries. The duration and timing of mouth opening events would also affect the species composition within an estuary and it may have been that the smaller OW was open for a shorter amount of time when compared to the other estuaries, thus resulting in fewer estuarine-dependent marine species recruiting into this estuary. Differences in the MAR between the estuaries may also have affected the nursery function of the estuaries. The importance of allochthonous cues to larval recruitment has been demonstrated in several estuarine studies within South Africa (Whitfield 1994a, Grange *et al.* 2000) and the MTN is the smallest of the three estuaries studied (15.7ha) but has the highest MAR (2.29 $x10^6 m^3$). It is therefore postulated that higher amounts of allochthonous cues may be emitted from this estuary when compared to the other two systems resulting in more juveniles being attracted to this system. Estuarine resident *G. aestuaria* juveniles have been documented as having peak abundances when high densities of zooplankton are recorded (Harrison & Whitfield 1990). High zooplankton densities are likely to have been present in the MTN estuary following elevated river flows during the summer, thus supporting the higher abundance of juvenile *G. aestuaria* recorded in this system.

Trophic integrity

The total number of fish caught in the EK and the MTN was far greater than that caught in the OW. However the OW scored better in the trophic integrity category than the other two systems because of the better 'balance' between the trophic groupings. The number of piscivorous and benthic invertebrate feeding species was similar for all three estuaries but when the overall catch is low within an estuary (as was the case in the OW) then the relative proportion of a species will become more important within that catch. The MTN had the highest number of benthic invertebrate feeding species of the three estuaries; however, because of the large overall catch, the relative abundance of these species is low.

The trophic integrity of a system requires that an ecosystem is in balance and contains representatives from the entire food chain (Belpaire *et al.* 2000). Trophic level shifts have been related to environmental degradation; e.g. Belpaire *et al.* (2000) undertook a community analysis of the Flandrian fresh water ecosystems and determined that a decrease in benthic invertebrate feeding fishes resulted from environmental degradation. These authors also proposed that for freshwater systems, piscivorous species should account for 3 – 5 % of the total catch. Trophic shifts in aquatic ecosystems have also been related to agricultural and industrial pollution, for example, heavily impacted sites along the Keum-Ho river in South Korea were found to be dominated by omnivorous fish species, whereas in relatively undisturbed sites, insectivores and carnivorous fish were more common (An *et al.* 2002). Rashleigh (2004) reported a similar trophic shift along a river that was linked to an increase in agricultural inputs into the river. The fish community shifted from specialised insectivores in the less impacted river zone to more generalised insect consuming fish and omnivores where agricultural inputs were greater (Rashleigh 2004).

In a South African context, Harrison & Whitfield (2004) determined threshold values for the number of piscivorous and benthic invertebrate feeding species which should be present in individual types of estuaries in particular biogeographic regions. Based on these reference conditions, it appears that the species in EK and MTN estuaries are 'unbalanced' due to an under representation in terms of the relative abundance of piscivorous and invertebrate feeding fishes. This is despite the fact that in terms of

presence/absence of piscivorous and benthic invertebrate species, these two estuaries scored moderately (Table 2.3.2). When compared to the Harrison & Whitfield (2006) study conducted in the same estuaries during the mid-1990s, the EK and OW contain fewer benthic invertebrate feeding species but more piscivorous species. The MTN has more benthic invertebrate feeding species and more piscivorous species. Therefore it appears that the estuaries have undergone minor trophic shifts since the Harrison & Whitfield (2006) study 10 years ago or that the different sampling seasons (spring versus autumn) are influencing trophic composition.

As previously discussed, pollutant related shifts can cause changes in trophic integrity. The fact that all the estuaries had an EFCI score at or above a 'moderate' condition does not mean that the trophic shifts observed during this study are unrelated to pollution. Indeed, it is quite possible that individual fishes may be affected by pollution, but unless fish perish or their behaviour is markedly influenced as a result of exposure to a pollutant, the EFCI score will tend to remain unchanged. Therefore conclusions about pollution related shifts in trophic levels within these three estuaries can only be substantiated by using complementary biomonitoring techniques (see Chapter 7).

2.5 Conclusion

Harrison & Whitfield (2006) conducted an assessment of 190 South African estuaries using the EFCI using data that was collected between 1993 and 1999. In the warm temperate zoogeographic zone, 30 estuaries were in good condition (included in these were the EK and MTN estuaries), ten estuaries were in moderate condition and five estuaries (which included the OW) were in poor condition. The results for the EK, OW and MTN were fairly dissimilar compared to the results from the present study. The EK scored 46 during the Harrison & Whitfield (2006) assessment compared to 48 during the present study, suggesting an improvement in the fish biota of this estuary since it was sampled in November 1995. Harrison & Whitfield (2006) found the OW had an overall score of 30 compared to 48 during the present study. These results suggest a significant improvement in the biota of the OW between September 1996 and April 2005. The MTN had an overall score of 46 for the biota sampled during September 1996 (Harrison & Whitfield 2006) and the present study found that this estuary had an overall score of 44, suggesting a slight degradation in estuarine health between the two sampling periods.

A few important issues need to be considered with regard to comparing the results from the present study to those of Harrison & Whitfield (2006). Firstly, the survey of the MTN in the current study was overwhelmed by the large number of *G. aestuaria* sampled which decreased the values for metrics 5 and 13. In addition the low numbers of piscivorous species in this estuary resulted in a low score for metric 14. It could have been that one of the factors which lead to such high numbers of *G. aestuaria* was that there were so few piscivorous species to prey upon them. This, coupled with high primary and secondary production, may explain the large hauls of *G. aestuaria* obtained. Secondly, in numerical terms, many more fish were caught in the EK and MTN than the OW in the present study, even if allowance is made for the fact that the OW has slightly lower MAR than the other two systems. Thirdly, the samples collected for the present study were collected during a different season to those used in the Harrison & Whitfield (2006) study. A validation study of the Sezela Estuary demonstrated that samples collected during October 1984 and January 1986 had the same score, indicating that this index is reproducible over different seasons (Harrison & Whitfield 2004); therefore this third point is possibly not a confounding factor.

The OW and EK communities scored well using the EFCI, indicating that the estuarine fish communities in these estuaries are in good condition. This is of particular relevance for the OW estuary which was given a 'poor' rating by Harrison & Whitfield (2006) 10 years prior to the present study. Superficially, the MTN appears to have decreased in biological integrity since September 1996, being rated as being in 'good' condition by the Harrison & Whitfield (2006) and 'moderate' in April 2005. However, as mentioned in preceding paragraphs, this result is biased by the overwhelming dominance of *G. aestuaria* in the fish assemblage, thus lowering metrics 5 and 6 in particular. This suggests that some of the metrics in the EFCI may be overly sensitive and may require some modifications. It should also be noted that in terms of actual scores for the EFCI, the MTN has only dropped by two points (this estuary scored 46 during September 1996 and 44 during April 2005).

Whitfield & Elliot (2002) state that geographical and hydrological conditions are major estuarine drivers which determine basic fish communities in South African estuaries. These drivers (which include factors such as estuary location, size, mouth state, fresh

water inflow, etc.) determine the basic species composition in an estuary. In addition, the community structure depends on what a species can physically tolerate in terms of daily fluctuations in temperature, salinity etc. Other major drivers influencing fish communities are biological and include inter- and intra-specific competition, predator/prey interactions, food availability, etc (Whitfield & Elliott 2002). In view of the above, it is noteworthy that the three estuaries investigated during the present study were dissimilar in terms of species composition, despite their geographical and physical similarities. From this it is suggested that the differences in community structures between the three estuaries arose from selected primary and secondary driving factors. Primary drivers may have included aspects such as the duration of mouth opening events differing between the estuaries (Harrison & Whitfield 1995). Secondary driving factors such as habitat and food availability may also have had an influence in differentiating the fish communities between these systems (Whitfield & Elliott 2002).

The EFCI has proved to be a useful bioindicator which was able to produce an overall picture of the health of the three estuaries analysed during this study. The outputs from the EFCI are easily understood by scientists, managers and the general public. This feature of the EFCI is important, since communicating information regarding the health status of estuaries is often difficult but essential to effective estuarine management schemes. The results from the EFCI provide an indication of the health status of the ichthyofaunal assemblages of each estuary by determining, for example, whether the populations present in an estuary occur in proportions similar to, or different to, a reference (pristine) fish community.

The EFCI is a rapid, cost effective measure of estuarine fish integrity. The data capture methods were straight forward and the subsequent metric calculations were simple. The fish identification requires some specialised knowledge but any unidentified taxa could be preserved in the field and analysed at a later stage in the laboratory with the aid of species keys. It is easy to use and does not require specialised technology. The above aspects are important factors when considering a rapid assessment method for estuarine health determination.

The EFCI also gives an indirect measure of the health status of invertebrate fauna, because if there were for example problems like anoxic conditions in the sediments, there would be no invertebrates and therefore no benthic invertebrate feeders. Problem 'areas' in terms of the four broad categories within the 14 metrics can be identified from the analysis. It is also capable of detecting recent water quality problems such as anoxic conditions which may lead to fish kills because the EFCI would record a dramatic loss in diversity and abundance in all species. The effects of anoxic conditions on fish would not be detected by biochemical measures such as the acetylcholinesterase assay (see Chapter 5 for more information on this assay). The EFCI also examines all the species within an estuary, which biochemical or histological markers rarely do (for logistical reasons).

Most of the fish sampled during an EFCI assessment are captured by seine netting. This is a non-destructive sampling technique where most of the catch is released alive and the method is therefore very suitable for long-term monitoring programs. In order to sample fish species that evade seine nets, it is necessary to use gill nets. Gill nets tend to be destructive, particularly if large panels of netting are deployed throughout an estuary. However, by limiting the size of the gill net panels and using a range of mesh sizes in selected strategic habitats, the number of fish caught using this method can be considerably reduced without compromising the value of the diversity information obtained.

Data on the frequency and duration of mouth opening events is very limited due to the isolated nature of many estuaries. In view of this paucity of information, the EFCI is a useful tool which can give a good indication of the status of an estuary without knowledge of past events. It does not require prior knowledge about an estuary and can rapidly portray the biotic integrity of a system using simple methods.

Negative aspects of the EFCI include the fact that this technique cannot always determine what causative agents are responsible for reported estuarine degradation. This biomonitoring technique only detects problems at the community level after they have occurred, rendering pre-emptive action difficult. Finally, this biomonitoring technique does not consider fish larvae, which have been shown to be sensitive indicators of ecological change (Neira & Sporcic 2002).

In terms of the national context, the Harrison &Whitfield (2006) study looked at 73 moderate to large closed estuaries in all three biogeographic regions and found that 41 (56%) of them were in a good condition, 17 (23%) were in a moderate condition, 14 (19%) were in a poor condition and one (1.3%) was in a very poor condition (Harrison & Whitfield 2006). None of the three estuaries in the current study was in a poor or very poor condition in terms of the biotic integrity within these types of systems. This has important management implications given the results which were found using other biomonitoring techniques discussed in subsequent chapters.

Chapter 3

Biomonitoring using organ level biomarkers

3.1 Introduction

Organ level biomarkers are a measure of the health of individual fish in an environment. The histopathological approach uses a sub-sample from a population to determine individual health rather than community health. The technique enumerates pathological changes in an organ's structure and describes the extent to which an organ has been affected by exposure to xenobiotics (Hinton & Lauren 1990). Pathological changes which occur when the teleost liver is exposed to a number of different contaminants have been investigated under laboratory conditions, and good correlations between the presences of lesions and exposure to contaminants have been found (Hinton & Lauren 1990). It is important to note that liver structure alterations from fish collected in the field may be a result of exposure to different combinations of pollutants; thus histopathology is not a diagnostic tool which is able to determine what pollutant caused the structural alterations.

The reasons for which the liver was chosen as the organ of this investigation were:

- It is the first organ to come into contact with absorbed material through a direct connection from the stomach wall to the liver via a portal vein (Naigaga 2002).
- It is a key organ in terms of fat and carbohydrate uptake and these are stored in hepatocytes (Hinton & Lauren 1990).
- It is the site of cytochrome P-450 mediated mixed function oxygenase system which detoxifies a number of xenobiotics and can form intermediary toxic substances (Hinton & Lauren 1990).
- Bile, which is synthesised in the hepatocytes, is used to excrete deactivated toxicants and also to aid in the digestion of fatty acids (Hinton & Lauren 1990).

- The liver is the site of vitellogenin synthesis and also binds estradiol, an essential hormone used during the initial development of reproductive organs (Hinton & Lauren 1990).

The liver is therefore of key importance to the overall homeostasis of the body in terms of nutrition, defence against toxicants and reproductive development.

Histological alterations[2] in the liver have been shown to provide definite biological end points of past exposure to a number of contaminants and have been successfully applied in several biomonitoring programs in Europe and America (Malins *et al.* 1988, Kohler 1989a, Hinton & Lauren 1990, Stentiford *et al.* 2003) Laboratory and field based studies have established relationships between contaminant exposure and toxicopathic lesion formation (Naigaga 2002, Stentiford *et al.* 2003, Van Dyk 2003). Van Dyk *et al.* (2007) exposed the southern African freshwater cichlid *Oreochromis mossambicus* to two concentrations of a combination of cadmium and zinc over long and short time periods. The study found that even a low dose of cadmium and zinc elicited a histological response, and that the response amplified over time (6 h, 12 h, 24 h, and 96 h). No dose-dependent differences were found in terms of the histological changes. A longer term experiment (672 h) was also conducted during the study and this showed that the liver lesions in these fish showed a degree of regeneration, indicating some form of adaptation (through regeneration of the affected tissues) to the chemical insult during periods of long-term exposure (Van Dyk *et al.* 2007). The findings of this study are significant given that anthropogenic pollution is generally low in concentration and many pollutants persist in the environment for long periods of time (Van Dyk *et al.* 2007). Therefore, it may be that fish which are exposed to contaminants in the wild, even at low doses, may be undergoing liver structural alterations which may be influencing the capability of the liver to undertake its normal functions.

Naigaga (2002) investigated the bioaccumulation and histopathological effects of copper on *O. mossambicus* on a short and long-term basis. Lesions were apparent in all the treatments in a dose-dependent manner (0.11 – 0.47 mg/L copper). A

[2] Appendix 1 contains definitions of different types of histopathological alterations discussed during the rest of this chapter

progressive transition through a range of liver structure alterations (hepatic vacuolar degeneration, fatty degeneration and necrosis), as the duration of exposure increased, was noted. Naigaga (2002) proposed that there were several phase of degeneration which were reflected in structural changes to the liver as a result of exposure to copper. The initial stages showed liver hyperfunction (characterised by vacuolar degeneration). This was thought to be an attempt by the organ to detoxify and remove the copper from the body, and may have been the reason for which, during the first 30 days of exposure, copper concentrations in the tissue were low. Following this, the liver went into hypofunction (characterised by fatty degeneration and necrosis) and copper levels in the tissue were found to increase (Naigaga 2002). This increase was attributed to the liver becoming overwhelmed by the metal exposure, resulting in the hepatocytes becoming necrotic (Naigaga 2002). Liver hypofunction may lead to impaired detoxification abilities in particular, but can also affect the overall health of the organism given the multiple roles the liver plays in the overall homeostasis of the body (Naigaga 2002).

The progression of liver lesion formation was investigated in the Elbe estuary according to 'exposure time' (Kohler 1989b). Kohler (1989b) found that liver damage in *Platichthys flesus* increased dramatically over the life of the species in the most polluted site (near a large city). The study also ran a concomitant laboratory experiment which demonstrated that if fish with known liver damage were placed in tanks containing sediment which was free of the major pollutant found in the study area, the liver was able to successfully regenerate. These results were also mirrored in the fish collected at the lower end of the pollution gradient in the field, where regeneration of lesions in the liver was apparent (Kohler 1989b).

Stentiford *et al.* (2003) successfully used histopathology in an estuarine monitoring program in the United Kingdom using three species of demersal fish (*Platichthys flesus, Pomatoschistus minutus* and *Zoarces viviparous*). In their study, several estuaries with different types of pollution contamination were sampled seasonally over a period of one year. Fish captured from sites which had high contaminant levels in the sediments had a greater number of pre-neoplastic and neoplastic lesions than fish captured at sites with lower contaminant levels (Stentiford *et al.* 2003). The authors conceded that the histological investigation which they undertook did not

determine the cause of the structural alterations detected, although it did provide a good indication of the health of the individual and this could be extrapolated to the population level (Stentiford *et al.* 2003).

A similar large scale investigation was undertaken in America where liver damage in the soleid *Parophrys vetulus* was highly correlated to the aromatic hydrocarbon content in the sediment where the animals were caught (Malins *et al.* 1988). Results also showed that aromatic compounds were traceable in the bile of the animals sampled, and where these concentrations were detected, a higher prevalence of hepatic neoplasms was found (Malins *et al.* 1988).

Thus, a number of studies have shown that certain chemicals can produce a range of lesions in the livers of fishes. Hibiya (1982) and Hinton & Lauren (1990) provide very thorough reviews of the appearance of damaged liver and also describe mechanisms by which these may have developed. Hinton & Lauren (1990) suggest a number of biostructural alterations which may be used as biomarkers of exposure to pollutants.

The aims of this study were to determine if any differences in terms of liver histopathology could be detected from fish collected from three intermittently open estuaries in the Eastern Cape. The null hypothesis was that there would be no difference in lesion type or in the extent of the lesions between the three estuaries. Three estuaries were selected based on morphological similarity. These were the East Kleinemonde, Old Woman's and Mtana estuaries (for more information on catchment characteristics and surrounding land use between these systems see Chapter 1). The Cape stumpnose, *Rhabdosargus holubi,* was selected as the indicator species for this study. The reasons for choosing this particular fish are also outlined in Chapter 1.

3.2 Methods

One hundred and thirty six *Rhabdosargus holubi* from the East Kleinemonde, Old Woman's and Mtana estuaries were weighed and measured prior to being killed. Their livers were dissected out and half of their livers immediately fixed in 10% buffered formalin (the other half was used for biochemical analyses (Chapter 4)). These were then transferred to 70% alcohol after 72h until further processing. The fixed livers

were sent to the National Health Laboratory Services (NHLS) in Port Elizabeth where the tissue preparation, staining and clearing processes are standardised and automated. Briefly, each tissue was dehydrated in increasing grades of alcohol from 70% to absolute. The tissues were transferred through serial solutions of xylene and then embedded in wax. The wax blocks were then sliced into 5µm thick sections and fixed on a glass slide. These sections of tissue were placed into serial solutions of xylene and were re-hydrated through serial dilutions of alcohol grades, from absolute to 70% alcohol. The sections were then placed into hematoxylin stain, rinsed with tap water and transferred into an Eosin stain. They were then rinsed with tap water again and transferred back through a series of dehydrating alcohol grades, from 70% to absolute alcohol. Finally the sections were transferred through a series of xylene solutions and covered using a cover slip.

The best slides were selected for detailed histological analysis after staining; six slides were selected from the Old Woman's estuary, nine from the Mtana estuary and 10 from the East Kleinemonde estuary. The reason for this small sample size was that most of the slide sections were either very torn during the sectioning process or had too many artifactual changes (from the staining and clearing process) to be used for detailed histopathological analyses.

The extent and degree of histological alterations was determined according to methods described in Van dyk *et al.* (2007) with one adaptation: in this study, congestion of the hepatic blood vessels was not described as mild, moderate or severe. This is because it was found that most of the nucleated red blood cells seen on the processed slides had been ruptured, rendering it very difficult to quantify the extent to which the hepatic blood vessels were congested. Thus this category was reduced to either congested or not. Congestion of the hepatic blood vessels was defined as the condition which occurred when all the hepatic blood vessels appeared full of blood and the sinusoids were also full of blood. This approach is supported by the fact that congestion of blood vessels is not necessarily pathological, and can also be a result of handling stress during the dissection of the tissue among other stressors (Van Dyk pers comm.). However, when the sinusoids and the blood vessels are congested it can be concluded that this is a pathological feature and occurred while the fish was still alive.

The method described in Van Dyk *et al.* (2007) was adapted from Pierce *et al.* (1978). Pierce *et al.* (1978) had grades ranging from zero to six, with six indicating the presence of neoplasms. Van Dyk *et al.* (2007) only contained the first four categories of the Pierce *et al.* (1978) method. The reason for using the Van Dyk *et al.* (2007) method is that most of the other categories present in Pierce *et al.* (1978) did not occur in any of the *R. holubi* samples collected. Myers *et al.* (1998) conducted a study on sub-adult English sole (*Pleuronectes vetulus*) and found that neoplasms rarely occurred in individuals younger than 4 years old. Similarly, the *R. holubi* used in this study were less than 2 years of age (< 14cm SL (Blaber 1974)), did not possess neoplasms and therefore the Van Dyk *et al.* (2007) method was considered more appropriate.

Table 3.2.1 indicates the various histological characteristics possible for each category and the subsequent value (or grade) that these characteristics represent. Grades 0-2 represent normal histological structure and any grades above three indicate pathological changes. Each fish was graded using one slice of tissue from one slide (grading was undertaken according to the categories in Table 3.2.1.) Other histopathological changes not listed on Table 3.2.1 were also recorded but were not used for the index calculation. The data were found to be not normally distributed (Kolmogorov-Smirnov, $P \leq 0.05$), therefore a non parametric Kruskal-Wallis test was performed. Statistical analyses were performed using a STATISTICA^{TM} version 7 statistical package

Table 3.2.1: Grading system for the determination of histological alterations observed in *R. holubi* liver sections from the East Kleinemonde, Old Woman's and Mtana estuaries (adapted from Van Dyk *et al.* 2007).

Grade	Histological characteristic
0	Typical hepatic cord structure and hepatopancreatic structure.
1	Typical hepatic cord structure and hepatopancreatic structure. Mild* lipid accumulation (vacuolation) within hepatocytes.
2	Typical hepatic cord structure and hepatopancreatic structure. Moderate* lipid accumulation (vacuolation) within hepatocytes.
3	Typical hepatic cord structure and hepatopancreatic structure with 2-5 of the following characteristics: Severe* lipid accumulation (vacuolation) within hepatocytes. Congestion of hepatic blood vessels. Increase* in perivascular connective tissue. Hyalinization within hepatocytes. Cellular swelling due to hydropic change. Increased* macrophage aggregates. Lymphocyte infiltration.
4	Loss of hepatic cord structure with 2-5 of the histological changes listed under category 3.

*The definitions of these categories were determined during discussions with Dr Van Dyk and other colleagues who were experienced in the field of histology of the liver.

3.3 Results

Generally, the histology slides of livers from *Rhabdosargus holubi* from the different estuaries differed from one another. Slides from fish in the East Kleinemonde estuary had a normal tincture, the hepatocytes were clearly stained and the hepatic cords were generally clearly visible. Slides from the Old Woman's estuary were pale in comparison and the hepatocytes appeared almost translucent in colour. However the hepatocytes, as well as the hepatic cords, were still clearly visible. Slides from the Mtana estuary tended to be darkly stained, and several had elongated cells which were light pink and smooth, characteristic of endothelial cells. Table 3.3.1 below shows the overall scores for each fish from each estuary. Figures 3.3.1 to 3.3.3 (Appendix 2) show the histology of normal unaffected liver parenchyma.

Table 3.3.1: Histological score for each fish from each estuary. Scoring calculated according to table 3.2.1 (adapted from Van Dyk *et. al.* 2007.)

East Kleinemonde		**Old Woman's**		**Mtana**	
Fish No.	Score	Fish No.	Score	Fish No.	Score
102	3	198	1	293	3
104	3	203	3	296	3
107	3	259	3	297	2
122	3	265	3	299	3
123	3	275	3	301	3
140	3			305	4
145	2			310	3
168	3			318	4
175	3			326	2
176	3				

Ten specimens were analysed from the East Kleinemonde estuary. An increase in melanomacrophage centres (MMCs) was observed in five of the specimens (Appendix 2, Figure 3.3.4). Five of the specimens showed vacuolation, one of which was quite severe (Appendix 2, Figure 3.3.5). Increased perivascular tissue was also observed in four of the specimens (Appendix 2, Figure 3.3.6) and in three of these specimens, focal areas of hypertrophy were observed (Appendix 2, Figure 3.3.7). One specimen also showed focal areas of necrosis (Appendix 2, Figure 3.3.8) and hydropic change was also observed in one of the sections (Appendix 2, Figure 3.3.9). One specimen showed an increase in mono-nuclear leucocytes (MNLs) (Appendix 2, Figure 3.3.11). Overall, nine out of the 10 specimens were assigned an index value of three, and one was assigned a value of two, giving a mean value for this group of 2.9.

Five specimens were analysed from the Old Woman's estuary and all were found to have an increased number of MMCs (Appendix 2, Figure 3.3.4). Two of the specimens showed areas of hydropic change (Appendix 2, Figure 3.3.9) and two of the specimens had vacuolated hepatocytes (Appendix 2, Figure 3.3.5). One specimen had increased perivascular tissue (Appendix 2, Figure 3.3.6) and one specimen had an increase in the overall size of the nuclei in patches. This specimen also had intracellular deposits in the cytoplasm and also showed signs of glycogen vacuolation (Appendix 2, Figure 3.3.10). Specimen number 203 contained few pathological changes and was assigned a value of one, with four other specimens assigned a value of three. The mean value for the Old Woman's group was 2.6.

Nine specimens were analysed from the Mtana estuary and seven of these showed an increase in MMCs (Appendix 2, Figure 3.3.4). Four specimens showed an increase in perivascular tissue (Appendix 2, Figure 3.3.6) and two specimens showed an increase in MNLs (Appendix 2, Figure 3.3.11). An overall loss of structure of the hepatic cells (Appendix 2, Figure 3.3.12) and an increase in endothelial cells was recorded in two of the samples. Two slides also had an increase in nuclear activity and one sample showed focal areas of necrosis (Appendix 2, Figure. 3.3.8). An increase in black structures of triangular shape was noted in two of the slides and may be Kupfer cells. One slide had congestion of the hepatic blood vessels (Appendix 2, Figure 3.3.13) and hyperplasia was noted in one of the slides (Appendix 2, Figure 3.3.14). Two of the slides had artifactual changes, one of which was a dry earth effect (Luna 1968) and one displayed hydropic change (Appendix 2, Figure 3.3.9). Overall the Mtana estuary samples were the most varied group of slides (Table 3.3.1), with the mean value for this group being 3.0.

A statistical analysis was undertaken using the scores for the slides and no significant difference was found in the histological state of the fish from the three estuaries (Kruskal Wallis, $H = 0.6378$, $df = 2, 24$, $P = 0.7269$).

3.4 Discussion

Despite there being no statistically significant differences between the samples, certain differences between estuaries as well as between individuals were apparent from the histological analyses. The following section will discuss each lesion identified in the samples from all the estuaries and will discuss their significance in terms of other fish health studies. The lesions identified from the index will be discussed first, followed by lesions observed which do not appear in the index. Finally, an overall assessment regarding the applicability of the index for this pilot study will be discussed and possible changes that could improve the index will be proposed.

Increase in MMCs

Half of the East Kleinemonde (EK) specimens had an increase in MMCs, all the Old Woman's specimens (OW) and seven out of nine of the Mtana (MTN) specimens showed an increase in MMCs. An increase in MMCs can be indicative of previous exposure to toxicants but may also be a result of parasitic infection (Hinton & Lauren 1990). A macroscopic inspection of each fish was conducted prior to removal of the liver and only one sample from the OW was found to have parasites in the stomach. Parasites can however also be present on the gills and in the muscle tissue of fish and these areas were not investigated due to time constraints in the field. Therefore it cannot be categorically stated that there were no parasitic infections of the fish used in this study. A study conducted using flounder (*Platichthys flesus*) from four estuaries in England found that MMCs occurred in samples collected from both the contaminated and uncontaminated estuaries. MMCs were more prevalent in the more polluted sites (containing PAH contaminated sediments) than in the control site (Stentiford *et al.* 2003). The authors highlighted the fact that MMCs play a role in other normal cellular mechanisms and therefore more research is needed to determine to what extent MMCs need to have increased in order for them to be indicative of previous exposure to contaminants (Stentiford *et al.* 2003).

Vacuolation of hepatocytes

Vacuolation of the hepatocytes was seen in half of the samples from the EK and two of the OW samples. None of the MTN samples had any signs of vacuolation. A study conducted by Wester *et al.* (1990) exposed *Oryzias latipes* to various concentrations of bis(tri-*n*-butyltin)oxide (TBTO) and di-*n*-butyltindichloride (DBTC). The authors found a dose dependent increase in glycogen related vacuolation in the liver occurred in response to exposure to both of these chemicals. The authors also found that at the highest concentrations (10 µg TBTO/L and 3200 µg DBTC/L) lipid vacuolation could be seen (Wester *et al.* 1990). Evidence from the above study suggested that glycogen vacuolation may be a precursor to lipid vacuolation, and therefore the glycogen vacuolation seen in the present study is proposed to be an antecedent form of lipid vacuolation. This hypothesis will need to be validated through laboratory testing.

A study conducted on the silver catfish *Rhamdia quelen* investigated the effects on liver histology and vacuole formation of short term exposure to the herbicide clomazone at various concentrations (Crestani *et al.* 2007). The authors exposed the fish to 0.5 and 1.0 mg/L of clomazone for 12, 24, 48, 96 and 192 h. Following the 96 h and 192 h exposures, the fish were then kept in herbicide free water for 96 h and 192 h, i.e. equivalent to their exposure duration (Crestani *et al.* 2007). The authors reported vacuolation of hepatocytes for both the exposure periods which were followed by recovery in herbicide free water, but no vacuolation was recorded in the shorter term exposures or in the controls. The above study suggests that vacuolation maybe indicative of long term toxicant exposure.

Hinton & Lauren (1990) suggest that lipid vacuolation is not a result of an increase in fat intake by the affected animal, but rather one of four possible biochemical anomalies: protein synthesis inhibition, energy depletion, disruption of pathways for the removal of fats from the cell, and a change in the substrate that usually catalyses enzymatic reactions. All of these biochemical interruptions may be a result of exposure to toxicants (Hinton & Lauren 1990). They may also be a result of the hormonal state of the animal (based on sex and sexual maturity of the fish), diet, and time of sampling (seasonality) (Hinton & Lauren 1990). In this project, neither the hormonal state of the fishes nor seasonality should be considered confounding factors for several reasons. Firstly, this study sampled all three estuaries at the same time thereby removing any seasonal bias when comparing the samples to one another. Secondly, by using only juveniles, and therefore reproductively immature fish, hormonal effects from this source were effectively removed. Although diet may have affected the results of the present study, it is suggested that the health of these fish is a proxy for the quality of habitat (biotic and abiotic) and food availability in each estuary. Therefore, should vacuolation be a result of food availability rather than toxicant exposure, this result is still meaningful because it highlights a difference between the state of the estuaries and the quality of habitat available for the ichthyofauna.

Increase in perivascular connective tissue

Four of the fish from the EK and MTN and one of the samples in the OW showed an increase in perivascular connective tissue. Hibiya (1982) states that an increase in connective tissue is a result of phagocyte infiltration to a focal area of necrosis. Connective tissue forms around the affected area and creates an envelope. From this, an increase in perivascular connective tissue is proposed to be indicative of prior necrosis. Van Dyk (2003) conducted a histopathological study on *Clarias gariepinus* collected from 3 sites: 2 were considered polluted and the 3rd was a control site which was considered to be in a pristine condition. He found an increase in connective tissue in 60% of the fish caught in one of the polluted sites and 35% of the fish caught from the second polluted site. None of the fish collected from the control site had an increase in perivascular tissue (Van Dyk 2003), suggesting that this lesion may be used as an indicator of exposure to xenobiotics; this will need laboratory testing for verification.

Hydropic change in the hepatocytes

One of the fish from the EK and the MTN and two of the fish from the OW showed hydropic change. Hydropic change (hydropic vacuolation) is a type of cellular swelling and is characterised by a cloudy cytoplasm and enlarged hepatocytes without a change in the diameter of the nucleus. Hydropic change was compared during a long term study of five study sites in Massachusetts (Moore *et al.* 1996). During that study, a comparison of neoplasia and hydropic vacuolation in the flounder *Pleuronectes americanus* was undertaken where specimens were collected once a year between 1987 and 1993. Samples collected during the 1980s showed a higher prevalence of neoplasia and hydropic vacuolation than samples collected in the 1990s. The authors attributed this difference to changes in sewage management practises between these periods, resulting in heavy metal and organic compound content in the sewage effluent decreasing by up to 70% (Moore *et al.* 1996). Another management action which was thought to be behind the decrease in the incidence of the pathological changes in the liver of *P. americanus* was removal of sludge from the sewage effluent at one of the sites. Despite the fact that these management actions were undertaken in 1991, fish samples from 1993 may still have been reflective of older sediments

containing toxic chemicals from the pre-1991 era, thus indicating a lag effect between management actions and the pathological changes seen in fishes. This lag effect was driven by the longer term bioavailability of certain chemicals which persist in the environment for extended periods of time (Moore *et al.* 1996).

Hydropic vacuolation in a flatfish (*Pleuronectes vetulus*) and in three estuarine species (*Platichthys flesus, Pomatoschistus minutus* and *Zoarces viviparous*) was described in two northern hemisphere studies and in both cases was shown to be correlated with exposure to hydrocarbons. Both studies highlighted that hydropic vacuolation responses seemed to be highly species specific and required laboratory based experiments to enable proper quantification of the responses to hydrocarbon toxicants (Stehr *et al.* 1998, Stentiford *et al.* 2003).

A study undertaken by Van Dyk *et al.* (2007) investigated the effect of cadmium and zinc on the liver of *Oreochromis mossambicus*. During their study only some of the animals from the intermediate exposure level (0.018mg/L cadmium, 0.16mg/L zinc) showed hydropic change after 96 h. No hydropic change was recorded at the higher exposure concentration nor during the shorter term exposure (6, 12, 18, 24 h) or the longer term exposure (672 h). The authors proposed that hydropic change was a transient state of cells exposed to a toxicant, and therefore it was uncommon to see this stage of cellular degeneration (Van Dyk *et al.* 2007). Due to this, it is not surprising that this pathology was infrequently recorded during the present study. Megalocytic hepatosis, which is another commonly reported type of cellular swelling involving the enlargement of the entire hepatocytes (nucleus included), and especially the endoplasmic reticulum and mitochondria (Myers *et al.* 1991) was not detected in the study.

Increase in mono-nuclear leukocytes (lymphocyte infiltration)

Only one fish from the EK and two fish from the MTN exhibited an increase in mono-nuclear leukocytes (MNLs or lymphocyte infiltration). None of the samples from the OW had this lesion. Lymphocyte infiltration occurs after an area of the liver has undergone focal necrosis. Phagocytosis by the lymphocytes eliminates the necrotic cells from the focal area (Hibiya 1982). An investigation into the effects of the

ingestion of methylmercury (a toxicant present in Amazonian waters as a result of gold mining) on the liver and kidneys of *Hoplias malabaricus* was undertaken in Brazil (Mela *et al. in press*). The authors found a significant increase in lymphocyte infiltration following a 70 day exposure to methylmercury, via contaminated food, compared to the unexposed control fish. The sites of lymphocyte infiltration were adjacent to or at the sites of focal necrosis. The authors also noted a significant increase in MMCs in the liver of the exposed fish when compared to the control fish livers, and attributed the elevated MMCs and the lymphocyte infiltration to an inflammatory response to heavy metal contamination of the liver (Mela *et al. in press*). The above study has shown that low doses of heavy metals which were bioconcentrated through the food chain can produce measurable effects on the liver histopathology of fish. This has important implications for the present study because one of the principle pathways of exposure (apart from direct exposure via the gills) is hypothesised to be through *R. holubi* consuming contaminated prey.

Congestion of hepatic blood vessels

Only one fish from the MTN showed complete congestion of the hepatic blood vessels while this damage was not found in the fish from the EK or OW. As mentioned in the methods, congestion was not described as mild, moderate or severe in this study. The reason for this was that these descriptions were too subjective because the nucleated red blood cells burst during tissue processing. This meant that quantifying the space that the whole blood cell would have taken up in the blood vessel would be difficult. If red blood cell nuclei and associated plasma were found in the sinusoids (as was the case with the above mentioned sample from the MTN), then it could be assumed that the hepatic blood vessels were full prior to death as these vessels fill up before the sinusoids do.

Congestion of the blood vessels was recorded during a study conducted by Zapata-Perez *et al.* (2000). The authors found that injecting the cichlid *Oreochromis niloticus* with contaminated sediments containing organochlorine pesticides, PCBs and PAHs resulted in congested sinusoids after a 24 hour exposure period. The fish were injected intraperitoneally with sediment extract which was diluted in 1 ml of corn oil (Zapata-Perez *et al.* 2000). Van Dyk *et al.* (2007) found congestion of blood vessels in

Oreochromis mossambicus following exposure over 6, 12, 18, 24, 96 and 672 h to a mixture of zinc and cadmium at medium and high concentrations. Congestion of the blood vessels has therefore been shown to occur following exposure to a wide range of chemicals and exposure durations, but is nevertheless a biomarker of exposure.

Overall loss of structure of the hepatic cord and hepatopancreatic tissue

One fish from the EK and two fish from the MTN suffered an overall loss of hepatic tissue structure. None of the fish from the OW were found to have this condition. In these three fish the normal, organised pattern of the hepatocytes in the hepatic cords was lost. The reason for this loss of structure is unknown. This lesion was classified as pathological by Pierce *et al.* (1978) because it was often associated with other pathologies. Van Dyk (2003) found a loss of overall structure of the liver in 30% of the fish captured from one polluted dam and in 35% of the fish from a second polluted site. The control site which he was using in the study showed cord disarray in 53% of the study animals but the overall structure of the liver was intact. A loss of structure within the liver parenchyma in the present study may therefore be as a result of exposure to pollutants as was the case in the Van Dyk (2003) investigation.

Hyalinization within the hepatocytes

Hyalinization is also listed in the current study index but it did not occur in any of the fish examined. Hyalinization was the predominant pathology described in a study using *O. mossambicus* exposed to differing concentrations of a mixture of cadmium and zinc over different periods (Van Dyk *et al.* 2007). The highest incidence of hyalinization was observed following a 96 h exposure to moderate and high concentrations of the zinc and cadmium mixtures but was not seen in the longer term (672 h) exposure. Based on the above evidence it was proposed that hyalinization may be a transient response to xenobiotics. The fish caught during the present study may have been exposed to xenobiotics for a period of time which was long enough to halt a hyalinization response from the hepatocytes. Alternatively, the fish in this study were simply not exposed to xenobiotics which results in hyalinization.

A number of histopathological changes were noted in the slides which were not used in the Van Dyk *et al.* (2007) index. Some of these histopathological changes were included in the original Pierce *et al.* (1978) index, however they were not included in the current index for reasons outlined below. A brief summary of the pathological changes recorded follows and comparisons with other studies which found similar results are presented. It is important to note that these histopathological changes were not used to calculate the index value assigned to each fish.

Focal areas of hypertrophy and hyperplasia of the hepatocytes

Hyperplasia was noted only in one of the fish from the MTN estuary and hypertrophy was recorded in three fish from the EK estuary. None of the fish from the OW showed signs of hyperplasia or hypertrophy. The reason for which these two conditions have been grouped together is because hepatic hypertrophy is usually a result of cellular hyperplasia (Hinton & Lauren 1990). Hyperplasia follows focal necrosis, which may be a result of disease or xenobiotics exposure. Pockets of hyperplasia (which is an abnormal multiplication of cells) are formed in a group of hepatocytes and for this reason is not the same as normal growth of the liver (Hinton & Lauren 1990). Hypertrophy results from hyperplasia and is an increase in overall numbers of cells or tissues. It is a type of cellular swelling but this swelling is a result of an increase in dry material, not fluids (as is the case when hydropic change occurs) (Hinton & Lauren 1990). This pathology was included in the Pierce *et al.* (1978) index, however, there was no indication of the extent of occurrence of this lesion in order to be considered a pathological change and therefore it was not included in the present index. More laboratory work is necessary to determine to what extent different types of hypertrophy and hyperplasia take place as a result of exposure to xenobiotics and from this, determine what is considered to be pathological levels of these lesions.

A study was conducted in America using several rivers which were polluted in different ways. Biliary cell hyperplasia was one of the most frequently occurring lesions from samples collected from polluted rivers but did not occur in the reference rivers. Redbreast sunfish (*Lepomis auritus*) collected from the Pigeon River showed diffuse biliary hyperplasia and this river was characterised by high loadings of chlorinated dioxins and furans due to the presence of a bleached kraft mill upstream

(Teh *et al.* 1997). This hyperplasia was thought to be the first step towards bile duct neoplasia. From this it can be seen that hyperplasia and hypertrophy are firstly indicative of prior focal areas of necrosis and secondly suggest that the affected cells may develop into more damaging lesions such as neoplasms.

Focal areas of necrosis

One fish from the EK and one fish from the MTN were found to have focal areas of necrosis. None of the fish from the OW had necrotic areas. According to Hinton & Lauren (1990), necrosis can take one of three forms: apoptosis, cytolytic and coagulative necrosis. Cytolytic necrosis is followed by replacement of the hepatocytes by reactive inflammatory cells and is a result of disease rather than exposure to toxicants (Hinton & Lauren 1990). Coagulative necrosis occurs when blood is no longer circulating in a particular zone of the liver or as a result of exposure to a toxin (Hinton & Lauren 1990). Apoptosis is not associated with inflammation and occurs under natural (cell death) circumstances and due to exposure to toxicants (Hinton & Lauren 1990). Differentiating between these three types of necrosis is difficult and requires experience in identifying hepatic pathological change. Hinton & Lauren (1990) highlight that coagulative necrosis can also occur under natural toxicant conditions such as during harmful algal blooms or as a result of bacterial or viral infections. They state that in the absence of signs in the field of these types of 'natural' insults to the organ, necrosis can be used as a biomarker of exposure to toxins. An infiltration of lymphocytes then removes the necrotic cells from the focal area of necrosis by phagocytosis (Hibiya 1982). This reaction normally occurs in tandem with the development of increased perivascular tissue around the areas (Hibiya 1982). This pathology was included in the Pierce *et al.* (1978) index, however, as was the case with the hypertrophy and hyperplasia, there was no indication of the extent of necrosis which was observed during the Pierce *et al.* (1978) study. The focal areas of necrosis observed during the present study were small, localised areas, however the necrosis described in Pierce *et al.* (1978) seemed to be more extensive and throughout the liver parenchyma. This uncertainty resulted in this pathology not being included in the current index. More laboratory work is necessary to determine to what extent necrosis arises as a result of exposure to xenobiotics and from this determine what a pathological level of necrosis is in *R. holubi.*

A study conducted by Zapata-Perez *et al.* (2000) found that injecting *Oreochromis niloticus* with contaminated sediments containing organochlorine pesticides, PCBs and PAHs induced necrosis after a 24 h exposure period (Zapata-Perez *et al.* 2000). Mondon *et al.* (2001) also found focal areas of necrosis in *Rhombosolea tapirina* following exposure to contaminated sediments containing PAHs, organochlorines and trace metals for 6 weeks. During the above study the authors also exposed *R. tapirina* to contaminants through food but focal areas of necrosis in the liver were only found in fish which were also exposed to disturbed sediment (Mondon *et al.* 2001). This is contrary to the findings of Mela *et al.*(*in press*) who recorded an increase in MNLs (as a result of necrosis) following consumption of contaminated prey by *H. malabaricus* (see section concerning MNLs above).

Nuclear change / nuclear activity in the hepatocytes

One fish from the OW and two fish from the MTN were found to have nuclear activity in some of the hepatocytes. None of the samples from the EK had this lesion. This condition is also known as nuclear pleomorphism and has been observed in several studies of fish suspected of being exposed to xenobiotics. Myers *et al.* (1992) conducted a study in the Puget Sound using three species of fish, English sole (*Parophrys vetulus*), the rock sole (*Lepidopsetta bilineata*) and the starry flounder (*Platichthys stellatus*). The above study was specifically aimed at detecting pre-neoplastic lesions in sub adult specimens as similar investigations had only found neoplastic lesions such as hepatomas in older fish (4+ years) (Myers *et al.* 1992, Myers *et al.* 1998). Samples of these species were collected from one reference (uncontaminated) site and seven contaminated sites over the period of a year. These authors found a significant correlation between fluorescent aromatic compounds (FACs) detected in the bile of the samples collected and the occurrence of nuclear pleomorphism (among other lesions) in the English sole and rock sole (Myers *et al.* 1992). Similarly, they found a significant correlation between PCBs and nuclear pleomorphism in the English sole (Myers *et al.* 1992). Nuclear pleomorphism has also been shown to be a precursor to megalocytic hepatosis and both of these lesions are considered indicators of exposure to xenobiotics and may be an early step in the sequence of the development of cancerous cells (Myers *et al.* 1991). The fact that nuclear pleomorphism is a precursor to megalocytic hepatosis may explain why no

megalocytic hepatosis was observed in *R. holubi* during the current study. It may be that xenobiotics concentrations were either too low or of too short a duration to trigger the formation of megalocytic hepatosis, but were sufficient to stimulate the development of nuclear pleomorphism.

The lesions described in the present study are all pre-neoplastic. This is in accordance with other research which suggested that younger fish do not show a high incidence of hepatic neoplasms, only the precursors to them (pre-neoplastic lesions) (Kohler 1989b, Myers *et al.* 1991, Myers *et al.* 1992). This is proposed to be a result of shorter exposure period because the fish are younger. Older fish that have been exposed to xenobiotics for a longer period of time will therefore show more advanced stages of liver degeneration (Au 2006).

3.5 Conclusion

All the fish from the EK had a good overall structure in the hepatic tissue except one. The mean index value for this group was 2.9 and the majority of the fish sampled from this estuary had an index value of 3. The most frequently occurring lesions were an increase in MMCs, an increase in perivascular tissue and vacuolation. The fish from the OW appeared to be very similar to the samples collected from the EK, with the most frequently occurring lesions being an increase in MMCs, hydropic change and vacuolation. The average index value for this estuary was 2.6, but the majority (4 out 5) of the fish had an index value of 3, while only one fish had a value of 1. None of the fish sampled from the OW showed a degeneration of the liver parenchyma (indicated by a score of 4). However, a larger sample size for this estuary would have given more information on the overall health of *R. holubi* in the system, but due to processing defects only five fish were used.

Fish from both the EK and the OW had an overall score which suggests normal histological structure. However, most of the individual fish in these estuaries had a score of 3 which is considered pathological. From this it is proposed that the fish from these estuaries may be being exposed to some sort stressor(s), possibly a xenobiotic(s), probably at a low dose and over a long period of time. If the stressor(s) occurred in high concentration, more pathological changes (such as necrosis or a loss

of structural integrity) would have been seen and if the stressor(s) had been present only for a short period of time, more regeneration would have been recorded. Hydropic change and increases in perivascular tissue have both been linked to xenobiotic exposure and both of these conditions were found in the fish from these two estuaries. The occurrence of these two lesions suggests that xenobiotics may have been stressing the fish from these two estuaries. Other lesions identified included an increase in MMCs and vacuolation, however these two lesions have been associated with normal liver function and therefore cannot be used to definitively state that the fish were exposed to xenobiotics.

The fish from the MTN estuary were the most variable from the three estuaries. The MTN had two fish which appeared to be healthy (grade 2), five fish were given a grade of 3 and two fish were grade 4. The grade of 4 indicates a degeneration of the liver parenchyma and is indicative of a fish which has been strongly affected by hepatic disease. The average score for this estuary was 3 which masks the fact that one estuary can contain fish with fairly severe hepatic damage and also contain fish that are healthy. In order to explain this finding, it is hypothesised that there may have been a stressor, possibly pollution, prior to the fish being sampled and some of the fish caught may have been nearer to this source than others, thereby explaining the differing extent of pollutant impacts seen in the histology.

Despite the literature available on the effects of xenobiotics on the lesions described in the current study, direct causative agents for the observed lesions are difficult to determine. Water samples collected for a multi-residue analysis of a range of contaminants from these estuaries did not detect any residues (see Chapter 5). Sediment samples need to be collected from these estuaries in order to determine whether they are contaminated and further laboratory trials need to be undertaken to determine the susceptibility of *R. holubi* to these contaminants. This study does suggest that there is a contaminant problem in all three estuaries and suggests that in one estuary at least, this may be a point source of pollution. Galloway & Handy (2003) state that histopathological damage on key organs such as the lymphoid tissues may impair an individual's ability to combat disease and infection due to possible immunosuppressant reactions caused by xenobiotics. It is proposed that similar problems may occur when liver impairment occurs. Fish examined during the present

study from all three estuaries are being affected at the organ level (the majority of individuals from all three estuaries obtained a score of 3 or above, which is considered a pathological condition). Using this as a proxy for the health status of an estuary, it is proposed that all three estuaries are under pollutant pressure, although the nature and extent of these stressor can only be determined using a combination of biomonitoring methods and laboratory studies. This will be discussed in Chapter 7.

The appeal of using histopathology to investigate the effects of xenobiotics on estuarine fish is that, unlike the community analysis, histopathology can be an early warning system that can identify detrimental effects which are occurring at the individual level before they become apparent at the population or community level. Liver histopathology is also a powerful tool because, essentially, it requires no prior knowledge of potential xenobiotics that may be in an estuary. Biochemical assays on the other hand are fairly limited in terms what an individual assay can detect. Histology is able to identify detrimental effects caused by a large range of xenobiotics and also enables the researcher to actually see what damage has occurred.

The index used in this study proved useful and most of the characteristics listed in the index were recorded in the fish sampled. It enabled a categorization of the lesions seen in a semi-quantitative manner, and so removing some of the subjectivity from the histological technique. A number of other lesions were also recorded and it is felt that these too should be included in this index if it is to be used in future estuarine studies. The weighting and placement in terms of grading for these lesions will require laboratory work in order to determine which category (2, 3 or 4) these should be listed under. For statistical analyses, it is felt that the weighting should be refined further, e.g. if a fish is listed under category 3 and has two of the listed lesions, it should be graded as 3.2. Similarly, a fish graded as 3 with four of the pathological features listed should be assigned a grade of 3.4. This additional information may lead to significant statistical differences being found between individuals and estuarine assemblages. Unfortunately this approach could not be used using the current data set because of the small sample size obtained.

Two major limitations were identified in this study. A hepatosomatic index (HSI), which is a simple measure of liver mass compared to the total mass of an individual

and can indicate atrophy or hypertrophy (Van Dyk 2003), would have added another dimension to the investigation. The equation for the HSI is simply liver mass divided by body mass multiplied by 100. Another limitation was the large number of slides which could not be used for histopathological analyses, probably as a result of poor initial preservation. Future studies should use Bouins solution as a fixing agent rather than 10% buffered formalin. Also, after removal from the fixative, the tissues should be slowly dehydrated through serial changes from 30% alcohol to 70% as this will lessen the damage to the tissue as the water is removed.

Chapter 4

Biomonitoring using the Lipid Peroxidation Assay (LPx)

4.1 Introduction

Oxidative damage or stress occurs when the antioxidant mechanisms within an organism's cells are overwhelmed by radical[3] (pro-oxidant) chemical species. Most of these radical species react with oxygen and are therefore termed Reactive Oxygen Species (ROS). Examples of ROS are superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxide radicals ($OH^{\bullet}$) (Kelly *et al.* 1998, Fatima *et al.* 2000, Valavanidis *et al.* 2006). ROS are unstable compounds which readily remove hydrogen atoms from substances such as polyunsaturated fatty acids (PUFAs). PUFAs are commonly found in cellular membranes, and the removal of hydrogen atoms from PUFAs alters the fluidity and permeability of the membrane (Kelly *et al.* 1998). ROS have four main sources in an organism, these being cellular respiration, certain oxidising enzymes, over-activation of phagocytes and certain xenobiotics (Kelly *et al.* 1998, Fatima *et al.* 2000). One to five percent of the ROS produced during the normal process of cellular respiration have been reported to not follow the normal electron pathway and cause damage to surrounding areas (Kelly *et al.* 1998). Certain oxidative enzymes such as cytochrome P450 also naturally produce ROS as part of normal enzyme function (Kelly *et al.* 1998). Phagocytes (e.g. monocytes, neutrophils and macrophages) produce ROS, which are potent bactericides, fungicides and virucides, to eliminate pathogens. The above listed sources of ROS occur naturally and a number of antioxidant systems are present in organisms to minimise any damage caused by ROS. Examples of such systems are superoxide dimutases (SODs), catalase (CAT) and glutathione peroxidase (GPx) (Kelly *et al.* 1998). All these mechanisms can be up-regulated under conditions of high oxidative stress. In healthy organisms, a balance exists between the production and neutralisation (by antioxidant systems) of ROS.

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[3] Radicals are molecule species with unpaired electrons. They are generally highly reactive and are therefore likely participate in chemical reactions with other molecules.

Xenobiotics can cause ROS in two ways: they can either over-activate the phagocytic response (Fatima *et al.* 2000) or they may be redox cycling compounds (Kelly *et al.* 1998, Valavanidis *et al.* 2006). ROS which originates from either of these two sources have the ability to overwhelm an organism's antioxidant systems, resulting in oxidative stress. Paper mill effluent containing dioxins, furans and pentacholorophenol, as well as other pollutants such as polycyclic aromatic hydrocarbons and organo-metallic compounds, have all been shown to affect the immune response (involving phagocytes) of fish (Fatima *et al.* 2000). Pulp mill effluent has been shown to over-activate the phagocytic response in fish, resulting in high levels of ROS damage in areas where phagocytes are most abundant, i.e. gills, kidneys and liver (Fatima *et al.* 2000).

Quinones, certain dyes, bipyridyl herbicides, certain transition metals, aromatic nitro compounds and some pesticides are examples of xenobiotics which are capable of redox cycling and enhance oxidative damage to cells (Kelly *et al.* 1998, Dorval *et al.* 2005, Valavanidis *et al.* 2006). Certain enzymes interact with these xenobiotics to form intermediate radicals. These intermediate radicals undergo chemical reactions with oxygen (O_2) to form superoxide ($O_2^{\bullet -}$) and subsequently return to their original form. Superoxide is also a radical and can therefore cause oxidative damage. The xenobiotics return to their original state during this process, therefore they are capable of forming numerous superoxide radicals, which increases the negative effect of these types of compounds (Kelly *et al.* 1998).

The lipid peroxidation (LPx) assay is designed to determine the extent of cellular oxidative damage which has taken place as a result of oxidative stress. Oxidative damage results in damaged lipids in cellular membranes and the formation of malondialdehyde (MDA). Phospholipases and glutathione peroxidase repair damaged lipids within the membranes of cells. The larger the chemical insult, the greater the amount of damage (and MDA formed) and the longer it will take for the cellular membranes to be restored (Kelly *et al.* 1998). The LPx assay is based on a series of reactions which ultimately detect the levels of MDA (Ringwood *et al.* 2003). It is determined by the levels of thiobarbituric acid reactive substance (TBARS) formed per hour per milligram of protein (Ferreira *et al.* 2005). This assay has been successfully used in both laboratory and field studies to detect oxidative damage

(Pandey *et al.* 2003, Dorval *et al.* 2005, Sole *et al.* 2006). It is important to highlight that this assay is regarded as a fairly crude measure of oxidative stress because thiobarbituric acid can also react with other chemicals and MDA is not only formed by lipid peroxidation (Janero 1990). It has nonetheless been successfully applied both in the field and in the laboratory to determine oxidative stress in fish (Pandey *et al.* 2003, Dorval *et al.* 2005, Lund Amado *et al.* 2006, Tejeda-Vera *et al.* 2007).

An aim of this study was to determine whether oxidative stress could be detected in *Rhabdosargus holubi* collected from three Eastern Cape estuaries using the LPx assay. The estuaries were selected based on their classification type (intermittently open) and proximity to one another and were the East Kleinemonde, Old Woman's and Mtana estuaries (for more information on catchment characteristics and surrounding land use between these systems see Chapter 1).

4.2 Methods

Sample collection

Rhabdosargus holubi of a similar size (n = 100) were collected over the period of one day from the lower reaches of each of the three estuaries during April 2005. Each fish was weighed and measured prior to being killed. The animals were juveniles and therefore not sexed. The liver was then dissected out of the fish, and approximately half of the liver was immediately snap frozen in liquid nitrogen. Samples were transferred to a -80°C freezer upon returning to the laboratory. Three surface water samples were also collected from each estuary in the mouth, middle and upper reaches, and arrived at the South African Bureau of Standards within 24 h of collection. A list of the chemicals that were tested for is presented in Chapter 5 (Table 5.2.1).

Lipid peroxidation (LPx) determination

Samples were assayed as reported by Ringwood *et al.* (2003). Each section of liver was weighed and homogenised using Teflon Eppendorf micropestles in four times the gram weight of the liver section in millilitres of cold 50 mM potassium phosphate

buffer (pH 7). The samples were then centrifuged for 5 minutes at 1300 g in a centrifuge at 4°C. An aliquot of 50 µl of sample supernatant was added to 700 µl of 10 mM 0.375% thiobarbituric acid (TBA) and 7 µl of 2% butylated hydroxytoluene (BHT). The samples were incubated in a 100°C dry bath for 15 minutes. The samples were then centrifuged for 5 minutes at 1300 g at 22 ± 2°C. An aliquot of 100 µl of supernatant was then placed in a microtiter plate (PowerWave X, Bio-Tek Instruments Inc, Winooska, VT, USA) and the absorbance was read at 352nm. A standard curve was prepared using serial dilutions of malondialdehyde tetraethylacetal (MDA). The concentration of the standard curve ranged between 6.25 and 3200 µM MDA. Sample MDA concentrations were calculated using this standard curve. All samples, standards and blanks were conducted in quadruplicate.

Sample protein content was determined using the method of Bradford (1976). A dilution was made using 2 µl of the original liver homogenate (containing four times the gram weight of the liver in millilitres of 50 mM potassium phosphate buffer (pH 7)) and 198 µl of 50 mM potassium phosphate buffer (pH 7). An aliquot of 20 µl of this diluted sample was placed in a microtiter plate and 230 µl of Bradford reagent was added. The plate was allowed to stand for 5 minutes at 22 ± 2°C after which a reading was taken at 595nm. Standards were prepared using commercial bovine albumin serum (BSA) and ranged between 0 and 0.4 mg/ml. This standard curve was used to calculate the protein content in each sample (Bradford 1976) and each protein level was multiplied by 100 to correct for the initial dilution of the sample homogenate. All samples, standards and blanks were performed in quadruplicate. Final MDA concentrations were expressed as nmol MDA formed/h/mg protein.

In addition to determining LPx activities, a condition factor was calculated for each fish using the following equation:

$$\left(\frac{weight}{(length)^b}\right)x100$$

where weight is in grams and length is in centimetres and b = 2.8512 in *Rhabdosargus holubi* (Blaber 1975). Condition factor is a biomarker which can be used as a measure of the feeding level of fish in one estuary compared to another. Under conditions of

low feeding levels, fish weight may decrease, thereby indicating lower food consumption in one system compared to another (Blaber 1975).

Statistical analyses

The LPx data were found to be non-normal (Kolmogorov-Smirnov, $P < 0.05$), therefore a non-parametric Kruskal-Wallis test was performed. Following this, multiple comparisons using Mann-Whitney U tests were conducted between each pair of estuaries to determine where significant differences occurred. The significance level was determined using a Bonferroni adjusted level of significance in order to avoid a type 1 error. The equation to calculate this is below:

$$\left(\frac{K(K-1)}{2}\right)$$

where K is the number of groups being compared (Zar 1999). The adjusted significance level was calculated to be 0.016.

The condition factor data was normally distributed (Kolmogorov-Smirnov, $P > 0.05$), and therefore an analysis of variance (ANOVA) was used to determine whether significant differences existed between estuaries in terms of fish condition factor. A post hoc Tukey test was used to determine where the differences occurred between the estuaries. A set of non parametric Spearman Rank order correlations were undertaken between condition factor and LPx levels in each estuary to determine whether any relationship existed between these two variables. All analyses were performed using a STATISTICA™ version 7 statistical package except for the correlations which were performed in Microsoft™ Excel.

4.3 Results

The results from the Kruskal-Wallis test for the LPx data are presented in Figure 4.3.1 below. Medians are used to describe the data rather than means because the data were not normally distributed. A significant difference in LPx levels in the fish was found between the three estuaries (Kruskal-Wallis, $H = 23.76$, $df = 287$, $P < 0.01$).

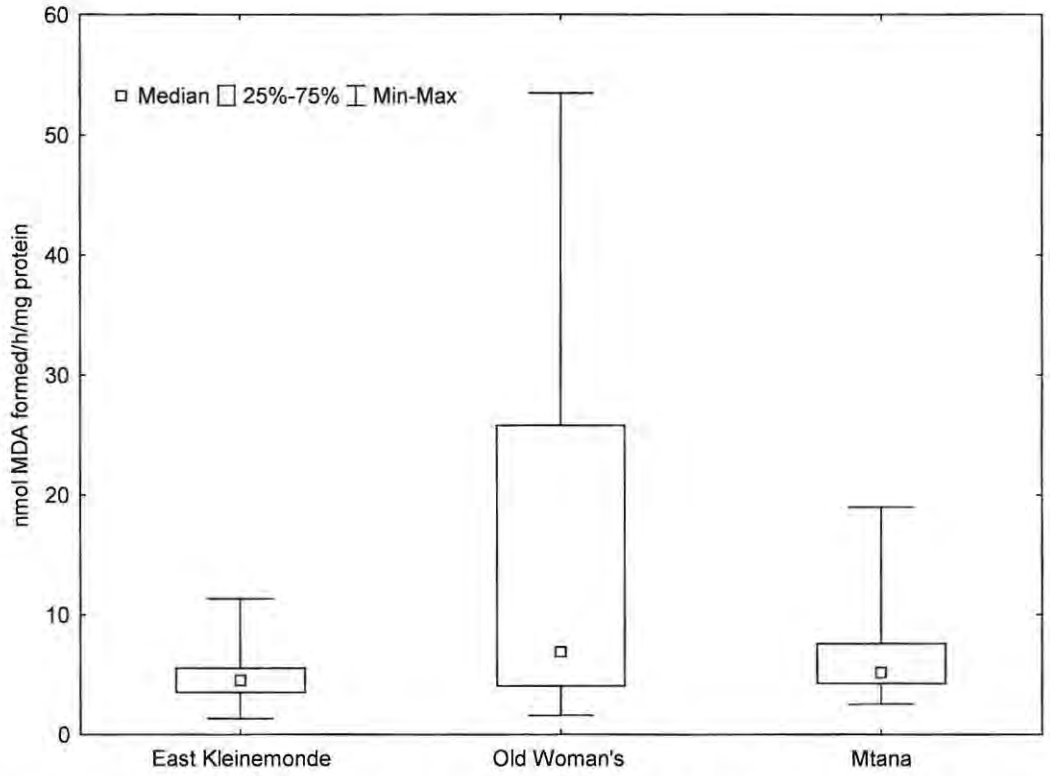

Figure 4.3.1: LPx levels for the East Kleinemonde (n = 97), the Old Woman's (n = 90) and Mtana (n = 100) estuaries. LPx levels are presented as nmol MDA formed/h/mg protein for each estuary. Error bars are minima and maxima values for each estuary and outer boxes are the upper and lower quartile limits for each estuary and the inner boxes are the median values for each estuary.

The LPx levels found in *R. holubi* from the East Kleinemonde (EK) ranged from between 1.32 and 11.33 nmol MDA formed/h/mg protein. Levels in unexposed fish from a laboratory experiment conducted during this study (Chapter 6) were found to range between 0.56 and 5.61 nmol MDA formed/h/mg protein and the range of LPx levels of unexposed fish is therefore hypothesised to range between zero and 6 nmol MDA formed/h/mg protein. Seventy nine percent of individuals from the EK had MDA concentrations of between zero and 6 nmol MDA formed/h/mg protein. LPx levels from the Old Woman's (OW) were from 1.59 to 53.47 nmol MDA formed/h/mg protein and 40% of the samples collected from this estuary occurred within the 0 – 6 nmol range. LPx levels in fish from the Mtana (MTN) estuary ranged between 2.52 and 18.97 nmol MDA formed/h/mg protein and 64% of the samples collected from this estuary fell within the range identified by the laboratory experiment.

A significant difference was observed in LPx levels between the EK and the OW (Mann Whitney U test, z = -4.329, P = 0.00001) and the EK and the MTN (Mann Whitney U test, z = -3.557, P = 0.0004), but no significant difference was found between the MTN and the OW (Mann Whitney U test, z = 2.206, P = 0.03).

A significant difference in fish condition factor was also found between all three estuaries (ANOVA, $F_{(2,296)} = 76.88$, $P < 0.0001$) (Figure 4.3.2). The results from the post hoc Tukey test showed that there were significant differences between all three estuaries (P = 0.0001, df = 284). Non parametric Spearman Rank order correlations were undertaken between LPx levels and condition factor between fish from each estuary but no significant correlations could be found ((P < 0.05) Figures 4.3.3 – 4.3.5).

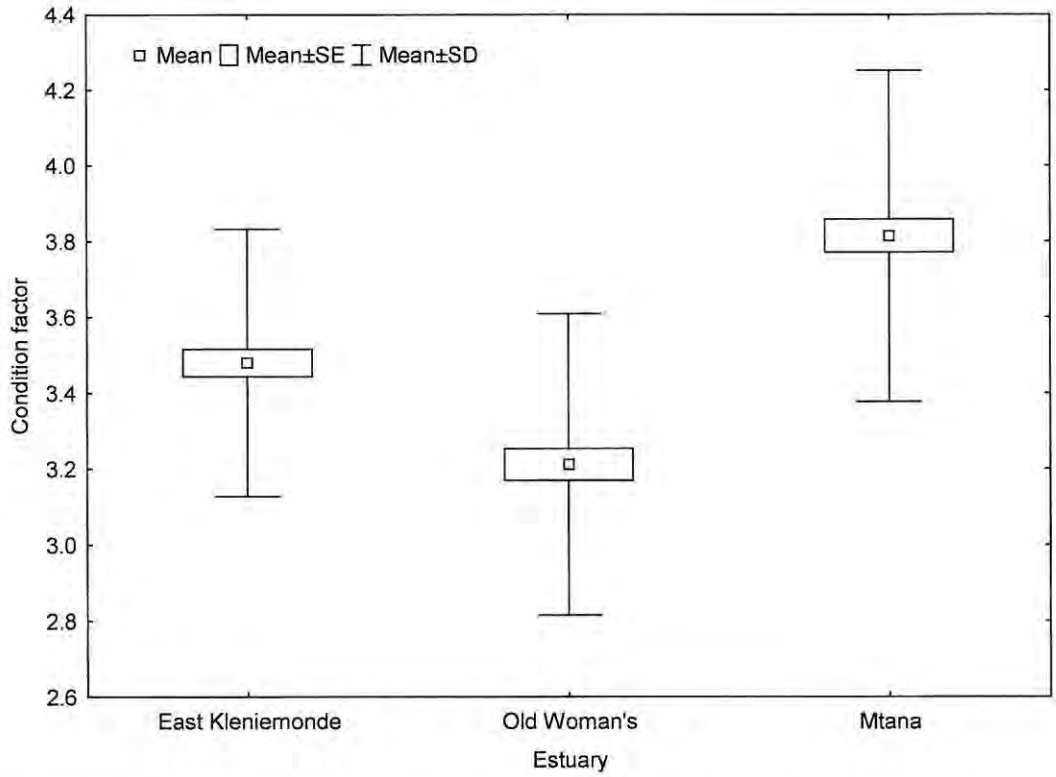

Figure 4.3.2: Comparison of condition factors obtained from fish sampled in the East Kleinemonde (n = 97), the Old Woman's (n = 90) and the Mtana (n = 100) estuaries. Inner boxes represent the means, outer boxes one standard error and error bars one standard deviation of the mean.

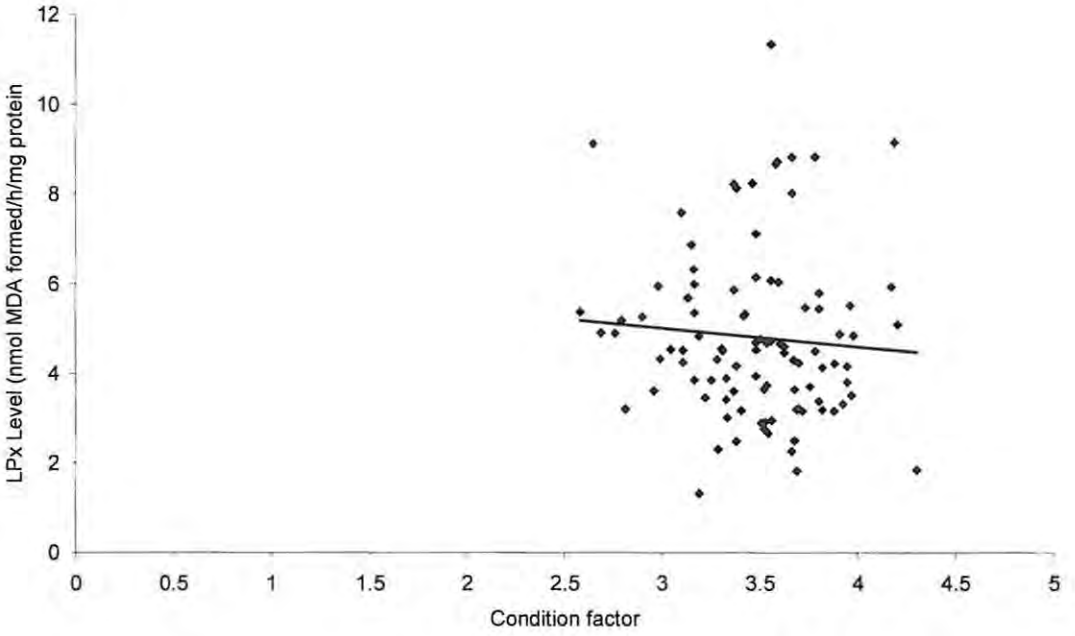

Figure 4.3.3: Data points indicating the relationship between condition factor and LPx levels for individual fish (n = 97) sampled in the East Kleinemonde estuary.

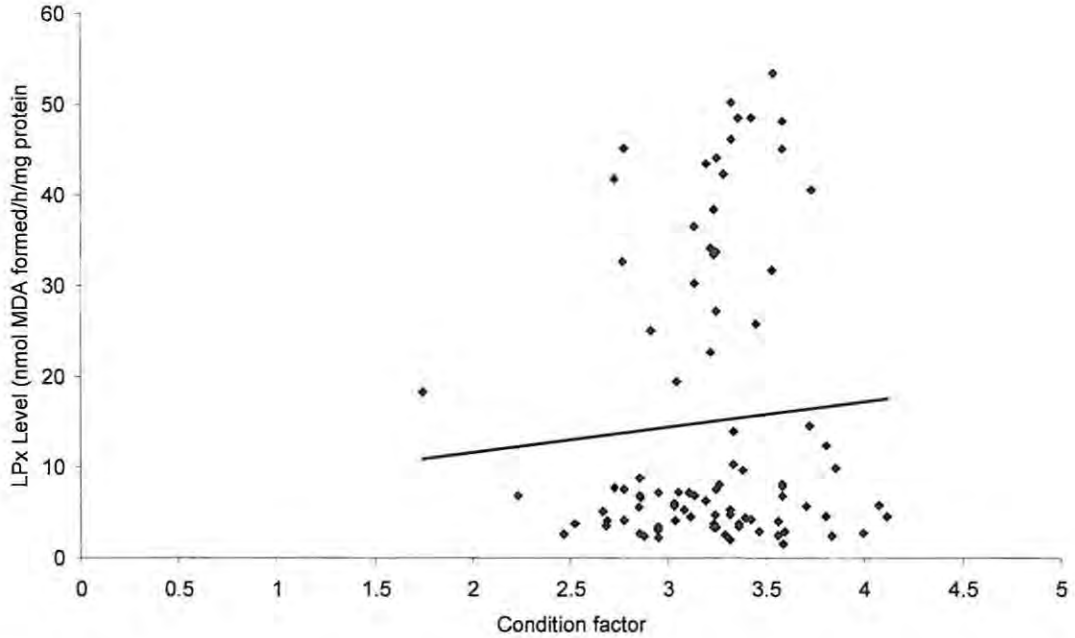

Figure 4.3.4: Data points indicating the relationship between condition factor and LPx levels for individual fish (n = 90) sampled in the Old Woman's estuary.

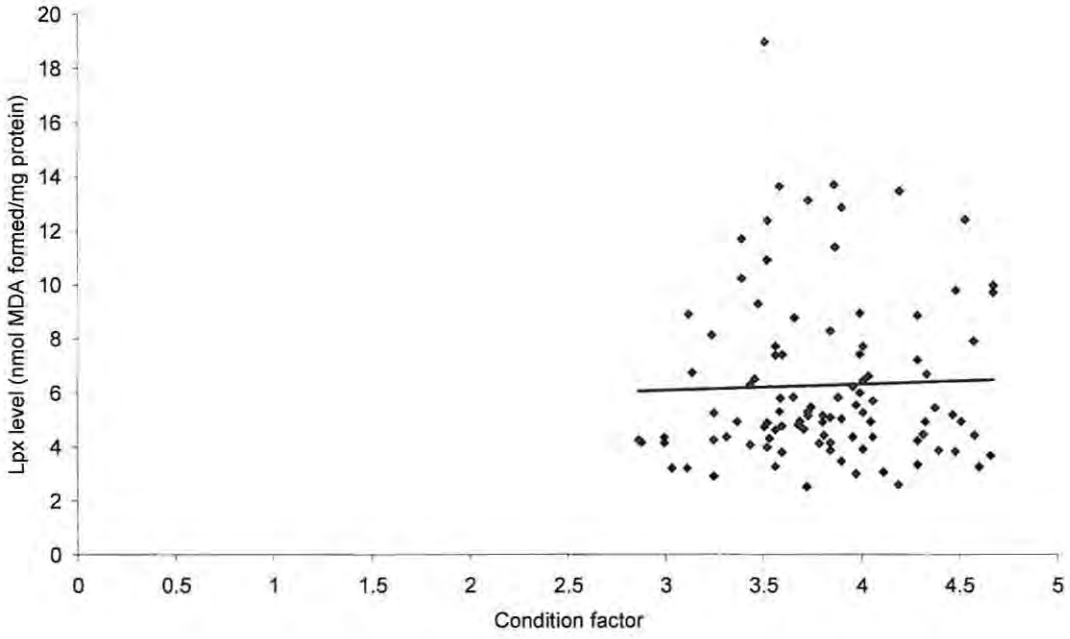

Figure 4.3.5: Data points indicating the relationship between condition factor and LPx levels for individual fish (n = 100) sampled in the Mtana estuary.

4.4 Discussion

Condition factor results

The three estuaries were found to be significantly different from each other in terms of the condition factor of *R. holubi* from these systems. Fish from the MTN had the highest overall condition factor, followed by the EK and then the OW. Considering the levels of development associated with these systems, the expected ranking of the estuaries in terms of fish condition factor would be MTN, followed by OW and then EK (i.e. simply looking at the systems from a development impact perspective). This expected ranking was not reflected in the *R. holubi* condition factor analyses from the three estuaries.

A possible reason behind the above result could be that the estuaries differ in terms of food availability and the presence of suitable submerged macrophytic habitats. Shallow water estuarine habitats that include submerged plant beds have been shown to be important nursery areas for the juveniles of fish species such as *R. holubi* (Blaber 1974). *R. holubi* feed on epiphytic diatoms which are present on aquatic macrophytes and filamentous algae, as well as epibenthic invertebrates found in the

littoral zone (Whitfield 1998). The low condition factor values obtained in the OW could be due to the fact that this estuary did not contain as much aquatic macrophyte habitat as the EK or the MTN. The reason behind the lower values in the EK when compared to the MTN could be due to the fact that the EK had lost most of its very extensive aquatic plant beds prior to 2006. However, at the time of sampling all three estuaries appeared to contain areas of submerged macrophyte beds; therefore this particular factor is unlikely to be the sole cause for the ranking in condition factor observed.

Another possible reason could lie with temperature differences between the estuaries. Blaber (1975) undertook a comparative study of the lipid content in the tissue of *R. holubi* collected from the West Kleinemonde estuary (a temporarily open/closed system) and the nearby Kowie estuary (a permanently open system). He found that the condition factor of *R. holubi* collected from the Kowie estuary was consistently high throughout the year, whereas the condition factor of *R. holubi* collected from the West Kleinemonde estuary varied seasonally. He proposed that the reason for this was that *R. holubi* from the Kowie system were feeding at a similar level throughout the year, whereas the fish from the West Kleinemonde were feeding much less during the winter months when compared to the summer months. Blaber (1975) attributed this difference in feeding rate between the two systems to temperature differences in the two systems, with the Kowie estuary receiving the moderating influence of marine waters throughout the year. During the present study, all three estuaries investigated were TOCEs, and all three would have water temperatures that were driven primarily by ambient land temperatures which are similar along this stretch of coast. Therefore, increased temperatures as a result of the influx of warmer seawater during winter (as was the case in the Kowie system) could not be the causative agent behind the higher condition factor in the MTN and EK when compared to the OW.

From the above it seems unlikely that either temperature or food availability were the primary drivers behind the differences in *R. holubi* condition factor between the three estuaries. As mentioned earlier, the expected condition factor ranking for these three estuaries was based solely on the level of development on the banks of each estuary and would have been the MTN followed by the OW and then the EK. However, the OW had a lower condition factor than the EK, thus suggesting that something else is

negatively impacting on the biota of the OW. It is proposed that pesticides which are being introduced into this estuary from the adjacent golf course could be the reason for the pattern observed. Exposure to pollutants often results in species specific reactions and it may be that the chemicals being applied to the golf course are entering the estuary and negatively impacting on the food sources of *R. holubi*, or directly on individual fish, thus resulting in a lower condition factor being recorded in this system. Further work is necessary before this hypothesis can be verified.

Lipid peroxidation assay results

A laboratory study (Chapter 6) was conducted using wild caught *R. holubi* which were held in uncontaminated seawater for six weeks prior to the exposure experiment. LPx levels for the unexposed fish ranged between 0.56 and 5.62 nmol MDA formed/h/mg protein. A laboratory study conducted using the liver of wild caught freshwater catfish *Heteropneustes fossilis* tested LPx levels of control (unexposed) fish over a period of 90 days (Fatima *et al.* 2000). The authors depurated the test fish for 15 days prior to the commencement of the experiment and measured LPx levels after 15, 30, 60 and 90 days during the experiment. They found no significant differences in the unexposed values over the course of the experiment (Fatima *et al.* 2000). Their study suggests that LPx levels stay constant under unstressed conditions and can be used as reference levels against which other levels (e.g. from wild caught fish) can be compared. LPx levels have however been shown to vary seasonally and depend on the nutritional state of the animal (Ahmad *et al.* 2004, Ferreira *et al.* 2005) and this could have affected the results obtained during the laboratory study of this the current study.

Ferreira *et al.* (2005) collected 12 flounder (*Platichthys flesus*) and 12 flathead mullet (*Mugil cephalus*) in various seasons and the fish were depurated for a month prior to LPx analysis. Flounder were found to have increased LPx levels following depuration during all seasons, possibly linked to the fact that *P. flesus* did not feed during the one month depuration period. The mullet, however, did eat for the one month duration of each experiment and showed higher LPx levels over the autumn and winter periods when compared to spring and summer. The lower LPx levels during the latter two seasons were proposed by the authors to be linked to higher temperatures associated these seasons.

In the current study, the laboratory fish were fed to satiation during the entire holding period, therefore nutritional state should not have affected the results obtained. However, the experiment was conducted during spring, whereas the field study was undertaken during autumn. Temperature and reproductive state are two of the major driving variables which create differences in LPx levels between seasons. The animals used during the present study were all juveniles (< 14cm SL (Blaber 1974)), and therefore reproductive state should not be a confounding factor. The holding tanks used were maintained at a constant temperature of 22°C. Estuarine temperatures fluctuate on a daily basis, as well as seasonally, and these fluctuations are therefore difficult to mimic under laboratory conditions. Although the results from the laboratory study cannot be used in a direct comparison with data from field caught individuals, they can be used to provide a guideline against which field values can be compared, and a range of 0-6 nmol MDA formed/h/mg protein was considered to provide baseline values for unstressed fish.

Using the 0-6 nmol MDA formed/h/mg protein bracket identified by the laboratory study, fish collected from all three estuaries appear to have been affected to varying degrees by oxidative stress. Twenty one percent of the fish from the EK exceeded this range but none of the individuals collected from this estuary had LPx values greater that 12 nmol MDA formed/h/mg protein. This suggests that even though some of the individuals from this estuary appear to have been affected by oxidative stress, the extent of this stress may have been relatively limited. It may have been that the individuals with slightly higher LPx levels were simply the more sensitive individuals of the population. Another possibility is that there was some point source of pollutants in the EK and the 21% of fish with higher LPx values were the ones closest to this point source and were therefore more affected than other fish from the same estuary. No correlations were found between condition factor and LPx levels in any of the estuaries, suggesting that nutritional status was not a factor driving the increased LPx levels observed.

In comparison to the EK, 60% of the individuals from the OW had LPx levels which exceeded 0-6 nmol MDA formed/h/mg protein. The data from the OW estuary was bimodal, with some individuals having LPx levels below 10 nmol MDA formed/h/mg protein and others having values between 20 and 60 nmol MDA formed/h/mg protein

(Figure 4.3.3). The estuary is known to have been closed for four months prior to sampling; therefore it is not possible that the less affected fish had recently entered the estuary from the marine environment. The data seems to point towards a localised pollution problem in this estuary which only affected a few individuals, resulting in high LPx levels in these fish. This hypothesis is supported by the fact that no pre-emptive or widespread spraying was conducted on the golf course, and sprays are only applied to specific areas after a problem occurs (Burger pers. comm., see Chapter 5 for more information). This may have resulted in patchiness in the chemical composition of runoff, creating localised patches of toxicant contaminated water.

Dorval *et al.* (2005) investigated LPx levels using the white sucker (*Catostomus commersoni*) between three polluted sites and one control site in Quebec. The authors found that the fish from the three polluted sites showed significantly higher LPx levels in the liver tissue than those taken from the control site. The authors attributed this to the presence of agricultural chemicals such as pesticides in the water at the polluted sites. Pesticides may have been affecting the fish from the OW. Although, water samples for pesticide residue analysis were collected from EK, OW and MTN estuaries (for more information on these analyses see Chapter 5), no residues were detected in any of the water samples. This may have been due to the short half life of most of the chemicals tested for in aqueous solution, or the chemicals may have been diluted in the water below the detection limit of the testing methods. Nonetheless, even brief exposures to chemicals can result in changes in LPx levels in fish over several days. *Gambusia affinis* was exposed to 20.49 mg/L monocrotophos (an organophosphate pesticide) for 96 h and LPx levels were reported to return to basal only after 8 days (Kavitha & Venkateswara Rao 2007). Therefore it is possible that the changes in LPx levels seen in the OW were as a result of exposure to pesticides. Thirty six percent of the fish from the MTN were outside the range identified by the laboratory study. The values reported in the MTN were not as high as those in the OW, with a maximum value of 18.97 nmol MDA formed/h/mg protein recorded in this estuary. The was no bimodal pattern in the data as was found in the OW, and most of the data points fell between 3.5 and 14 nmol MDA formed/h/mg protein. A significant difference in LPx levels was found between the EK and MTN, suggesting that these two systems are different despite having similar LPx levels. There is no development in the MTN catchment; therefore the reason behind the elevated LPx

values is unknown. The water samples collected in this estuary also showed no traces of any of the chemicals tested for in the pesticide residue analysis. One hypothesis is that free ranging cattle from the catchment that are treated with a pesticide to combat ticks may be introducing pollutants into the estuary by wading through the water when crossing the estuary.

Overall, the EK appears to be the least affected of the three estuaries because the fish from this estuary were found to have the lowest LPx values. The MTN was found to be significantly different to the EK and from this is it proposed that some of the individuals from the MTN estuary were affected by ROS producing xenobiotics. The origin of these chemicals is unknown but may be related to the consequences of cattle dipping in the catchment. The OW had bimodal data, which suggests a point source of pollution within the estuary and this pollution may be originating from the adjacent golf course.

The use of the liver as a biomarker organ for lipid peroxidation

In the present study, the liver was analysed for signs of oxidative damage. The liver is an important organ in terms of detoxification of xenobiotics because it is the first organ to come into contact with absorbed material from the stomach via the portal vein (Naigaga 2002). The liver is one of the primary organs involved in the overall homeostasis of the body in terms of nutrition, defence against toxicants and reproductive development (Hinton & Lauren 1990). The liver's key role in the overall homeostasis of an animal was therefore the principal reason for which this organ was selected for the present study. The liver was also chosen to determine whether signs of stress at the biochemical level are also seen at the organ level using histological techniques (see Chapters 3 & 7).

Several studies have investigated the effects of a range of pollutants on other organs of fish such as the kidneys and the gills (Fatima *et al.* 2000, Ahmad *et al.* 2003, Ahmad *et al.* 2004). A study by Fatima *et al.* (2000) showed that upon exposure to pulp mill effluent, freshwater catfish (*Heteropneustes fossilis*) showed tissue specific oxidative damage as a result of phagocytic over-activation. The main affected areas were the kidneys and the gills, despite the fact that the liver is also a site of high

phagocyte concentration. They hypothesised that the reason for the liver being less affected compared to the other organs was because the liver is the site of metallothionein production. Metallothioneins are strong antioxidant compounds and therefore are able to limit oxidative damage (Fatima *et al.* 2000). The liver is therefore more protected from oxidative stress than the other two organs.

Ahmad *et al.* (2004) found that eels (*Anguilla anguilla*) exposed to polluted harbour waters showed a time dependent reaction to ROS compounds in the kidney, gills and liver. The gills showed signs of oxidative damage following 8 h of exposure to contaminated water whereas the liver and kidneys only showed these signs after 48 h (Ahmad *et al.* 2004). Their study focused on the role of the natural defence mechanisms against ROS damage. The authors demonstrated that upon exposure to contaminants, phagocytes were activated and an antioxidant potential was developed concomitantly in the gill, kidney and liver. The liver was the site of highest antioxidant potential activation and therefore this was the site of lowest LPx levels and this was hypothesised by the authors to be a result of metallothionein production. Metallothioneins were proposed to have reduced the impact of the pollutants from the harbour water on the liver for an initial period. Following this, the liver became overwhelmed, and all three organs showed similar levels of oxidative stress. Thus, the authors demonstrated that following a long duration of exposure, all organs show exposure of some extent to contaminants (Ahmad *et al.* 2004). From the two studies described above, it appears that the liver is slower to show signs of oxidative stress during the initial phase of a toxic insult, however if the insult persists, the liver reacts in a similar manner to other organs.

Relationships between antioxidant systems and lipid peroxidation

Ferreira *et al.* (*in press*) conducted a laboratory study using the mullet *Mugil cephalus* exposed to mixed contaminants in the Duoro Estuary, Portugal. The authors collected 22 fish from the estuary and measured LPx levels following 24 h, 1 month, 4 months and 8 months in captivity. During the captivity, the fish were maintained in contaminant free water. The authors recorded a decrease in LPx levels with increasing captivity time (Ferreira *et al. in press*). However, following eight months in captivity, the authors recorded an increase in LPx levels and attributed this to a recorded

decrease in glutathione peroxidase (GPx) activity. GPx is a ROS scavenging complex, which is in the form of a glutathione redox equilibrium and oscillates between the reduced state of glutathione (GSH) and the oxidised state glutathione disulphide (GSSG) (Pena-Llopis *et al.* 2003). Ferreira *et al.* (*in press*) concluded that there was an inverse relationship between LPx and GPx, with the latter being a protective mechanism to avoid oxidative damage measured by the former (Ferreira *et al. in press*). Similar results were found by Li *et al.* (2003) who carried out an exposure experiment using *Carassius auratus* to determine oxidative stress responses. The authors found an increase in LPx with increasing toxicant concentrations of 3,4-Dichloroaniline (a common ingredient in pesticides and herbicides) accompanied by a decrease in GSH levels (Li *et al.* 2003).

Antioxidants may explain the results found in the present study in the MTN and the OW. It may be that some of the individuals from the MTN had been exposed to a point source of pollution, but unlike the individuals with high levels from the OW, the fish from the MTN were able to combat the oxidative stress. It may also be that these mechanisms were overwhelmed in the OW individuals because the stressor was too strong or too prolonged. Roberts & Oris (2004) exposed rainbow trout (*Oncorhynchus mykiss*) to 10.2 mg/L hexavalent chromium for 0, 1, 2, 7, 14, 21, and 28 days. LPx responses fluctuated over this period, initially increasing until 168 h, after which they returned to basal levels. The authors attributed this levelling off to mediation between biochemical effects. The authors proposed that metallothionein (MT), LPx and superoxide dimutases (SODs) were all interdependent, with MT and SODs reducing LPx levels because they are antioxidants (Roberts & Oris 2004). These results suggest that there may be a whole range of biochemicals which are compensating for each other. The extent of variability seen in the OW results, when compared to the variability in the MTN and the EK, suggests that at least some of the individuals in the OW had been strongly affected by oxidative stress. Therefore it is suggested that in order for the OW samples to show such high values, all the compensatory mechanisms which are able to combat oxidative stress may have been overwhelmed.

Biomonitoring using the lipid peroxidation assay

The LPx assay is a general biomarker of effect and is useful because it can give an indication of the overall water quality with regards to xenobiotics which can cause oxidative stress. It has been successfully used in field and laboratory studies to determine sub-cellular stress levels using freshwater, estuarine and marine species (Fatima *et al.* 2000, Ahmad *et al.* 2004, Dorval *et al.* 2005, Ferreira *et al.* 2005, Ferreira *et al. in press*). One of the major short comings of the present study was that no concomitant antioxidant responses were measured to compare against LPx levels. A good antioxidant biomarker of exposure is the glutathione-*S*-transferase assay, and results from this assay can give an indication of the extent of antioxidant production which is taking place within an animal (Lund Amado *et al.* 2006).

The LPx assay was shown to be sensitive enough to distinguish between three estuaries with different catchment activities. It provides a longer term record of past pollution events than water chemistry analyses. This was shown in the present study, where signs of oxidative damage were found despite there being no detectable chemical residues in the water samples that were collected. It is a relatively straight forward assay to perform, is repeatable and many samples can be assayed at once, which means that large sample sizes can be used. Finally, because this assay uses the liver, it is possible to compare the results from this assay with analyses conducted on the entire organ, such as histopathological studies of the liver. This can enable researchers to use a weight-of-evidence approach when attempting to identify causative agents of pollution in the field.

There are a number of negative aspects related to using this assay. Field studies using the LPx assay for biomonitoring purposes have had results which highlight areas where knowledge about the mechanisms of oxidative stress is lacking. For example, Pandey *et al.* (2003) conducted a field study of the freshwater fish *Wallago attu* on the Yamuna River, comparing two sites which were under different types of pollutant pressure. The authors used lipid peroxidation to detect whether there were signs of oxidative stress in fish. Chemical analyses revealed that one of the sites was more polluted than the other (Pandey *et al.* 2003) but no statistically significant differences in terms of LPx levels were found between the two sites. The authors suggested that

this may have been because LPx levels had reached saturation in both sites. The authors measured a number of other antioxidant biomarkers and proposed that over-activation of these mechanisms may also have contributed to levelling off of LPx at both sites (Pandey *et al.* 2003). A shortcoming highlighted by this study is that there is no laboratory data which shows at what point oxidative stress levels off.

Schmitt *et al.* (2007) used a battery of biomarkers to determine whether differences in oxidative stress levels existed between a number of varyingly impacted sites (including three reference sites). Their results detected no difference between reference and control sites using LPx; however other biomarkers, e.g. δ-aminolevulinic acid dehydratase (ALA-D), iron and zinc protoporphyrin in the blood, were statistically correlated to elevated concentrations of metals in the water (Schmitt *et al.* 2007). The main reason proposed for there not being any significant differences using the LPx assay was because the river was contaminated with lead-zinc mining tailings, and lead has been shown not to be a redox cycling chemical (Campana *et al.* 2003, Schmitt *et al.* 2007). Zinc on the other hand is a transition metal and has been shown to cause oxidative stress in humans (Lin *et al.* 2005). It is interesting that the lead-zinc combination did not affect LPx levels in the Schmitt *et al.* (2007) study and this result highlights the need for investigation into synergistic and antagonistic effects of pollutant mixtures on LPx levels.

Finally, Lund Amado *et al.* (2006) found a decrease in LPx levels in polluted sites compared to reference sites in *Micropogonias furnieri*. The authors attributed this decrease in LPx levels between the sites to an increase in PUFAs within the membrane of the fish caught in the reference site, which increases the vulnerability of a membrane to oxidative stress (see introduction). More information is required on the mechanisms which increase the occurrence of PUFAs in order to be able to better interpret these types of results. From the three studies described above, it can be seen that many factors influence LPx levels. This means that supporting laboratory work is necessary in monitoring work and also means that the results are not necessarily straight forward to interpret.

Recommendations and future research

Future biomonitoring programs using the LPx assay should include a concomitant antioxidant assay to give an indication of both the level of oxidative stress occurring in an organisms and the amount of repair which is occurring. An interesting connection between oxidative stress and endocrine disruption has emerged. Dorval *et al.* (2005) investigated this relationship and found that prolonged exposure to agricultural chemicals increases oxidative stress and results in impaired endocrine function in terms of thyroid hormone plasma levels. This has important implications in terms of the estuarine management, since South Africa imports 60% of all pesticides used in sub-Saharan Africa (Naidoo & Buckley 2003). The thyroid endocrine system has been shown to control neural development and metabolism in marine fish, as well as to have influences over other endocrine systems (Matthiessen 2003). Therefore, any changes to this system can have direct effects on neural development but also indirect effects on other endocrine systems.

Investigations should also be conducted into the potential success of management interventions, such as limiting the amount of chemical use allowed during rainy seasons, since this action may alleviate oxidative damage to estuarine biota. LPx levels were used to determine the extent of oxidative damage which had occurred in two fresh water species (*Ameca splendens* and *Goodea atripinnis*) in Mexico by comparing a polluted site containing waste water from a sugar processing industry and a control site (Tejeda-Vera *et al.* 2007). Results from the study showed that LPx levels were highest during the rainy season (September) and lowest during the dry season (May) (Tejeda-Vera *et al.* 2007). This may also be the case in regions of South Africa where rainfall is seasonal and a limitation on the use of chemicals during these periods could reduce oxidative stress effects on the aquatic biota.

The above study also highlighted that endemic species may be more sensitive to chemicals than more cosmopolitan species (Tejeda-Vera *et al.* 2007). During the present study, an endemic species was used (*R. holubi*); however this fish is regarded as being relatively hardy and resilient to physiological stresses (Blaber 1975, Whitfield 1998). Therefore future research should investigate the pollution

sensitivities of different species in order to determine which taxa are the most appropriate for estuarine monitoring in South Africa.

4.5 Conclusion

The LPx assay was successfully applied to three Eastern Cape estuaries to determine the extent of oxidative stress in individuals collected within them. The most affected estuary (showing the highest LPx levels) was the OW and the origin of the chemicals is hypothesised to be the golf course fairways and greens adjacent to the estuary. Thirty four percent of the fish from the MTN estuary also had elevated LPx levels but the reason for this is unknown because there are no industrial or agricultural activities within the catchment. It is hypothesised that some form of cattle dip is being used to treat cattle for tick infestations, after which the cattle may walk along or through the estuary, in the process releasing this product into the water. The samples from the EK mostly fell within the range of unexposed laboratory held specimens. Approximately 20% of the fish examined were above this limit but the LPx levels were not as high as those found in the MTN or the OW. From this, it is hypothesised that the fish from this estuary are generally unaffected by oxidative stress.

The present study was conducted within the same biogeographical area and in the same month. Due to this, problems regarding comparability of data in terms of geography and season should not affect the results. All the fish used for the study were sexually immature and therefore hormonal status should not have affected the results either. LPx levels have been shown to be altered by nutritional status of the animals under investigation however, the role of an estuary as a food rich environment is fundamental to its role as a nursery area. For this reason, if the LPx values recorded are elevated as a result of lower food or habitat availability in one estuary compared to another, then this is proposed to be an indication of the differential status of the two estuaries and is therefore a meaningful result in terms of the aims of this project.

The LPx assay has been shown to be a useful indicator of biological health and some future areas of investigation have been suggested. These include investigating the effects of spraying chemicals less frequently during rainy seasons to see if this

reduces the impact on oxidative stress in aquatic animals. Different species should be investigated in order to determine whether *R. holubi* is sensitive enough for biomonitoring schemes using the LPx assay. Investigations should be undertaken into the links between oxidative stress and endocrine disruption because should such effects exist, these would have additional important estuarine management implications. This is primarily because estuaries are important nursery areas for many recreationally and commercially important species and, if their reproductive potential is being adversely affected by the environmental conditions within estuaries, then the stocks of the species need to be managed accordingly.

Chapter 5

Biomonitoring using the Acetylcholinesterase assay (AChE)

5.1 Introduction

Acetylcholine (ACh) is a neurotransmitter found in brain and in muscle tissue (Mathews & Van Holde 1990). In muscle it acts as an inhibitor and in the brain it acts as a stimulant (Fulton & Key 2001). Neurotransmission is achieved by the release of an electrical signal from the synapse into the axon. This signal travels to the axonal terminal and is converted into a chemical signal. This chemical signal then travels to the following neuron (to the post synaptic receptor) and is converted back into an electrical signal. The chemicals which transmit across the spaces between neurons are neurotransmitters. Acetylcholinesterase (AChE) is an enzyme which regulates the duration of the neurotransmitter acetylcholine (ACh). AChE breaks down ACh at the post synaptic receptor, effectively switching off the delivery of the chemical message by ACh. This terminates the transmission of the nerve impulse across the synaptic cleft (Mathews & Van Holde 1990). ACh is the primary neurotransmitter that controls skeletal muscle contractions and cardiac muscle contractions. Inhibition by xenobiotics of the AChE enzyme can result in muscle spasms in the affected animal because the ACh neurotransmitter is not 'switched off', resulting in the chemical message being continuously transmitted. A classic example of AChE inhibition in humans is the effect of the nerve agent Sarin on muscular functions. The AChE assay measures levels of the AChE enzyme to detect the extent of acetylcholinesterase inhibition which has occurred as a result of exposure to a range of anticholinesterase compounds. Inhibition of AChE enzymes exposed to these types of chemicals is generally irreversible (Sturm *et al.* 1999) and thus in order for neurotransmission to resume, new AChE enzymes must be formed.

Measuring enzyme inhibition caused by neurotoxins gives an indication of the motor abilities of the animal in question. This measurement can be obtained using the acetylcholinesterase (AChE) assay. AChE controls a large proportion of physiological and behavioural responses in fish and is therefore of importance to the normal

functioning of an animal and because of this any changes to these regulatory abilities could potentially be detrimental to the animal (Pan & Dutta 1998, Kirby *et al.* 2000). The AChE assay is regarded as a good biomarker of exposure and has been successfully used in several monitoring programs (both in the laboratory and in the field) to detect previous exposure to a range of anticholinesterase compounds (Kirby *et al.* 2000, Roex *et al.* 2003, Eder *et al.* 2004, Lau *et al.* 2004).

The use of pesticides in coastal areas has raised questions about the effects of these products on nearby aquatic systems such as rivers, estuaries and ocean waters. Pesticides are generally introduced from non-point sources such as agricultural runoff, spray drift and urban development sites (Fulton & Key 2001). Insecticides are generally in the form of organophosphorous (OP), carbamate (Cs) and synthetic pyrethroid compounds. OP, Cs and pyrethroids degrade rapidly in the environment (Fulton & Key 2001), however they are non-specific in action and can be toxic to a range of organisms (Kirby *et al.* 2000, Fulton & Key 2001). South Africa is the main pesticide user in sub-Saharan Africa (London *et al.* 2005) and therefore there is a need to monitor the effects of these chemicals on the biota of South African estuaries. OPs, Cs and pyrethroids are neurotoxic, and acetylcholinesterase activity (AChE activity) can be rapidly inhibited by exposure to these chemicals (Sturm *et al.* 1999, Fulton & Key 2001). The AChE assay can be used as a biomarker of exposure to pollutants such as OPs and Cs in freshwater, estuarine and marine teleosts (Sturm *et al.* 1999, Fulton & Key 2001, De la Torre *et al.* 2002). Until recently, the AChE assay was considered to be a specific biomarker of exposure to pesticides and insecticides. However recent studies have shown that AChE activity is also inhibited by metals, organochlorines and surfactants (Guilhermino *et al.* 1998, Guilhermino *et al.* 2000, Corsi *et al.* 2003). The assay is now considered a more general biomarker for exposure to neurotoxins.

The test species used in this study was *Rhabdosargus holubi*. It is a category IIa estuarine dependent marine species (Whitfield 1994b) and as such is representative of a number of other species such as kob (*Argyrosomus japonicus*) and white steenbras (*Lithognathus lithognathus*). This group of fishes is important to both subsistence and recreational fisheries in South Africa. *R. holubi* is also readily adaptable to laboratory conditions. Few South African studies on fish have been conducted using the AChE

assay, and all of these studies used the Mozambique tilapia *Oreochromis mossambicus* (Joubert 2000, Slabbert *et al.* 2004). *O. mossambicus* would have been an ideal candidate for estuarine biomonitoring because, despite the fact that it is freshwater species (category IV according to the estuarine association system (Whitfield 1994b)) it has been reported in salinities reaching 100 psu (Whitfield 1998). This species is also readily adaptable to laboratory conditions (Joubert 2000, Naigaga 2002, Slabbert *et al.* 2004) but it does not occur in Eastern Cape estuaries during winter months (Ellender, unpub.) and is therefore not an appropriate candidate for estuarine studies in this region.

The aims of this study were therefore to determine whether different levels of AChE inhibition could be detected in *R. holubi* collected from three estuaries using the AChE assay. Three intermittently open Eastern Cape estuaries were selected based on proximity to one another and topographical similarity. The selected systems were the East Kleinemonde, Old Woman's and Mtana estuaries (for more information on catchment characteristics and surrounding land use between these systems see Chapter 1).

5.2 Methods

Sample collection

Rhabdosargus holubi of similar size (n = 100) were collected over the period of one day (in April 2005) from the mouth region of each estuary. Each specimen was weighed and measured prior to being killed. All the fish were juveniles and were therefore not sexed. For each individual, the spinal cord was severed and the brain material was immediately removed, rinsed in a 0.02M phosphate buffer (pH 8.0) and snap frozen in liquid nitrogen. The brain material was stored at -80°C until it was analysed. Vertebrates generally contain two types of cholinesterases, Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) and questions have been raised about the reliability of the AChE activity assay due to its non-selectivity between AChE and BChE. Several studies have shown that fish brain tissue contains only AChE and therefore only brain tissues were used for AChE activity determination during this study (Sturm *et al.* 1999, Varo *et al.* 2003).

Pesticide analyses

Three water samples were collected from each estuary at the surface of the water column. Samples were collected from the mouth, middle and upper reaches of each system and reached the South African Bureau of Standards for pesticide analyses within 24 h of collection. A list of the chemicals that were tested for is presented in Table 5.2.1.

Table 5.2.1: List of chemicals which were tested for in water samples from the East Kleinemonde, Old Woman's and Mtana estuaries.

	Chemical type			
	Polychlorinated biphenyls (PCBs):	**Organochlorines**	**Organophosphorous pesticides**	**Pyrethroids**
Chemical name	PCB-52	Alpha-BHC	Chlorpyrifos-Me	Cyhalothrin
	PCB-87	Lindane	Fenitrothion	Cyfluthrin
	PCB-153	Heptachlor	Malathion	Cypermethrin
	PCB-138	Aldrin	Chlorpyrifos	Deltamethrin
	PCB-180	Heptachlor epoxide	Chlorfenvinphos	
		pp'-DDE	Profenophos	
		Dieldrin	Dichlorvos	
		Endrin	Sulfotepp	
		β-Endosulfan	Diazinon	
		Methoxychlor	Parathion,	
		p-p'-DDT	Mevinphos	
		o-p'-DDT	Pirlmiphos-methyl	
		p-p'DDE	Parathion-methyl	

The analytical method used is a generic test for South African waters (OACA International 1995). The greens manager at the Fish River Sun, where the Old Woman's estuary is situated, was able to provide some information on possible contaminants in the water based on the spraying regime the company uses. No information regarding the presence of any chemical pollutants was available for the other two estuaries. Due to this lack of knowledge about possible contaminants in the EK and MTN estuaries, this generic test was deemed the most appropriate for pesticide analyses in these three estuaries.

Acetylcholinesterase activity determination

Methods for the determination of AChE activity in the brain tissue were optimised for *R. holubi* based on methods described by McLoughlin *et al.* (2000). Briefly stated, each brain was homogenised on ice in 30 µl cold phosphate buffer (pH 8.0) containing 1% (v/v) Triton X-100, using Teflon Eppendorf micropestles. The homogenate was then diluted with 270 µl phosphate buffer (pH 8.0) and centrifuged at 4°C at 13000 x *g* for 20 minutes. Supernatant (50 µl) was then diluted with 950 µl of cold phosphate buffer (pH 8.0) containing 0.1% (v/v) Triton X-100. Standards were prepared using commercial acetylcholinesterase at a concentration of 1 mg/ml. Standard curves were undertaken between 0 mg/ml and 0.125 mg/ml. An aliquot of 100 µl of 8 mM 5,5'-di-thiobis (2-nitrobenzoic acid) (DNTB) was added to each microtiter plate well, along with 50 µl of the diluted supernatant or commercial acetylcholinesterase and kept on ice. The microtiter plate was then incubated at 30°C in a microtiter plate reader (PowerWave X, Bio-Tek Instruments Inc, Winooska, VT, USA) for 3 minutes. An aliquot of 50 µl of 16 mM acetylthiocholine iodide was then added to each well after which a reading was taken at 405 nm every 30 seconds for 10 minutes (McLoughlin *et al.* 2000). Each sample, standard and blank reaction, was carried out in quadruplicate and the average of these four readings was taken for each sample to calculate enzyme activity. Enzyme activity was calculated using the following equation:

$$\text{Activity}(\mu\text{mol/ml/min}) = \left(\frac{\Delta abs}{\varepsilon \times L}\right) \times (df) \times 10^3$$

Where an extinction coefficient (ε) value of 1.36 x 10^4 mol/l/min, and where L is the pathlength, Δabs is the change in absorbance and df is the dilution factor (Ellman *et al.* 1961).

Protein content in the brain tissue was determined according to methods described in Bradford (1976). An aliquot of 20 µl of a solution containing 50 µl sample homogenate supernatant and 950 µl phosphate buffer (at a pH of 8 with 0.1% (v/v) triton X-100) solution was placed in a microtiter plate well. Bradford reagent (230 µl) was added. The plate was allowed to stand for 5 minutes at 22±2°C after which a reading was taken at 595nm. Standards were prepared using commercial bovine albumin serum (BSA) and ranged between 0 and 0.4 mg/ml. The standard curve was used to calculate the protein content in each sample (Bradford 1976). All microtiter

plates contained at least two standards which were compared to the standard curve to check repeatability of the results. All samples, standards and blanks were performed in quadruplicate.

All AChE activities are presented as nmol/min/mg protein. In addition to determining the AChE activity, a condition factor was calculated for each fish using the following equation:

$$\left(\frac{weight}{(length)^b}\right)x100$$

where weight is in grams and length is in centimetres and b = 2.8512 for *Rhabdosargus holubi* (Blaber 1975).

Statistical analyses

The AChE activity data were found to be non-normal (Kolmogorov-Smirnov, $P \leq 0.05$), therefore a non-parametric Kruskal-Wallis test was performed. Following this, multiple comparisons using Mann-Whitney U tests were conducted between each pair of estuaries to determine where significant differences occurred. The significance level was determined using a Bonferroni adjusted level of significance in order to avoid a type 1 error. The equation to calculate this is below:

$$\left(\frac{K(K-1)}{2}\right)$$

where K is the number of groups being compared (Zar 1999). The adjusted significance level was calculated to be 0.0166.

The condition factor data was normally distributed (Kolmogorov-Smirnov, $P > 0.05$), and therefore an analysis of variance (ANOVA) was used to determine whether significant differences existed between estuaries in terms of fish condition factor. A post hoc Tukey test was used to determine where the differences occurred between the estuaries. A set of non parametric Spearman Rank order correlations were undertaken between condition factor and AChE activity in each individual estuary to determine whether any relationship existed between these two variables. All analyses were

performed using a STATISTICA™ version 7 statistical package except for the correlations which were performed in Microsoft™ Excel.

5.3 Results

The water samples contained no detectable levels of any of the chemicals tested for (detection limits for the analyses were 0.5µg/l).

AChE levels are presented in medians rather than means because all the data were found to be not normally distributed. Median brain AChE values of 54.82 nmol/min/mg protein, 36.57 nmol/min/mg protein and 55.94 nmol/min/mg protein were obtained for the East Kleinemonde (EK), Old Woman's (OW) and Mtana (MTN) estuaries respectively (Figure 5.3.1). AChE activity was inhibited by 2% in the EK compared to the MTN estuary, and was inhibited by 35% in the OW relative to the MTN estuary. A significant difference in AChE activity was found between the between the three estuaries (Kruskal-Wallis, $H = 44.08899$, $df = 293$, $P < 0.0001$) (Fig 5.3.1). A significant difference in AChE brain activity was observed between the EK and the OW (Mann Whitney U test, $z = 4.781$, $P = 0.000002$) and the MTN and the OW (Mann Whitney U test, $z = -6.409$, $P = 0.000001$). However no significant difference was found between the EK and the MTN (Mann Whitney U test, $z = -1.476$, $P = 0.140$). Variability was lowest in the OW estuary, and highest in the MTN (Figure 5.3.1).

A significant difference in fish condition factor was also found between all three estuaries (ANOVA, $F_{(2,296)} = 76.88$, $P < 0.0001$) (Figure 4.3.2). The results from the post hoc Tukey test showed that there were significant differences between all three estuaries ($P = 0.0001$, $df = 284$). Due to this, non parametric Spearman Rank order correlations were undertaken between AChE activity and condition factor between fish from each estuary. Significant correlations between AChE activity and condition factor in fish from the EK ($P < 0.5$, $r = 0.256$) and the MTN ($P < 0.5$, $r = -0.207$) using Spearman Rank order correlations (Figures 5.3.2 & 5.3.4). No significant relationship was found between these variables in the OW ($P > 0.5$, $r = -0.048$) (Figure 5.3.3).

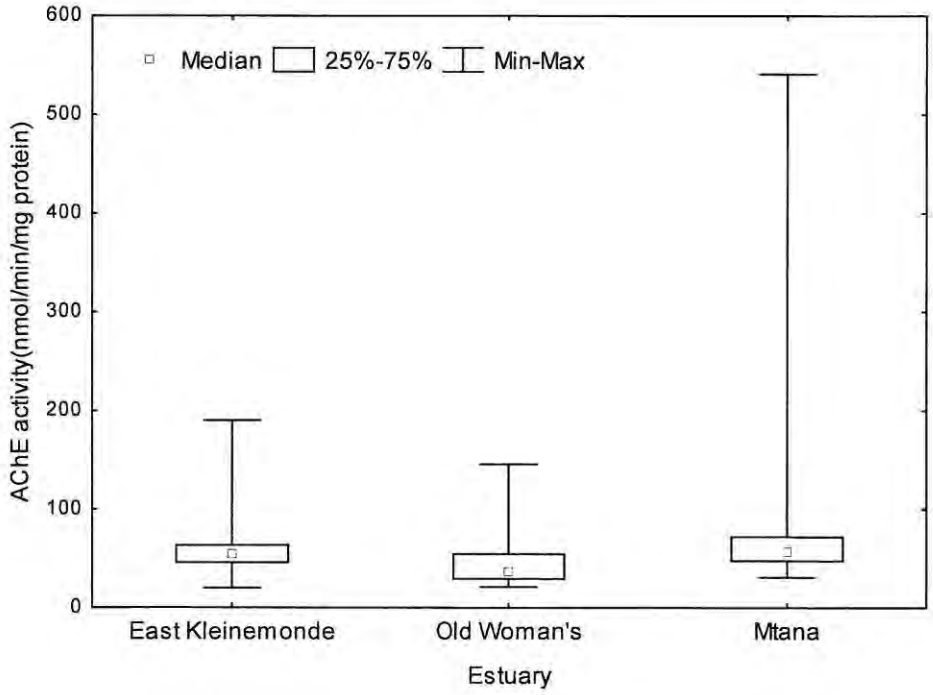

Figure 5.3.1: AChE values for the East Kleinemonde (n = 100), Old Woman's (n = 99) and Mtana (n = 94) estuaries. AChE activity is presented in nmol/min/mg protein for each estuary. Error bars are minima and maxima values for each estuary, outer boxes are the upper and lower quartile limits for each estuary and the inner boxes are the median values for each estuary.

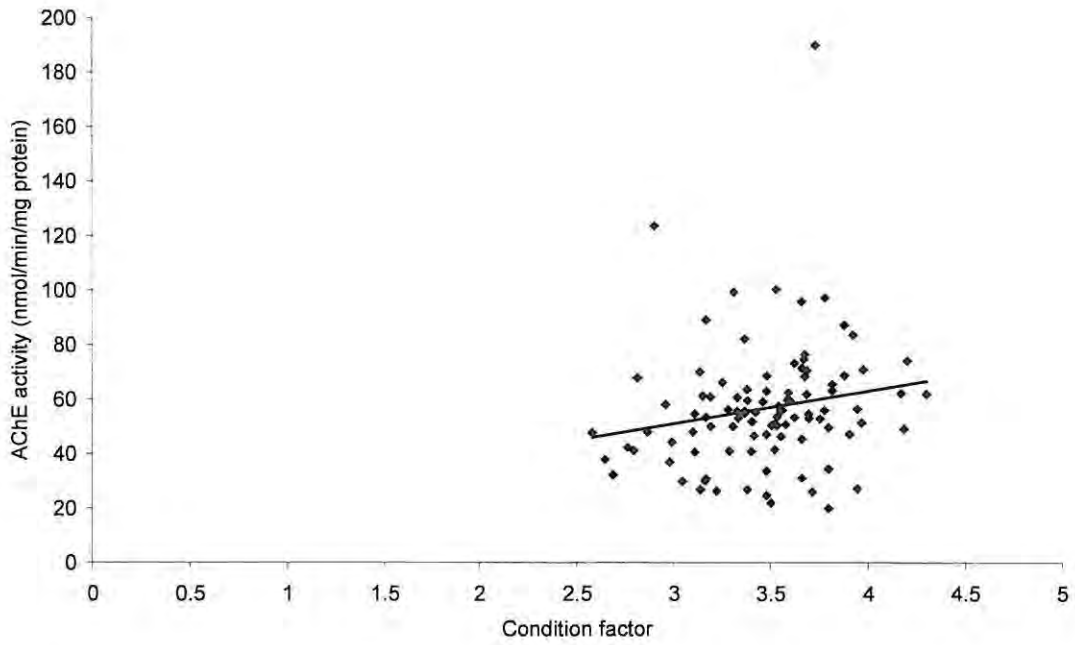

Figure 5.3.2: Correlation between condition factor and acetylcholinesterase activity for all fish (n = 100) from the East Kleinemonde estuary.

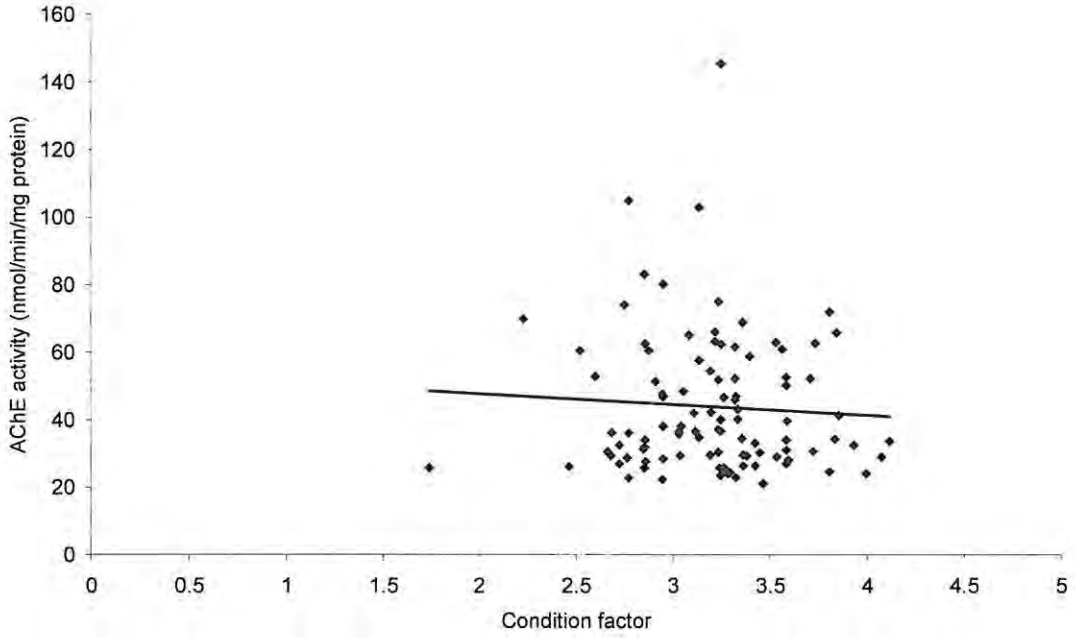

Figure 5.3.3: Correlation between condition factor and acetylcholinesterase activity for all fish (n = 99) from the Old Woman's estuary.

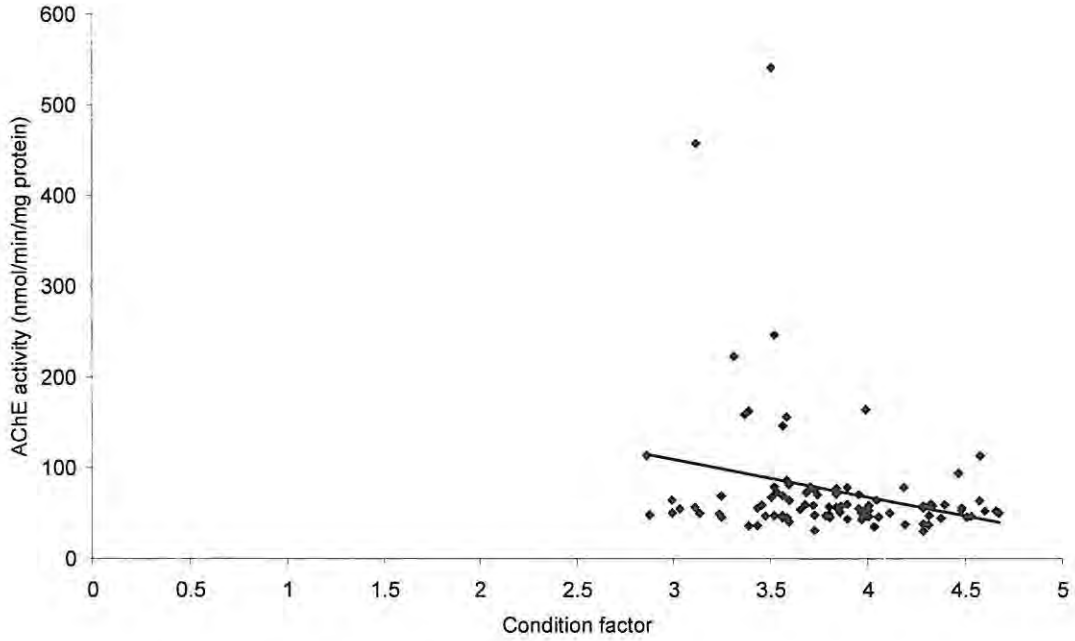

Figure 5.3.4: Correlation between condition factor and acetylcholinesterase activity for all fish (n =94) from the Mtana estuary.

5.4 Discussion

Pesticide analyses

Information obtained from the greens maintenance company of the golf course situated on the banks of the Old Woman's estuary (Fish River Sun Resort) confirmed that the company had applied a number of chemicals on the golf course in the year prior to sampling. These were 2,4 Dimethylamine salt, glyphosate, ferrous sulphate, ammonium sulphate, metribuzin and triadimefon - all which are listed as slightly toxic to fish on the PAN pesticide database (Pesticide Action Network Pesticide database). Isoxaben, tebuconazole, DDVP, Propiconzale and copper sulphate - which are documented as moderately toxic by the Pesticide Action Network Pesticide database - were also used. Products such as chorothalonil, hydramethylnon, cyfluthrin and trifluralin (which are listed as highly toxic to aquatic organisms on the Pesticide Action Network database) were also infrequently applied to the golf course. The golf course greens managers used these products when a pest or problem appeared, and not in a pre-emptive manner.

The fact that none of the chemicals tested for were detected in the water samples collected from the estuaries may be due to the time of sampling, as there had been no recent rainfall event to wash any chemicals into the estuaries. Chemicals may have already been washed into the river and diluted or degraded before sampling took place. Other factors to consider are the short half life of pesticides and other similar chemicals in the aqueous solutions (De la Torre *et al.* 2002), or that the chemicals were present in levels below the detection limit of the analyses. These possibilities highlight the short comings of using water sample chemical analyses as the results provide limited insight into the longer term effects of harmful chemicals on the biota of an ecosystem. Chemical analyses simply provide a 'snap shot' of the water quality at the time of sampling. Biomonitoring integrates recent pollution events and can be used to determine the effect of the pollution problem on the aquatic biota.

Acetylcholinesterase assay results

The time it takes for brain AChE levels to return to basal levels following exposure to anticholinesterase has been reported to take up to six weeks (Fulton & Key 2001). A laboratory study (see Chapter 6) was conducted during the present study using *R. holubi* which were kept in holding tanks for 6 weeks in uncontaminated seawater prior to any analyses being undertaken. The median brain AChE value for control (unexposed) *R. holubi* specimens was 55.75 nmol/min/mg protein following this six week depuration. The median brain AChE value for the MTN was 55.94 nmol/min/mg protein and was 52.82 nmol/min/mg protein for the EK. The median brain AChE values obtained for the control specimens from the laboratory experiment and the brain AChE values obtained from wild caught *R. holubi* in the MTN and the EK were very similar. It is therefore hypothesised that the test species from these two estuaries were unaffected by anticholinesterase substances in the immediate period prior to their capture for this study.

Similar results were seen in two studies conducted in Portugal. In a laboratory study undertaken in 2005, Monteiro *et al.* (2005) determined that *Pomatoschistus microps* taken from uncontaminated sites had a mean AChE activity value of 85 nmol/min/mg protein. During a subsequent laboratory study using wild caught *P. microps*, Monteiro *et al.* (2007) determined that brain AChE values for unexposed fish after a two week depuration in the laboratory was 83.09 nmol/min/mg protein (Monteiro *et al.* 2007). From the above mentioned studies, it is proposed that there is little variability over time in unexposed fish brain AChE levels in a species. Therefore, if wild caught samples are within 2 or 3% of acetylcholinesterase concentrations obtained from unexposed fish in laboratory studies, the samples are unlikely to be have been affected by anticholinesterase substances.

Results from the current study showed that the AChE levels in fish from the OW were decreased by 35% compared to values obtained for fish from the EK and MTN. Inhibitions greater than 20% are considered to be indicative of exposure to anticholinesterase chemicals (Varo *et al.* 2003). The results therefore suggest that fish from the OW were exposed to anticholinesterase agents although the percent inhibitions recorded during the current study are lower than those found in related

studies which used similar techniques (Coppage & Braidech 1976, Kirby *et al.* 2000, Minier *et al.* 2000, De la Torre *et al.* 2002). For example, Coppage & Braidech (1976) found differences in inhibition rates of 205% between carp (*Cyprinus carpio*) caught upstream and down stream of an organophosphate and carbamate pesticide manufacturer on the Missouri River. De La Torre *et al.* (2002) reported 65% inhibition of AChE in fish from a contaminated site on the Reconquista River compared to laboratory controls. Both of these studies were undertaken in heavily industrialised rivers, and this is thought to be the primary reason for the high inhibition rates recorded during these studies. In the present study, the estuaries sampled are not heavily impacted by land-use developments (see Chapter 1 for more details), therefore such high inhibition rates would have been surprising.

A study conducted using three marine coastal sites (two polluted and one reference) in France using flounder (*Platichthys flesus*) found that muscle ChEs underwent a 40-45% decrease in activity in the polluted sites compared to the reference site (Minier *et al.* 2000). These observations were attributed to the fact that the two polluted sites were known to have polycyclic aromatic hydrocarbons (PAHs) in the sediments due to an along shore drift of dredged material from a nearby port. Fifteen estuaries in the UK were sampled using *P. flesus* muscle tissue for an evaluation of neurotoxic contamination in the estuaries (Kirby *et al.* 2000) and inhibition rates of up to 68% inhibition were recorded. It should be noted that both the above studies used muscle ChE levels, and these tissues include BChE which is not present in brain tissue (Sturm *et al.* 1999). This may, in part, account for the slightly higher inhibition rates in muscle evaluations compared to the current study.

None of the four studies mentioned above could guarantee that the fish sampled had not migrated between sites. The Eastern Cape study was conducted in intermittently open estuaries, and therefore has the advantage of ensuring that the estuaries were closed (and thereby prohibiting any migration of the test species between estuaries) for at least four months prior to sampling. The principle pollution source in the OW is the golf course adjacent to this estuary. It is suggested that the fish brain AChE inhibition reported in the present study may be a result of pesticide and other anticholinesterase products being applied on the golf course and subsequently being washed into the system during rainfall events. The brain AChE samples obtained from

the EK showed no signs of exposure to anticholinesterase pollutants, despite the fact that there are housing developments along the banks of this estuary. No fish brain AChE inhibition was recorded in the MTN either, suggesting that anticholinesterase pollution was not affecting the fish from this estuary at the time of sampling.

Acetylcholinesterase activity and condition factor

Brain AChE activity was found to be positively correlated with condition factor in the EK, negatively correlated in the MTN and not orrelated in the OW (see Chapter 4, Figure 4.3.2; refer to Figures 5.3.2-5.3.4 for more information on condition factor). A number of laboratory studies have been conducted to determine the effect of fish size/age classes on AChE activity. Varo *et al.* (2003) investigated the effects of dichlorvos on brain AChE in European sea bass (*Dicentrarchus labrax*). They found mean values of brain AChE for *D. labrax* of 58.05 nmol/min/mg protein for control fingerlings (length = 7.88 ± 0.72 cm) and 43.32 nmol/min/mg protein for control juveniles (length = 19.93 ± 0.1.26 cm) (Varo *et al.* 2003). Thus it appears that *D. labrax* fingerlings have higher AChE levels than juveniles.

Oliveira *et al.* (2007) examined brain AChE levels in 20 marine species and found levels varied substantially between species (from 145 to 530 µmol/min/g protein). These authors found that fish size significantly affected AChE levels. In their study, size was negatively correlated to AChE activity and the authors concluded from this that larger fish contain less brain AChE than smaller fish (Oliveira *et al.* 2007).

The Oliveira *et al.* (2007) study was conducted in the marine environment where a broad range of size classes are available for each species. In the current study, the size range of *R. holubi* specimens collected from the estuaries was limited because adults of this species only occur at sea. However, a limited size range of juveniles was purposefully selected in order to try to limit interference from confounding factors such as size on the results obtained from each estuary. A negative correlation was recorded between AChE activity and condition factor in the MTN estuary, which would agree with the Oliveira *et al.* (2007) study. However, a positive correlation between AChE activity and condition factor was recorded in the EK and no correlation was found in the OW. The overall lack of pattern in the data set and the

lack of other abiotic parameters which were not recorded during this data collection renders any conclusioins on the effect of size on AChE activity (or vice versa) in *R. holubi* unclear. More research is necessary in order to elucidate any interactions between these or other parameters.

Biomonitoring using the acetylcholinesterase assay

The appeal of using the AChE assay is that it is a biomarker of effect (Peakall & Walker 1994), and only a limited range of pollutants, namely organophosphorous pesticides, organochlorines, carbamates, pyrethroids, surfactants and certain metals, can affect AChE levels. Due to this, the AChE assay is a powerful monitoring tool since it gives an indication of the type of pollutant in the system. This assay has been successfully used in field studies to determine the health status of a suite of different types of habitats, ranging from the freshwater to marine environment under a wide range of pollution scenarios using both fish and invertebrates (Forget *et al.* 2003, Robillard *et al.* 2003, Varo *et al.* 2003, Oliveira *et al.* 2007). It is a reliable indicator of exposure and is more informative than simple water chemistry samples because it indicates the extent to which an animal has been affected. Levels of chemical which affect AChE levels mostly have short half lives within the water column (apart from certain metals and some persistent organic pollutants) and are therefore difficult to detect using water chemistry methods (Fulton & Key 2001, Abdel-Halim *et al.* 2006). This was shown during the present study, where water samples showed no traces of a range of chemicals (see Table 5.2.1), however the fish sampled from the OW estuary had been exposed to anticholinesterase agents as indicated by to the lowered AChE activities in the fish sampled from this estuary. The AChE assay integrates pollution effects over longer periods (up to 6 weeks following exposure (Fulton & Key 2001)) than water samples. It is an easy, repeatable, quick assay to undertake and many samples can be analysed in one batch ($\approx$ 45 samples at one time). There is a wealth of literature on the use of this biomarker over a number of different scenarios which can also aid in the interpretation of results.

In South Africa, very little information regarding chemical contamination of estuaries is available for the majority of estuaries. Given this paucity of information, it is very difficult to carry out water quality testing using water chemistry techniques because

researchers do not know for which chemicals to test. The AChE assay can be used to detect whether an effect is apparent and, if so, there is a limited range of chemicals which could be tested for in the water column or in the sediments. This is a far more time and cost effective approach than testing for an inclusive range of possible contaminants.

There are, however, several problems associated with using the AChE assay because results from the assay can sometimes be inconclusive. The effect of interactions between chemicals on AChE levels is not always straight forward. For example, a laboratory study conducted using the European sea bass *Dicentrarchus labrax* found significant decreases (76%) in AChE activity in the brain of fingerlings exposed to 1mg/L of dichlorvos for 96 h. However, they observed a 62% decrease in brain AChE activity after exposure to 4mg/L dichlorvos for 96 h (Varo *et al.* 2003). Therefore two different concentrations of the same pollutants have relatively similar effects on brain AChE levels, despite the fact that they are four orders of magnitude apart. The fact that many biomarker responses are not dose dependent, meaning that the biomarker cannot identify the concentration of pollutant which has resulted in the effect measured, is a limitation of these monitoring tools. The slightly higher levels of AChE reported at the 4mg/L exposure, when compared to AChE at 1mg/L exposure, could be indicative of compensatory mechanisms at the higher concentration which are combating the toxicant insult. Laboratory testing is necessary in order for such compensatory mechanisms to be quantified under different pollution scenarios and with different species.

Another type of problem which occurs when interpreting data from the AChE assay is that different combinations of pesticides in the field can have the same overall effect on AChE levels in fish. This renders the interpretation of inhibition rates between sites considerably more difficult under circumstances where pollution scenarios are known to be different between different sites. In order to assist with the interpretation of such cases, studies have investigated correlations between pesticide concentrations in the tissues of test species with AChE activity. A study conducted in the Gulf of Taranto, Italy, using *Mullus barbatus* and *Trachurus mediterraneus* brain AChE activity found no significant difference between polluted and control (pristine) sites for both species (Lionetto *et al.* 2004). AChE values for values *M. barbatus* were

between 80-90 nmol/min/mgprotein at the control and exposed sites and AChE values for *T. mediterraneus* were between 130-140 nmol/min/mgprotein at the control and exposed sites (Lionetto *et al.* 2004). These results were complemented with tissue analyses which found 0.01 ppm of organophosphate and organochlorine pesticide residues in fish at each site (Lionetto *et al.* 2004). Using tissue burden analyses in tandem with AChE activity enabled the authors to conclude that the site which they believed to be uncontaminated was in fact was also contaminated. From the study described above, it appears that tissue burden levels are a good proxy for AChE determination.

However, the above findings are contrary to a field study conducted in Egypt which found that tissue burden levels were not a good proxy for AChE activity (Abdel-Halim *et al.* 2006). Abdel-Halim *et al.* (2006) compared a range of anticholinesterase agents present in water, sediment and tissue samples of *Tilapia* spp. against AChE activity. When total organophosphorous pollutants (OPP) were at a concentration of 43 ppb in the fish tissue, AChE activity levels were depressed by 22.8%. When the total concentration of a different range of OPP measured in the tissue was 52 ppb, AChE activities were 21.3% (Abdel-Halim *et al.* 2006). Therefore, different combinations of different OPP within the tissues of fish showed similar effects on the AChE activities (Abdel-Halim *et al.* 2006).

Another example of complicated interactions between xenobiotics and AChE levels was demonstrated during a laboratory study conducted using the fresh water species *Gambusia yucatana*. Rendon Von Osten *et al.* (2005) tested for the toxicity of carbofuran, chlorpyrifos, and a mixture of chlorpyrifos and glyphosate. AChE inhibition was 60% after 24 h exposure to chlorpyrifos alone at an EC50[4] value of 0.011mg/L but *G. yucatana* appeared to be less susceptible to chlorpyrifos when it was mixed with glyphosate as the fish were able to tolerate an EC50 concentration of 0.7mg/L.

[4] EC50: Effect concentration: dosage at the which the desired effect is present in 50% of the population being tested

The major problem with these types of scenarios arises when it comes to determining which toxicant or combination of toxicants is causing the observed decrease in AChE activity. Pollution management policies are difficult to formulate if a biomarker is unable to pinpoint which toxins are responsible for measured effects. There are a number of factors at play in the natural environment which may not be simulated accurately in laboratory studies. There may be antagonist and synergistic effects between different pollutants when they are all present in the water at the same time. The AChE assay is therefore useful for measuring effect, however it is limited because it cannot identify what the causative agents were that triggered the measured effect.

All the studies discussed in this section highlight the complexity of the reactions between pollutants in the aquatic environment and the biota which reside within this environment. Water chemistry samples could be misinterpreted in these circumstances in terms of the effect of the measured chemicals on the biota because they would not take into consideration antagonistic or synergistic effects. A biomarker based approach which monitors pollutant effects in environmental monitoring is imperative in order to be able to detect 1) that a pollution problem is present and 2) to what degree the biota are being affected. Chemical mixing processes in the aquatic environment, particularly in dynamic environments such as estuaries, are very difficult to mimic in laboratories. This is why traditional methods which determine lethal end points are of limited use because many toxicants may be rapidly diluted in these environments and only affect estuarine biota at a sublethal level. Approaches such as these are restricted in terms of what they can explain because they only test for one chemical and are then related to measured levels in the field. These tests do not take into consideration reactions between several chemicals in the environment. The biomarker based approach is one tool which can be used to quantify the effects of a number of chemicals on estuarine biota in the natural environment (*i.e.* incorporating mixing effects etc).

Recommendations for future studies in estuaries

The AChE assay has been successfully used to monitor longitudinal gradients of pollution along estuaries. Kirby *et al.* (2000) found decreasing inhibition of AChE

along the Tyne estuary in the UK. The authors attributed this result to a down stream gradient of anticholinesterase chemicals which were affecting their test species less and less as they moved away from the point source of the contaminant. This type of research would be useful in South Africa, particularly in heavily industrialised estuaries such as the Swartkops system. This type of study could, for example, help identify particularly harmful sources of pollutants along an estuary.

A study conducted in Portugal looked at the effect of both seasonality and longitudinal gradients on AChE activity. Monteiro *et al.* (2007) investigated the seasonal variability in the estuarine fish, *P. microps* brain AChE activity over the period of a year in three Portuguese estuaries. They determined that AChE inhibition changed significantly with season in each estuary, depending on the catchment activities over the different seasons (Monteiro *et al.* 2007). However, it was unclear from this study whether this seasonal effect was a result of inherent seasonality in the fish AChE levels or whether this effect was due to, for example, an increase in tourism activity at one site which may have led to an increase in pollutants entering the system at that time. In one of the estuaries, the Aveiro lagoon, AChE inhibitions of 20% were recorded in all 3 sites during the winter sample. It is interesting that the AChE inhibition values were similar at all the sites investigated because the sites in this lagoon were quite far apart and showed no longitudinal decrease in inhibition as in the Kirby *et al.* (2000) study. Seasonal responses in biomarkers in South African estuaries would be interesting to determine, for example whether higher rainfall during summer results in increased runoff of toxicants into estuarine systems compared to dry (winter) conditions.

In other studies, the AChE assay has been combined with other biomarkers of reproductive health and correlations have been found. This is an important result as any impairment of the reproductive capacity of a species increases its vulnerability to over-exploitation. A study conducted by Corsi *et al.* (2003) proposed a possible link between AChE inhibition and irregular ovarian development in two estuarine species *Mugil cephalus* and *Zosterisessor ophiocephalus*. They found unusually small oocytes in areas where *Z. ophiocephalus* had the lowest brain AChE values (Corsi *et al.* 2003). In *M. cephalus* they found that females with higher gonad somatic indices (GSI) had proportionally smaller oocytes to females with lower GSI values. Furthermore, the

females with high GSIs also had the lowest AChE values (Corsi *et al.* 2003) and the authors proposed that AChE inhibition negatively impacted ovarian and oocyte development. Studies should be initiated to investigate whether the above relationship is also true of fish species found in South African waters. Many commercially and recreationally important species use South African estuaries as nursery areas and if these areas are negatively impacting the reproductive potential of fish using these areas, species need to be managed accordingly in terms of minimum size limits, total allowable catches, closed seasons etc.

Further research also needs to be undertaken in the above area because other studies have reported no effect of AChE on reproductive activity. One such study was conducted using the zebrafish *Danio rerio* to determine the effect of exposure to parathion for 250 days on food consumption, survival, growth and reproductive health (Roex *et al.* 2003). No significant differences in survival, growth or reproductive health were found between the different treatments but a significant increase in food consumption was recorded (Roex *et al.* 2003). Results from the two studies above are contrasting and it would be interesting to determine whether AChE activity and other biological effects are linked in South African estuarine species.

5.5 Conclusion

In conclusion, the significantly decreased brain AChE activity in the fish captured in the Old Woman's estuary suggests previous exposure to anticholinesterase compounds which may have originated from the chemicals applied on the golf course adjacent to the estuary. The fish from the Old Woman's estuary showed a 35% inhibition of cholinesterase activity compared to the fish from the Mtana and East Kleinemonde estuaries. From a parallel laboratory experiment conducted using *R. holubi*, the animals sampled from the Mtana and East Kleinemonde have AChE levels which are comparable to values of fish which are known to have been unaffected by anticholinesterase agents. The AChE assay is well documented as a detection method for OPs and Cs (Sturm *et al.* 1999, Kirby *et al.* 2000, Fulton & Key 2001, De la Torre *et al.* 2002) and in this study was demonstrated to be a useful biomarker because water samples gave no indication of a pollution problem in the Old Woman's estuary.

This study has therefore shown the applicability and practicality of the AChE assay for biomonitoring in South African estuaries using *R. holubi.*

Future work using this assay should include investigations into physiological effects associated with AChE activity, seasonal variations of AChE levels, the effects of fish size on AChE levels, and differences in AChE levels using fish species from different trophic levels. In addition, comparisons could be made between highly mobile species and those that are territorial or associated with specific habitats. Other analytical techniques including sediment and tissue analyses may also be useful in future studies to determine whether any chemical residues can be detected in the fish or other sessile organisms on which the fish prey. Longitudinal and spatial gradients of pollutants also need to be investigated in order to identify sources of anticholinesterase agents in estuarine systems.

Chapter 6

Biomarker validation experiment

6.1 Introduction

The present biomonitoring study using selected biochemical markers is the first investigation that has been conducted in South Africa using the estuarine species *Rhabdosargus holubi*. It was therefore necessary to undertake a laboratory experiment which could be used as a positive control to show that this species can be used in such biomonitoring studies.

A laboratory experiment was designed to determine the effect of a known cholinesterase inhibitor, chlorpyrifos, on brain acetylcholinesterase activity in *R. holubi*. The acetylcholinesterase assay (AChE assay) measures the amount of inhibition the acetylcholinesterase enzyme has undergone following exposure to anticholinesterase compounds. Examples of anticholinesterase compounds include pesticides, insecticides, metals, organochlorines and surfactants (Guilhermino *et al.* 1998, Guilhermino *et al.* 2000, Fulton & Key 2001) (for more background on this assay see Chapter 5). Chlorpyrifos was chosen as a toxicant because it was one of the products used on the golf course adjacent to the Old Woman's estuary.

The effect of chlorpyrifos on lipid peroxidation levels in the liver of *R. holubi* also required investigation. Lipid peroxidation results from oxidative stress (caused by xenobiotics) damaging the lipid components of cellular membranes (Kelly *et al.* 1998). The lipid peroxidation assay (LPx assay) measures a by-product of lipid peroxidation, malondialdehyde, and can be used as a quantitative measurement of the extent of oxidative damage an individual organ has undergone. Examples of redox cycling compounds include quinones, certain dyes, bipyridyl herbicides, certain transition metals, aromatic nitro compounds and certain pesticides (Kelly *et al.* 1998, Valavanidis *et al.* 2006) (for more background on this assay see Chapter 4). Chlorpyrifos is also a redox cycling compound, and is therefore capable of causing oxidative stress (Goel *et al.* 2005).

The primary aims of this study were to determine whether measurable effects to AChE activity and LPx levels could be detected in *R. holubi* following a six hour exposure to 3 µg/L of chlorpyrifos, as a positive control. No histological analyses were performed because it was felt that the duration of exposure (six hours) would be too short to illicit a response at the organ level. An ancillary aim of this study was to determine baseline levels of AChE activity and LPx levels in *R. holubi* following six weeks depuration in controlled laboratory conditions.

6.2 Methods

Fish collection and holding conditions

Sixty *R. holubi* were captured from the West Kleinemonde estuary (an estuary considered to be unaffected by pollutants) using a 30 m x 1.7 m x 15 mm bar mesh seine net fitted with a 5 mm bar mesh cod end. These fish were transported back to the laboratory in oxygenated water and placed in a 1 m^3 holding tank where the salinity was gradually increased to 35 psu. The sea water used during the holding period and the experimental exposure was collected from a clean seawater source which was transported to the laboratory on a weekly basis. Water temperature was maintained at 22°C and lighting was set to 12 h light:dark cycle. Ammonia was measured every second day and maintained below 0.25 ppm. A 10% water change was undertaken once a week for the 6 week acclimation period. Fish were fed to satiation daily on commercial trout pellets but were starved for 24 h prior to the commencement of the experiment. No fish mortalities were recorded during the acclimation period.

Exposure experiment and biochemical assays

Following a series of range finding experiments, it was determined that *R. holubi* could survive in 3 µg/L of chlorpyrifos for short periods of time (the recommended application dose for chlorpyrifos on garden 'pests' is 0.96mg/ml). Twenty fish (mean length 8 cm ± 0.74, mean weight 17.32 g ± 4.7) were exposed to this concentration for 6 hours and 20 fish (mean length 7.93 cm ± 0.87, mean weight 16.39 g ± 5.2) were used as unexposed controls. Each fish was randomly assigned to a bucket containing 10 L of fresh seawater (35 psu) in a controlled temperature room (maintained at 22°C)

and aerated with an airstone throughout the experiment. Chlorpyrifos was added to the seawater 5 minutes prior to a fish being placed in a bucket to obtain a nominal concentration of 3 µg/L of chlorpyrifos. The experiment was staggered to enable an exact exposure time of 6 h for each fish. Following exposure, fish were euthanized by severing the spine and immediately excising the brain. The liver was dissected out and both the brain and liver were snap frozen in liquid nitrogen and stored at -80°C until analysed in the laboratory.

AChE activity was determined and calculated according to the methods described in Chapter 5. The LPx assay and calculations were carried out according to the methods described in Chapter 4. Results for the AChE assay are presented as nmol/min/mg protein. Results from the LPx assay are presented as nmol MDA formed/h/mg protein.

Statistical analyses

The results from the AChE assay and LPx assay were not normally distributed (Kolmogorov-Smirnov, $P \leq 0.05$), therefore a non-parametric Mann-Whitney U test (one tailed) was performed to compare AChE and LPx levels in exposed (experimental) and non-exposed (control) groups.

6.3 Results

Two mortalities were noted following the exposure experiment and were therefore not used for the biochemical analyses. The airstones in the buckets containing these two fish had poor air pressure and it is presumed these individuals died due to a combination of asphyxiation and chlorpyrifos poisoning. The remaining 38 fish survived the duration of the experiment.

A significant difference was found in the brain AChE activity between the control and exposed groups (Mann Whitney U test, $z = 3.65$, $n = 38$, $P < 0.001$) with AChE significantly reduced (82% inhibition) in individuals exposed to chlorpyrifos (Figure 6.3.1). Median values of 55.75 nmol/min/mg protein and 10.19 nmol/min/mg protein were obtained for the control and exposed treatments respectively. However, no significant differences were found between treatments using the LPx assay (Mann Whitney U test, $z = -0.56$, $n = 38$, $P > 0.05$) (Figure 6.3.2). Median levels of 1.4 nmol

MDA formed/h/mg protein and 1.7 nmol MDA formed/h/mg protein were obtained for the control and exposed treatments respectively.

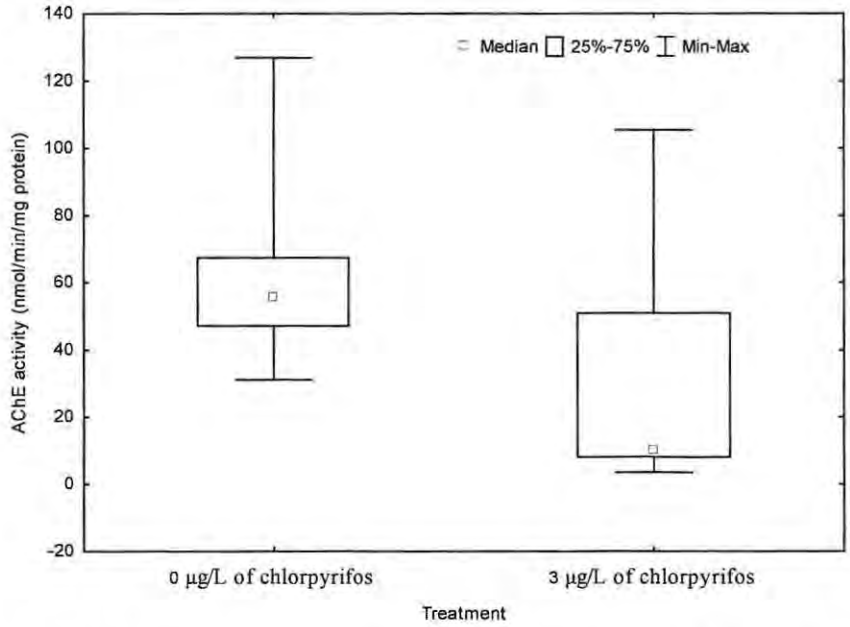

Figure 6.3.1: AChE values for the control (n = 20) and exposed (n = 18) treatments. AChE activity is presented in nmol/min/mg protein. Error bars are minima and maxima values for each treatment, outer boxes are the upper and lower quartile limits and the inner boxes are the median values.

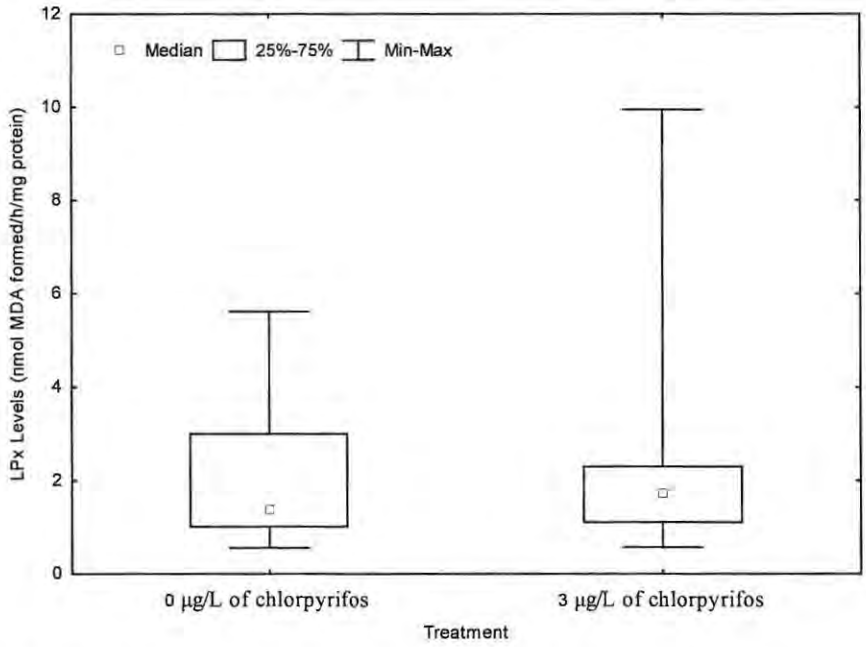

Figure 6.3.2: LPx values for the control (n = 20) and exposed (n = 18) treatments. LPx levels are presented in nmol MDA formed/h/mg protein. Error bars are minima and maxima values for each treatment, outer boxes are the upper and lower quartile limits and the inner boxes are the median values.

6.4 Discussion

AChE assay results

Fulton & Key (2001) reported that AChE inhibition and subsequent recovery (in the absence of a toxicant) is a function of the degree of initial inhibition and the recovery duration following this. Following an extensive literature review, Fulton & Key (2001) stated that it may take up to six weeks in uncontaminated water for brain AChE levels to return to basal levels following exposure to anticholinesterase compounds. The fish used in this study were held for six weeks prior to any experimentation; therefore it is unlikely that there was any inhibition in the control fish due to prior exposure to anticholinesterase compounds.

From the above it is proposed that the control values obtained during this experiment can be used as a baseline, against which values obtained from wild caught samples can be measured. The holding conditions were controlled, such that temperature, photoperiod, feeding and selected water quality parameters stayed constant during the exposure period. Due to this, the AChE values obtained from the unexposed fish during this experiment should not be used as cut off values for AChE basal levels because all the above mentioned parameters fluctuate on a daily basis in the natural environment and may affect AChE activity. The results can, however, be used as a reference value, against which values measured in wild caught samples can be compared.

The median control brain AChE activity (55.75 nmol/min/mg protein) obtained for *R. holubi* during the present laboratory study was similar to the AChE activity for *G. yucatana* (a freshwater species) reported by Rendon von Osten (2005) where the value for unexsposed fish was 58 nmol/min/mg protein. Similarly Varo *et al.* (2003) determined the effects of dichlorvos on *Dicentrarchus labrax* (a marine species) and found similar (52 nmol/min/mg protein) unexposed values for brain AChE activity. However, Karen *et al.* (1998) reported AChE activity values of 310 nmol/min/mg protein for unexposed individuals of the estuarine species *Fundulus heteroclitus* and Crestani *et al.* (2007) reported control AChE activity levels of 400 nmol/min/mg protein in the freshwater silver catfish (*Rhamdia quelen*). From the studies described

above it can be seen that AChE activity levels are clearly species specific and independent of whether the species is freshwater, estuarine or marine. This highlights the importance of laboratory studies in order to obtain species specific control levels of AChE activity, from which to make comparisons with AChE activity levels, either in field-caught organisms or those used in laboratory experiments under varying exposure scenarios.

The group of *R. holubi* exposed to chlorpyrifos had 82% inhibition of brain AChE activity when compared to the unexposed fish. These results are in agreement with Eder *et al.* (2004) who investigated the effects of 1.2, 7.3 and 81 µg/L chlorpyrifos on juvenile Chinook salmon (*Oncorhynchus tshawytscha*) for 96 h. These authors found that at 81 µg/L total mortality of the exposed specimens occurred. At 7.3 µg/L brain AChE activity was reduced to 15% activity (85% inhibition) and at 1.2 µg/L brain AChE activity was reduced to 92% (8% inhibition) activity compared to control specimens (Eder *et al.* 2004). Thus it would seem that juvenile Chinook salmon are more tolerant to chlorpyrifos than *R. holubi* because at 3 µg/L *R. holubi* showed AChE activity of 18% (82% inhibition).

Rendon Von Osten *et al.* (2005) exposed *Gambusia yucatana* to chlorpyrifos concentrations ranging from 6 µg/L to 100 µg/L for 96 h. *G. yucatana* brain AChE activity was significantly inhibited by chlorpyrifos concentrations greater than 6 µg/L, with 50% AChE inhibition being reported at that concentration (Rendon Von Osten *et al.* 2005). This concentration is double the exposure concentration used during the current study, yet the inhibition was much less than that observed with *R. holubi* at 3 µg/L for only 6 hours.

A study conducted by Karen *et al.* (1998) investigated the effects of pulses of chlorpyrifos on the estuarine species *Fundulus heteroclitus*. These authors were trying to mimic salinity changes under natural estuarine conditions by pulsing 5 psu chlorpyrifos water for 6 h into the test aquaria, followed by 18 h of pulsing with clean 20 psu water. The 5 psu water pulsed into the different aquaria contained 0.63, 1.25, 2.5, 5.0 and 10.0 µg/L chlorpyrifos. *F. heteroclitus* samples were collected from each aquarium after 48 h and 96 h. Karen *et al.* (1998) reported an increase in AChE inhibition both over time and with increasing chlorpyrifos concentration.

As mentioned in the introduction, the present experiment did not investigate dose-response relationships between AChE activity and chlorpyrifos. Rather this experiment demonstrated that *R. holubi* is sensitive to chlorpyrifos (a known cholinesterase inhibitor) and can therefore be used as a sentinel species for biomonitoring of this pesticide in South African estuaries. The above mentioned studies seem to suggest that a dose-response relationship in AChE inhibition may exist however more laboratory testing will be required to determine whether this is the case for *R. holubi.*

Lipid peroxidation assay

There appears to be a lack of literature dealing with how long the liver takes to recover from oxidative stress. Kelly *et al.* (1998) describe the mechanisms by which damaged lipids in the cellular membrane are repaired but do not state the duration of this process. Some studies have investigated recovery time in LPx levels following exposure to toxicants, e.g. Kavitha & Venkateswara Rao (2007) exposed *Gambusia affinis* to an organophosphate pesticide (monocrotophos) for 96 h and recorded their subsequent recovery. They found a 120% increase in LPx levels following the 96 h exposure, with levels returning to levels in unexposed fish only after 8 days (Kavitha & Venkateswara Rao 2007).

Ferreira *et al.* (2005) determined LPx levels in mullet (*Mugil cephalus*) following one month depuration in contaminant free water over four seasons. The authors reported an increase in LPx levels in summer and autumn and a decrease in winter and spring following the depuration period. The authors attributed the increase in LPx levels during summer and autumn to increases in water temperatures in the laboratory during this period (Ferreira *et al.* 2005). During the current study, temperatures were held constant throughout the holding period so this factor should not have affected the fish. Results from Kavitha & Venkateswara Rao (2007) suggest that LPx levels return to basal levels relatively quickly, therefore the 6 week depuration period in the laboratory prior to the exposure experiment was likely to be long enough time to allow any damaged lipids in *R. holubi* to recover back to basal levels.

Several redox cycling chemicals have been found to increase LPx levels in fish, including quinones, certain dyes, bipyridyl herbicides, certain transition metals, aromatic nitro compounds and some pesticides (Kelly *et al.* 1998, Dorval *et al.* 2005, Valavanidis *et al.* 2006). Included in this list is chlorpyrifos, which has been shown to increase LPx levels in rat liver (Goel *et al.* 2005). Despite the fact that chlorpyrifos is known to increase hepatic LPx levels, no significant difference was found between unexposed and exposed fish LPx levels during the present laboratory study. It is likely that the duration of exposure (6 h) in the current study may not have been long enough to elicit a response.

Similar results have been found in several other exposure studies. For example, Crestani *et al.* (2007) measured LPx levels following exposure of *Rhamdia quelen* after 12, 24, 48, 96 and 192 h to clomazone, and exposed the fish to 500 and 1000 μg/L of this herbicide. The authors found that following 12 h of exposure to 500 μg/L clomazone, no significant differences could detected between exposed and unexposed groups. However, following 24 h of exposure at the same concentration, a significant difference was recorded (Crestani *et al.* 2007).

Ahmad *et al.* (2004) conducted a study using European eels (*Anguilla anguilla*) exposed to contaminated harbour water during an *in situ* experiment in the Aveiro lagoon, Portugal. *A. anguilla* were collected following 8 h and 48 h exposure and the results showed an increase in LPx levels in the liver only with the latter exposure. The authors hypothesised that the liver had a lag response time to xenobiotic exposure (Ahmad *et al.* 2004).

This was also the hypothesis proposed by Santos *et al.* (2006) who found that LPx showed variable responses during short term exposure. In their study, the authors placed *A. anguilla* in cages along a pollution gradient in the Vouga River in Portugal. The sites were chosen according to distance (50 m, 100 m and 2000 m) from a disused bleached kraft pulp mill effluent outlet which had been out of operation for 25 months. The eels were exposed for 8 h and 48 h. The result from the LPx study showed that the only site where a significant increase in LPx levels occurred was at a site 100 m from the outlet following a 48 h exposure (Santos *et al.* 2006). The authors explained this pattern by suggesting that the sites differed significantly in the terms of

the content of the sediments between the sites. They suggested that the sediments from the 100 m site contained oxidative compounds which may have been removed from the 50 m site due to hydrological processes. This hypothesis was supported by work which the same authors had carried out earlier at the same sites looking at an overall antioxidant decrease in *A. anguilla* over the same period of time (Santos *et al.* 2004).

A study undertaken by Amaral Monteiro *et al.* (2006) investigated the effects of an organophosphorous insecticide on the freshwater characid *Bryon cephalus* LPx levels. During this study the authors exposed the fish for 96 h to 2000 µg/L of methyl parathion and then investigated the effects of this insecticide on the liver, white muscle and gills. Their study found significant differences between the gills and white muscle which had been exposed when compared with control fish; however no significant difference was seen in the liver. The authors attributed this difference to a greater antioxidant capability of the liver when compared to the other two organs, suggesting that the liver more successful at combating the effects of lipid peroxidation (Amaral Monteiro *et al.* 2006).

A study conducted by Roberts & Oris (2004) supports this hypothesis. They exposed rainbow trout (*Oncorhynchus mykiss*) to a known redox cycling chemical (hexavalent chromium) over a period of 28 days. Samples were collected at the start of the exposure and subsequently after 1, 2, 7, 14, 21 and 28 days for LPx, superoxide dimutases (SODs) activity and metallothionein (MT) gene expression assays. One of the purposes of this experiment was to determine the extent of interactions between antioxidant systems within trout in relation to oxidative stress. Significant increases in gill LPx levels were reported during the initial 14 day exposure period, and LPx levels gradually returned to basal levels over the following 14 days of exposure (Roberts & Oris 2004). Increases were also recorded in the liver which followed the same pattern as those recorded in the gills but at a lower level, and the authors were able to conclude that the biochemical reactions were probably working in tandem against the toxicant. They proposed that the lower LPx damage in the liver compared to the gills could have been a result of increased MT gene expression and SODs activity, both of which are powerful antioxidants (Roberts & Oris 2004).

Using the information contained in the Roberts & Oris (2004) and Amaral Monteiro *et al.* (2006) papers, the most likely reason for which no significant difference was found between the control and exposed fish during the present laboratory study, is most likely due to a combination of the activation of antioxidant enzymes which were able to reduce the oxidative stress the liver was undergoing and the short exposure duration. Had the fish been exposed to chlorpyrifos for a longer period of time (>24 h), these antioxidant systems may have become overwhelmed, as was found in the Crestani *et al.* (2007) and Ahmad *et al.* (2004) studies.

6.5 Conclusion

This study has shown that *R. holubi* is readily adaptable to laboratory testing methods and may therefore be a useful sentinel species for ecotoxicological studies in South African estuaries. The study validates the use of acetylcholinesterase as a biomarker in the field component of this study because *R. holubi* was demonstrated to be a sensitive indicator of exposure to organophosphorous pesticides. Although no significant changes were found in LPx levels during this study, it appears from the literature that the liver needs to be exposed for longer periods of time to elicit a response in this biomarker. This is hypothesised to be a result of antioxidant systems that are present in the liver and which assist in maintaining low LPx levels following the initial insult.

Chapter 7

The development of an index of the health status of South African estuaries using multiple levels of biological organisation

7.1 Introduction

Biomonitoring using biomarkers and bioindicators at multiple levels of biological organisation is based on the concept of using assessment methods that are sensitive to different types of stressors and react over different time frames. When a combination of biomarkers and bioindicators is used, an overall picture of the long and short term stressors which have affected a biological community can be generated. Biomonitoring using this approach in fish has been successfully applied in assessing aquatic ecosystem health in the freshwater, marine and estuarine environments (Ham *et al.* 1997, Elliott *et al.* 1988, Minier *et al.* 2000, Adams *et al.* 2005, Sanchez *et al.* 2007).

Elliott *et al.* (1988) investigated the pollution status of the Firth of Forth estuary in Scotland and measured community, individual and cellular health parameters. At the community level, the authors used a community index which looked at ratios between primary, secondary and tertiary consumers. In terms of individual health, the authors focused on flounder (*Platichthys flesus*) and eelpout *(Zoarces viviparous*), looking at tissue mercury concentration, skeletal deformities, skin and fin lesions and parasitic load. The authors also looked at stress at the cellular level using the mixed function oxidases (MFO) system assay and hepatic aryl hydrocarbon hydroxylase (AHH) assay which, in combination, can give an indication of exposure to polynuclear aromatic hydrocarbons (PAHs) and other contaminants. Elliott *et al.* (1988) were able to trace changes in the community composition using historical data which showed that certain species which had previously been excluded from the Firth of Forth estuary due to, for example the development of anoxic conditions, were now able to use the estuary as a result of pollution control measures. The authors were also able to track changes in individual fish species health, in terms of tissue mercury concentration, in

relation to pollution management actions that had considerably improved the condition of the fish sampled between 1981 and 1988 (Elliott *et al.* 1988).

A similar, longer term study was conducted by a series of authors on the East Fork Poplar Creek in the USA (Ham *et al.* 1997, Adams *et al.* 2000, Adams *et al.* 2005). These studies used a wide range of biomonitoring techniques to describe the recovery, over the period of 15 years, of a previously heavily impacted river. During the studies periphyton, benthic invertebrates and fish communities were investigated in terms of diversity and composition, as well as individual fish health measures such as blood serum analysis for indications on creatinine levels, urea nitrogen and serum triglycerides. The above authors also investigated a range of cellular biomarkers such as the MFO system, albumin and alanine aminotransferase (ALT which is a measure of the health of the liver), and 7-ethoxyresorufin *O*-deethylase (EROD) which is a biotransformation enzyme indicative of exposure to PAHs and polychlorobiphenyls (PCBs). Fish liver, visceral and spleen condition factors were measured and studies also conducted age and growth analyses of *Lepomis auritus* (redbreast sunfish) using scales. During the various investigations, clear temporal and spatial improvements in the biomarkers levels were recorded by all the studies, and these results were related to improvements in water quality and sediment contaminant loading. The above data sets are very powerful in terms of demonstrating the potential for a multi-level biomarker and bioindicator approach to monitoring. These studies provide a weight-of-evidence approach to finding causal relationships in observed alterations to fish communities and biomarker responses, which can be related to contaminant loadings in the environment.

The present study investigated the applicability of various biomonitoring techniques to assess the health status of South African estuaries. Previous chapters have shown that these methods were successfully applied to three Eastern Cape estuaries. The Estuarine Fish Community Index (EFCI) was applied to the estuaries in order to determine the status of the fish communities, and the results showed that the East Kleinemonde and Old Woman's estuaries were in 'good' condition (EFCI score of 48) whilst the Mtana estuary was in a 'moderate' condition (EFCI score of 44) (Chapter 2).

Community indices have often been used as bioindicators to give a measure of long term stressors on the biota of an ecosystem. Frequently used indicators include the presence or absence of susceptible or fragile species because these taxa are often the most sensitive to environmental change and will be the first to be forced out of an environment (Harrison & Whitfield 2004, Adams *et al.* 2005). A stressed fish community has the ability, to a limited extent, to absorb change within biological levels of organisation (Whitfield & Elliott 2002). This environmental homeostasis is able to compensate for short-term localised stress such as disease or low dose and/or infrequent pollution events. However, if this stress is prolonged or too intense, the biological community will change, with vulnerable and fragile species disappearing first (Harrison & Whitfield 2004). This will eventually result in a change from a community that was relatively complex and diverse to one with simple trophic level interactions and being dominated by a few pioneer species. Due to this, abundance and diversity indices are good measures of the health of a fish community (Whitfield & Elliott 2002).

At the individual level, a condition factor was calculated for each fish. This is a coarse measure of the feeding level of an animal and has been used in other studies for comparisons between systems (Blaber 1975, Suliaman *et al.* 1991, Kirby *et al.* 2000). Condition factor has not been shown to be directly correlated to pollution contamination, however, it is felt that it this is a good indication of the extent to which an estuary is fulfilling its role as a food rich environment for juvenile fishes and was therefore included as a biomarker of estuarine health.

At the organ level, histopathological studies were conducted on the livers of a sub-sample of *Rhabdosargus holubi* from each estuary (Chapter 3). The overall score for the fish from the EK was 2.9, but the majority of these fish (nine out of ten) showed pathological changes (score of 3), therefore this result may be biased by the small sample size. A slightly lower score (2.6) was obtained for the fish from the OW, indicating that the fish from this estuary were also in good condition. However, it was felt that the small sample size biased this result with the majority (four out of five) of the fish from this estuary showing pathological changes to the liver. The overall score for the MTN was 3, indicating that the majority of fish from this estuary showed minimum pathological changes to the liver.

Organ level biomarkers are measures of the extent to which organ processes have been affected in an individual. Liver histopathology can give an indication of the detoxification capabilities of an individual because the liver is the primary organ of detoxification in fish (Hinton & Lauren 1990). The ability to detoxify xenobiotics, of natural or anthropogenic origin, is essential to fish. Therefore histopathological biomarkers provide a good indication of previous exposure to xenobiotics, and can also indicate the ability of an individual to resist future insults which in turn provides insight into the susceptibility of a population to pollution events.

The fish from the EK were found to have the lowest LPx levels in the liver, followed by the fish from the MTN (Chapter 4). The LPx levels from the OW were found to be bimodal, indicating that only some of the individuals in this system were being affected by oxidative stress. These results suggest that the fish from the OW were affected by oxidative stress and those from the MTN were affected to a lesser extent. In terms of the AChE assay, the fish from the OW were found to have significantly reduced AChE activity compared to fish from the EK and MTN (Chapter 5). These results suggest that the fish from the OW were exposed to anticholinesterase compounds.

Cellular level biomarkers such as the AChE and LPx assays respond rapidly to exposure to xenobiotics and are therefore good indicators of recent pollutant events (Fulton & Key 2001, Van der Oost *et al.* 2003). Depending on the extent and duration of exposure, these markers will not be detectable at higher levels of organisation and as such are used as 'early warning systems'. Cellular level biomarkers are powerful because they can be relatively pollutant specific and can give insight into the type of pollutant which is affecting a system.

Each of the monitoring tools described above were successfully applied to fish from the study estuaries and were used to describe the health of these systems. However, an index that incorporates the results from all the techniques would be useful because it would provide a more holistic description of the health of each estuary.

The following sections will discuss whether the cellular and individual biomarkers were significant contributors to detecting differences between the three estuaries.

Following this, a composite index was developed using all the biomarkers and the bioindicator used, thus assisting in identifying problem areas in the estuaries studied and to determine possible management intervention measures that could ameliorate the state of these systems. A critical evaluation of the index is undertaken, with recommendations of other biomarkers which would strengthen the approach being proposed. Finally, the suitability of *R. holubi* as an indicator species for South African estuaries was discussed.

Assessing three temporarily open/closed Eastern Cape estuaries using multiple biomarkers

An important consideration when using biomarkers for biomonitoring purposes is biomarker redundancy (O'Connor *et al.* 2000). If two biomarkers are very similar in terms of their response to contaminants and are indicative of exposure to the same class of contaminants, then it can be argued that it is unnecessary to measure both biomarkers. This can save time and money in terms of determining the ecological health of a system and is an important consideration when deciding on the composition of a monitoring package (O'Connor *et al.* 2000). Principle Component Analysis (PCA) can be used to determine whether biomarkers are redundant or not. If two biomarkers which measure damage to the same organ vary in the same direction, to the same extent, and on the same principle component (PC) axis, then it can be concluded that one of the biomarkers is redundant. The simplest, quickest and cheapest of the two should be selected as the biomarker to measure (O'Connor *et al.* 2000).

Sanchez *et al.* (2007) investigated the response of three-spined stickleback (*Gasterosteus aculeatus*) to a range of contaminant types in several rivers in northern France. The exposure sites differed in terms of development pressure (urban, agricultural and mixed) and were ranked in terms of a biotic index according to a previous study that used a fish based index. The authors measured two biotransformation enzymes (EROD and gluthatione–*S*-transferase), three biomarkers of oxidative stress (glutathione peroxidase, total glutathione content, lipid peroxidation), as well as a range of morphological indices such as the liver somatic index (HSI) and condition factor (CF). They undertook a PCA and determined that the

biomarkers used during the study were able to distinguish between similarly impacted sites, e.g. two separate sites which receive similar amounts of urban runoff but from different types of activities. The authors found that despite having several types of oxidative stress and biotransformation measures, any reduction in the number and type biomarkers used during the study would have resulted in a loss in the ability to distinguish between sites using the PCA method (Sanchez *et al.* 2007). Based on this, a PCA analysis was conducted using the data from the present study using the AChE activities, the LPx levels and the condition factor for each individual fish in order to determine the relative importance of each biomarker in the present study (Figure 7.1). The histological results were not included in the analysis due to the small sample size used in this study.

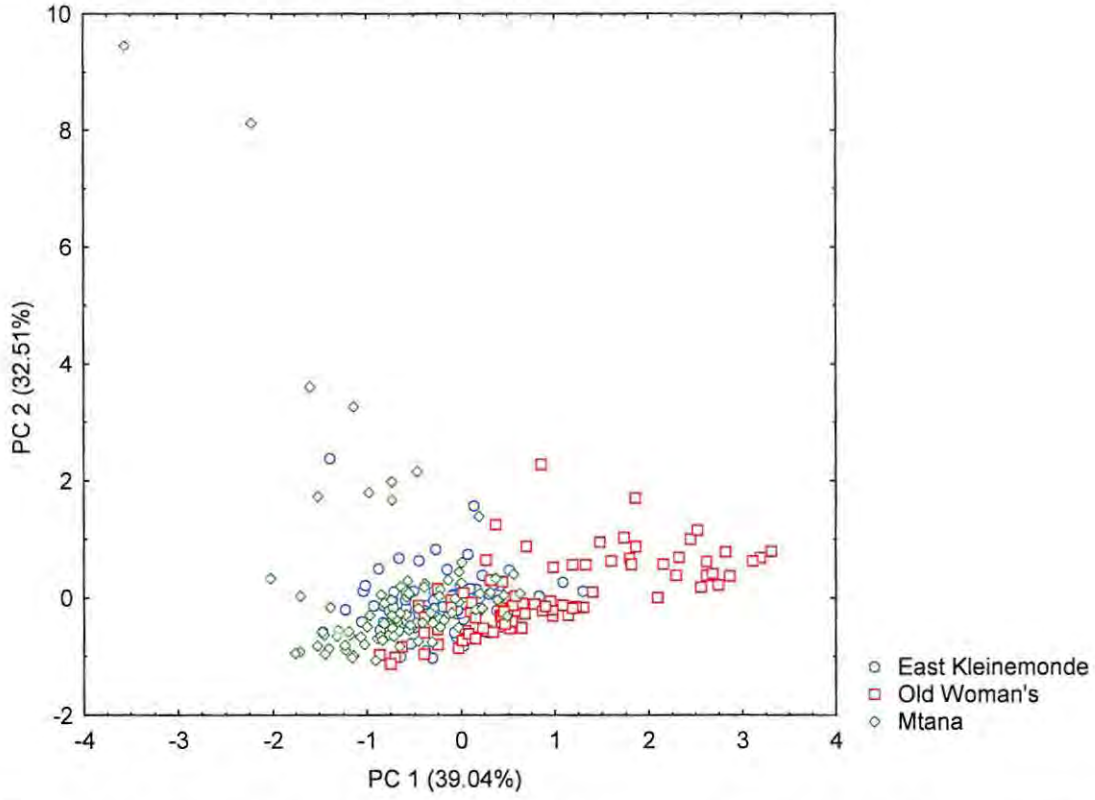

Figure 7.1: Plot of principle component 1 (39.04%) against principal component 2 (32.51%).

There were several outliers in Figure 7.1 which made distinguishing between the points around the origin difficult; therefore the PCA results are presented at a smaller scale in Figure 7.2. The first two principal components explained 71.55% of the variance. Eigen vectors for PC 1 were dominated by LPx levels and CF, and the factor projections show that these two determinants vary in opposite directions (Figure 7.3).

PC 2 was mostly explained by AChE activities and the factor projection shows that AChE activities vary perpendicularly to both the LPx and CF factor projections (Figure 7.3).

In general, most of the samples from the EK (circles, Figures 7.1 & 7.2) were found to occur around the origin of the graph. Some of the samples from the OW (squares, Figures 7.1 & 7.2) were also found around the origin, however a number of the samples also occurred to the right of the origin. The samples from the MTN estuary (diamonds, Figures 7.1 & 7.2) had fewer samples removed from the origin, but had greater distances from the origin when compared to the OW samples. These occurred between -1.5 and -6 on the x axis and 1.8 and 9 on the y axis.

In terms of the biomarkers, generally samples with high CF were more towards the negative region of the x axis while samples with low CF were more towards the positive side of the x axis, but there were some exceptions to this pattern. Samples with low AChE activities occurred towards the positive region of the x axis and negative area of the y axis, and samples with high AChE occurred in the negative area of the x axis and positive area of the y axis (Figure 7.2). Generally, samples which had LPx level between 0 and 15 nmol MDA formed/h/mg protein occurred around the origin, and samples with higher values than this were located outside this area.

Overall, the PCA seems to suggest that individuals near the origin are in better overall condition (in terms of the biomarkers measured) than individuals further away from the origin. The fact that PC1 and PC2 explain 71.55% of the variance indicates that these three biomarkers appear to describe the health of the individual estuarine systems fairly well. Had two of the biomarkers varied in the same direction and to the same extent, this would have suggested that one of the measures was redundant as both biomarkers would have explained the same variance. All three biomarkers varied in different directions, which suggest that there are no redundant biomarkers.

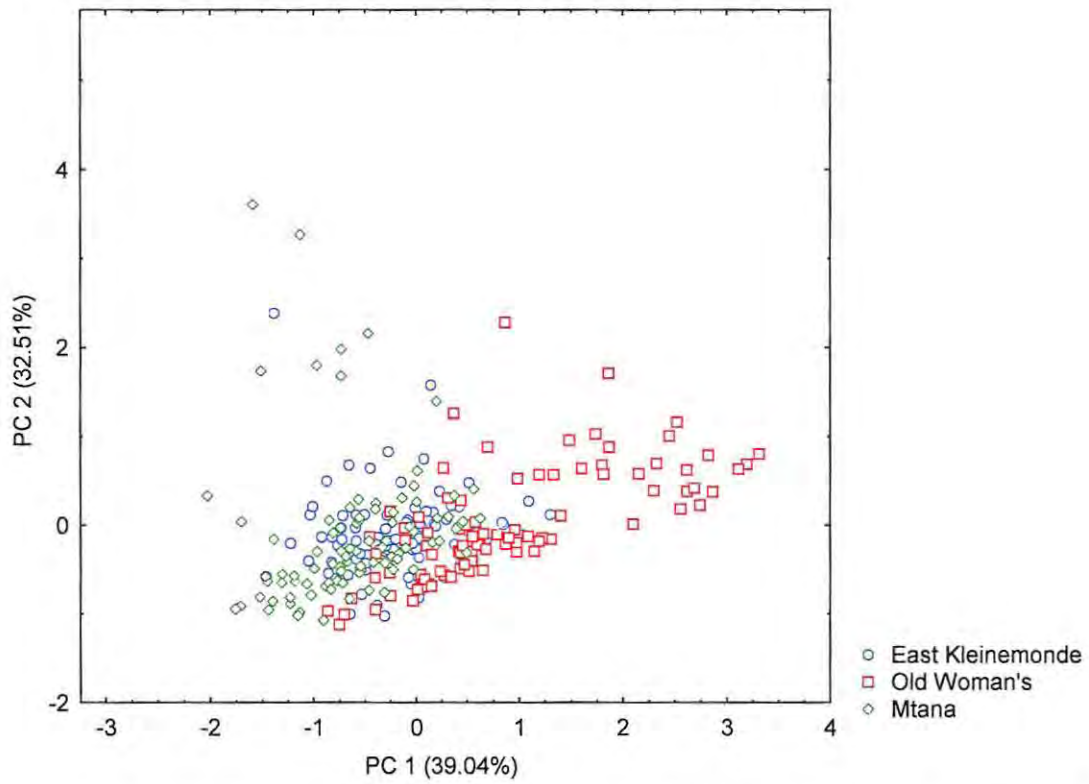

Figure 7.2: Plot of principle component 1 (39.04%) against principal component 2 (32.51%) at a smaller scale.

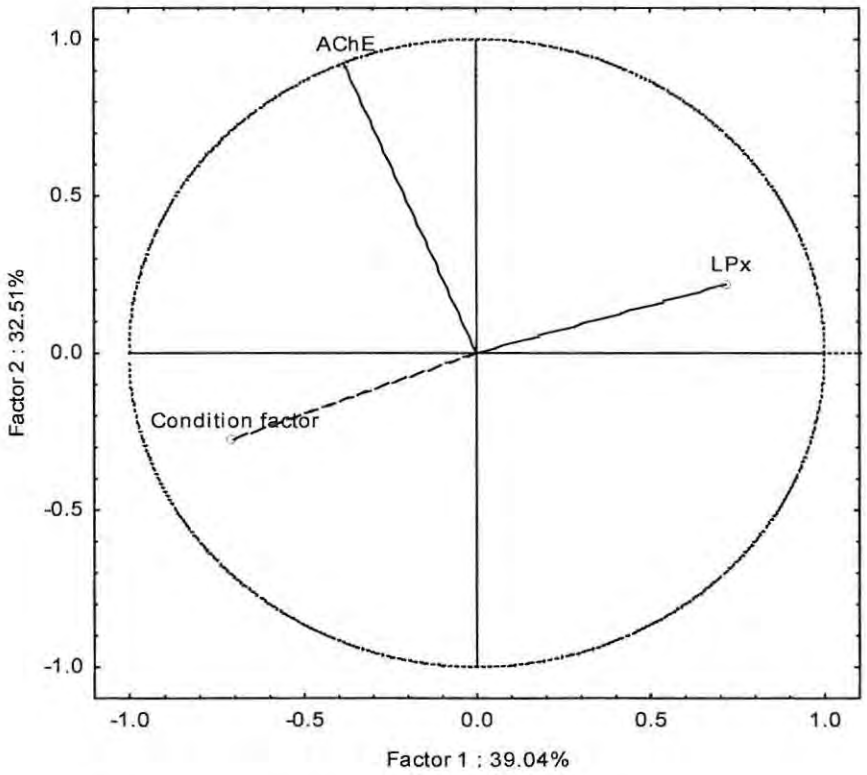

Figure 7.3: Factor projections of PC 1 and PC 2.

Development of an index of biological health for South African estuaries based on monitoring multiple levels of biological organisation

An important responsibility of scientists is to communicate their findings in a clear and simple manner to others, who are not necessarily trained in the scientific field (Ramm 1990, Harrison & Whitfield 2004). A simple way to do this is through the use of indices (Ramm 1990, Harrison & Whitfield 2004). An index was therefore developed using all the data collected during this pilot study, as a model for further estuarine studies, using multiple levels of biological organisation. It was also developed to give managers an indication of the types of management interventions that an estuary may require. The overall index is calculated using a combination of all the biomarkers into a single score together with a score for the EFCI.

Biomarker scoring

The AChE activity threshold value was determined from the laboratory study (Chapter 6), where the median values for control AChE activities from unexposed fish was found to be 55.75 nmol/min/mg protein. Inhibition levels of 20% or more of AChE activities are indicative of exposure to anticholinesterase compounds (Varo *et al.* 2003); thus a threshold of 44.6 nmol/min/mg protein (20% of 55.75 nmol/min/mg protein) was used for this assay. AChE data were found not to be normally distributed, therefore a median (rather than a mean) value of AChE activity was calculated for each estuary. Estuaries with an overall median AChE activity value lower than 44.6 nmol/min/mg protein were assigned a score of one. Estuaries with an overall median AChE activity value higher than 44.6 nmol/min/mg protein were assigned a score of two (Table 7.1). In the case of the present study, the EK scored two (median brain AChE activity was 52.48 nmol/min/mg protein), the OW scored one (median brain AChE activity was 36.57 nmol/min/mg protein) and the MTN scored two (median brain AChE activity was 55.94 nmol/min/mg protein).

Table 7.1: Index scoring system for individual biomarkers (1 indicates a good value and 2 indicates impairment).

Biomarker	Threshold value	Index score for values above the threshold	Index score for values below the threshold
AChE activity	44.6 nmol/min/mg protein	2	1
LPx levels	6 nmol MDA formed/h/mg protein	1	2
Condition factor	144	1	2
Histology	3	1	2

The threshold value for the LPx values was determined by the laboratory study (Chapter 6). Values between 0 and 6 nmol MDA formed/h/mg protein were obtained for fish which were not exposed to chlorpyrifos. The LPx data were found not to be normal, thus medians were used to describe the data (Chapter 4). Therefore the median value for each estuary was taken, and if this value was less than 6 nmol MDA formed/h/mg protein, the estuary was given a score of two. If the median value for an estuary was greater than 6 nmol MDA formed/h/mg protein the estuary was given a score of one (Table 7.1). In the case of the present study, the EK scored two (median LPx levels were 4.81 nmol MDA formed/h/mg protein), the OW scored one (median LPx levels were 6.88 nmol MDA formed/h/mg protein) and the MTN scored one (median LPx levels were 6.28 nmol MDA formed/h/mg protein).

Equal samples were used to compare CF from all three estuaries (n = 89 fish/estuary, extra samples were randomly chosen and removed). The mode and upper and lower quartile ranges of the entire data set were then identified. Each individual fish from each estuary that was in the lower quartile range was assigned a value of one and those above the lower quartile range were assigned a value of two. Following this, a score combining the values assigned to all the individuals from one estuary was calculated for each estuary. The lowest possible score was 111 and the highest possible score was 178. The scoring categories were calculated simply by determining the difference between the lowest and highest scores and dividing that into two equal categories. An estuary which scored between 111 and 144 was given an overall score of one. An estuary which scored between 145 and 178 was given an overall score of

two. In the case of the present study, the EK scored two, the OW scored one and the MTN scored two (Table 7.2).

Table 7.2: Condition factor scoring scheme.

Estuary		EK	Score	OW	Score	MTN	Score
Number of samples in	Lower quartile	21	21	40	40	6	6
	Middle/ upper quartile	68	136	49	98	83	166
	Total		157		138		172
	Overall index score	2		1		2	

The methods described in Van Dyk *et al.* (2007) provide a measure of pathological changes in the liver (Chapter 3). A mean value was calculated for each estuary using all the data collected within that estuary. If the mean was three or greater, the estuary was assigned a score of one (because grades of three or above in the rating scale are indicative of pathological changes) and if the mean was less than three the estuary was assigned a value of two (Table 7.1). In the case of the present study, the EK and OW scored two and the MTN scored one.

The scores for each biomarker were then added up to obtain a total biomarker index score. Scores between 7 and 8 were given a biomarker index score of three. A score of 6 was given a biomarker index score of two and scores between 4 and 5 were given a biomarker index score of one (Table 7.4).

EFCI scoring

Harrison & Whitfield (2004) provide six possible rankings for an estuary using the EFCI. These were; very good, good, moderate, poor, very poor and critical (Harrison & Whitfield 2004). The 'critical' category was not included because this category only occurs when there are no fish in an estuary and therefore no biological material can be collected under these circumstances. The remaining categories were combined into three categories, which were ranked as one, two or three. Categories 'very good' and 'good' were assigned a score of three. The 'moderate' category was assigned a score of two and the 'poor' and 'very poor' categories were assigned a score of one (Table 7.3).

Table 7.3: EFCI scoring scheme based on the EFCI rating.

EFCI score	EFCI rating	Index score
16 - 20	Very poor	1
22 - 38	Poor	1
40 - 44	Moderate	2
46 - 62	Good	3
62 - 68	Very good	3

The reason for grouping the two poor categories together, and only having one rating (moderate) for a score of two, was because if a community is found to be in 'poor' condition this suggests that the estuary has been strongly affected by a stressor and therefore requires a score that reflects this in the index. In the case of the present study, the EK and OW scored three and the MTN two.

The overall biomarker index score and the EFCI index score are then added together (Table 7.4). The biomarker index and the EFCI index are given equal weighting because the EFCI is a combination of 14 metrics which look at various aspects of community health. Also, changes at the community level are of high ecological significance because this implies that certain species have been excluded from an environment, resulting in a loss of diversity. Damage at the individual and cellular levels are important measures of individual health, but most damage is reversible if the stressor is discontinued and therefore changes in the diversity of a community do not necessarily take place.

Table 7.4: Overall scoring scheme for the index using all the levels of biological organisation.

	EFCI Score		**Biomarker index score**						**Total index score**	
	EFCI Rating	**EFCI index score**	Histology	CF	LPx Levels	AChE activity	Total score	**Biomarker index score**	**Total score**	Management intervention?
EK	48	**3**	2	2	2	2	8	**3**	**6**	No
OW	48	**3**	2	1	1	1	5	**1**	**4**	Yes
MTN	44	**2**	1	2	1	2	6	**2**	**4**	Yes

Any estuary which has a total score below five requires management intervention because a stressor is either affecting the lower levels of organisation or the fish

community has changed markedly from the reference condition (Figure 7.4). Because the index is composed of many components from different levels of biological organisation, it may be possible to determine what type of stressor is present. Using the results from the MTN for example, this estuary scored well for the condition factor and the AChE, but scored poorly in terms of the histology and the LPx assay (Table 7.4). From this, it can be proposed that the stressor is unlikely to be a pesticide (because it is not affecting AChE activity) and may have been either a persistent chemical or could have been introduced into the estuary in a semi continuous manner. This type of information can help researchers and managers identify possible pollutants. The index is therefore a reflection of the health of individuals within a system as well as the overall fish community.

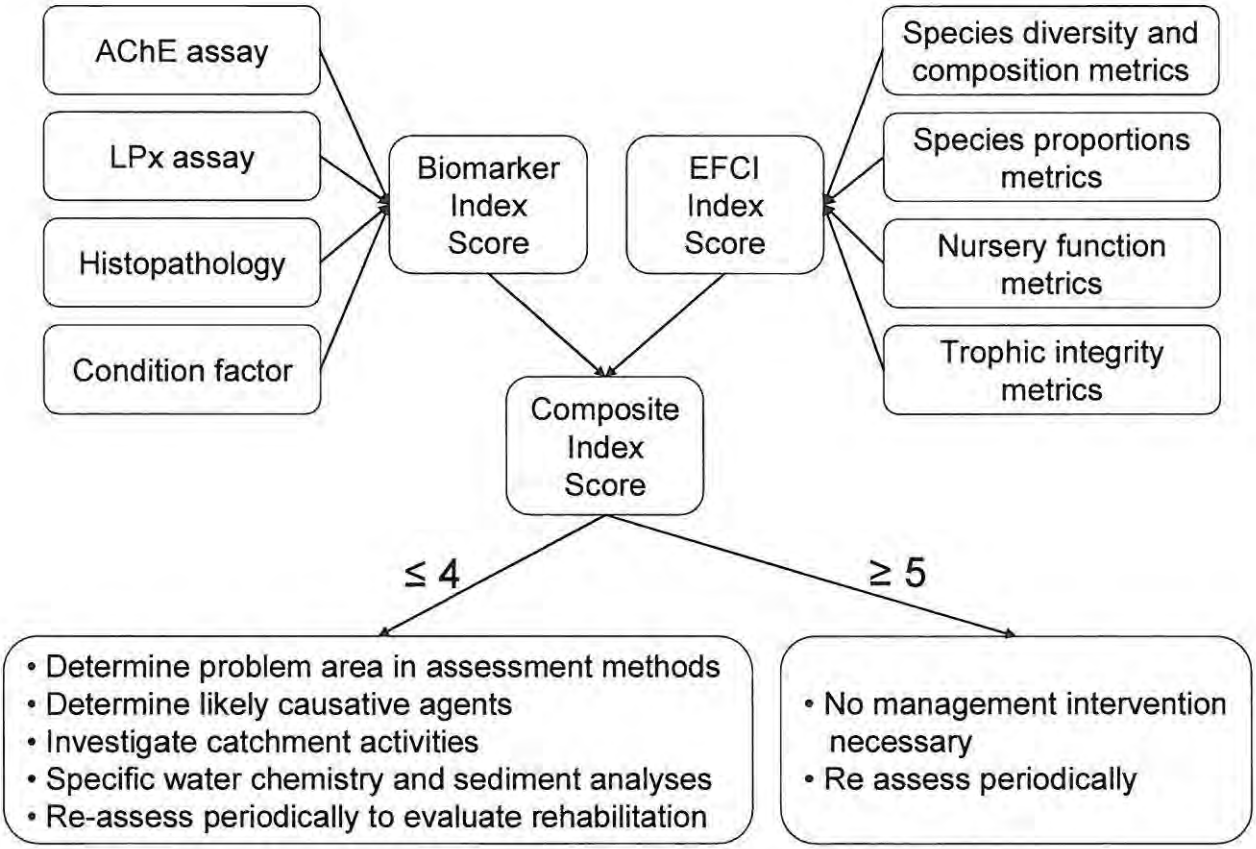

Figure 7.4: Schematic representation of the index.

Interpretation of the index applied to the three estuaries investigated

Table 7.4 shows the how each estuary investigated during the present study scored for each biomarker. The EK achieved the highest possible score in terms of the overall index with the EFCI indicating that the estuarine fish community is in 'good' health when compared to reference conditions. AChE levels were comparable to laboratory generated unexposed levels and LPx levels were mostly within the thresholds determined by a parallel laboratory study. The condition factor of *R. holubi* from this estuary was lower than in the MTN but higher than fish in the OW (Table 7.2). As mentioned earlier in this section, the majority of the samples from this estuary showed pathological changes to the liver (in terms of the histology). This is not reflected in the overall index score. The main reason for this is due to the small sample size used for the histological procedure. It is interesting to note that despite many of the histological analyses showing pathological changes to the liver, the LPx level results do not mirror this stress. This could suggest the fish from the EK had previously been exposed to a toxicant (which resulted in the histopathological changes) but that the toxicant has now ceased (which may be why the LPx levels are low). More monitoring will be required to determine whether this is true or not. Based on results obtained from the overall index, the EK appears not to require management intervention in terms of the parameters measured during the present study, but the estuary should be re-assessed periodically to ensure that no deterioration occurs and to measure the recovery of the liver in terms of the histopathology.

The OW estuary scored the lowest of the three estuaries investigated in terms of the biomarker index but scored the same value as the MTN in terms of the overall index (Table 7.2). The EFCI score for the fish community in the OW was 'good', indicating good species representation in terms of diversity and abundance. AChE levels of fish in this estuary were low, indicating exposure to anticholinesterase compounds. This estuary contained fish with the highest LPx levels recorded over the entire study, and the data were bimodal (Figure 4.3.4), suggesting that only a portion of the population were affected by a toxicant. The histopathological findings from this estuary showed that most of the samples examined had signs of pathological alterations occurring in the liver tissue, but a small sample size limited the interpretation of these results. Although the histology assessment was good (2), it is probable that a larger sample

size would have resulted in a lowering of this score. The condition factor of *R. holubi* from the OW were the lowest of all three estuaries (Figure 4.3.2.), possible reasons for this being the lack of macrophyte beds and associated epifauna, as well as possible insecticide impacts on the benthic invertebrate fauna within the system (Chapter 4). The overall index score was lower than five and the estuary therefore requires management intervention. The fact that negative effects of contaminants were measured at the individual and at all the cellular levels, but not at the community level, suggests that the fish are being stressed at a sub-lethal level and that this stress has not yet become apparent at the community level. Since *R. holubi* are showing signs of stress in all the biomarkers measured it is probable that this has a compounding effect on the fish. These results also indicate that the fish population in the OW may be more susceptible to other stressors such as disease or further exposure to xenobiotics, since the fish are already stressed at the sub-organism level.

A pilot mark-recapture study was undertaken in 2000 in the OW by a number of trained ichthyologists who had conducted a successful mark-recapture study in the EK (Cowley & Whitfield 2001a). A number ($n \approx 100$) of apparently healthy (externally) fish from several species were fin clipped and quickly released back into the OW. The following day, almost all the clipped fish were found dead in the estuary but there were no mortalities of non-clipped fish. It was hypothesised at the time that the reason for the fish kill was that the individuals were already under stress at a sub-organism level, and the handling stress overwhelmed the fish, resulting in an almost 100% mortality. Although the present study was conducted six years later, the fish have been shown to be stressed at a sub-organism level which tends to support the hypothesis proposed for the deaths of the fish during the 2000 mark-recapture exercise.

The OW scored less than five using the overall index; therefore management intervention in terms of water quality management (because the assays indicate that the fish are being exposed to xenobiotics) is required in this estuary. In terms of the flow chart presented earlier (Figure 7.4), pesticides and other chemicals that are used on the golf course are probable causative agents, simply because there are no identifiable anthropogenic activities in the upstream catchment. Golf course and littoral estuarine sediment samples should be collected to determine which chemicals

are most persistent and encourage the golf course manager to discontinue the use of these products. Intensive chemical sampling could be undertaken following rain events to determine the presence or absence of chemical runoff from the golf course into the estuary. Another management intervention could include extending the 'rough' area which is adjacent to the estuary, thus increasing the buffer zone for chemical runoff from the greens/fairways. Continuous monitoring of the health status of fish from this estuary following these interventions would be necessary to assess the success or otherwise of these strategies.

The MTN estuary scored moderately in terms of the EFCI. This was mainly attributed to the dominance of *Gilchristella aestuaria* in the catch and the low numbers of piscivorous species recorded (Chapter 2). There was no sign of acetylcholinesterase inhibition in *R. holubi* from this estuary; however LPx levels were significantly higher than the levels found in fish from the EK. The histology mirrored the LPx assay findings, with many tissue samples from this estuary showing pathological changes in liver structure. The fish from this estuary had the highest overall CF of all the estuaries sampled. However, in terms of the overall index the MTN requires management action because there are signs, both at the cellular and organ level, of problems in the liver of *R. holubi*.

The liver is the primary detoxifying organ in fish; therefore any lesions to this organ have negative consequences for the overall health of the individual. Liver structure alterations can occur as a result of continuous low dose exposure to xenobiotics. Naigaga (2002) reported necrosis in *Oreochromis mossambicus* following exposure to different copper concentrations in the water column. Concentrations of 0.47 mg/L copper resulted in diffuse necrotic areas becoming apparent after only 4 days. Parallel copper treatments were run at 0.29 mg/L and 0.11 mg/L and necrosis was only observed following 16 days of exposure at 0.29 mg/L concentration and no necrosis was recorded at 0.11 mg/L after 62 days. Thus it is apparent that more severe lesions such as necrosis occur relatively quickly following exposure to high levels of contaminants, and slowly under low dose conditions over long periods of time. Based on the above it is proposed that the contaminants in the MTN may not have been present at high concentrations but may have been chronic in nature. Had the contaminants been present in high concentrations, severe necrosis (and possibly

hepatomas) may have been recorded. The LPx assay measures the rapid response of the cells to ROS damage and the levels measured in the *R. holubi* sampled in this estuary indicate that the fish had been exposed immediately prior to capture. In addition, the results from the histology and the LPx assay suggest that there is a low dose chronic source of pollution in the MTN.

The water samples collected from the MTN did not show any pesticide residues. This may have been because they were present in concentrations below the detection limit of the analyses undertaken or another type of contaminant (not a pesticide) may have been affecting the liver of the fish. As mentioned in the introductory chapter, there are no formal developments within the catchment of the MTN, therefore the source of these contaminants is not currently apparent. However, a possible source of contaminants could be cattle belonging to local inhabitants wading in the estuary (as was witnessed during the fish survey) and thus introducing pollutants. Cattle are routinely 'dipped' in a biocide solution to prevent parasites by the state veterinary services. Dipping occurs every 2-3 weeks in winter and every 1-2 weeks in summer. Plunge dips are used and the dip solution is Amitraz (chemical name N,N'-[(methylimino) dimethyllidyne] di-2,4-xylidine) (Grahamstown State Vetenarian, Dr Pretorius *pers. comm.*). Amitraz has been shown to be moderately toxic to fish (Pesticide Management Education Program website) and it is proposed that the cattle may be the source of xenobiotics causing liver stress in MTN fish. Confirmation of the impacts of Amitraz on *R. holubi* needs to be undertaken under laboratory conditions. A management intervention to avoid further damage to the MTN could be to prevent the cattle from entering the estuary immediately after the dipping procedure. Amitraz has been shown to breakdown in soil after one day in oxygenated conditions (Pesticide Management Education Program website) and from this it is proposed that preventing the cows from entering the estuary for two days following dipping should reduce the negative impacts of this chemical on the biota of the estuary. Continuous monitoring would be required to assess the success of this management strategy.

Shortcomings of the index

The proposed index is a 'snapshot' indication of estuarine condition and because of this is limited in what it can describe. It is therefore proposed that any formal assessment of an estuary would require repeat sampling in order to ensure that the values obtained are representative of estuarine condition over time. The frequency at which an estuary should be re-sampled depends on the nature and extent of negative impacts. Monitoring should focus on the area which the composite index has identified as stressed. For example, if the biomarkers are scoring poorly but the community scores well, then the biomarkers would need to be monitored more frequently than the entire community. A severely impacted estuary (at biomarker level) should be sampled relatively frequently (e.g. every three months) to ensure that the management interventions which have been undertaken are adequate to reverse the negative impacts reported. If the community of an estuary is also found to be severely impacted then an assessment of the community using the EFCI should be conducted once every six months because the community will take longer to recover than the biomarkers. If an estuary is found to be in good condition (*i.e.* not requiring immediate management interventions, score of five or higher using the index) then monitoring should be undertaken on an annual basis to ensure that the system remains in good health in terms of the parameters being measured.

The index relies on TOCEs being closed immediately before and during the investigation, primarily because when TOCEs breach there is a large efflux of species out of the estuary and influx of larvae into the system. This can result in very low catches in terms of the EFCI since the majority of immigrating 0+ fish are too small to be sampled by the seine or gill net. In terms of the biomarkers, sampling the immigrating fish will give little indication of the health of the estuary since these individuals have spent very little time in the estuary.

The overall index relies on fish populations and does not look at plant, invertebrate or bird populations of an estuary which are also important components of estuarine biota. Certain pollutants may, for example, be affecting the invertebrates, but fish may be insensitive to these pollutants and therefore these are not being identified as contaminants. Niemi *et al.* (2004) state that the use of multiple taxa with different life

history strategies is a promising approach to biomonitoring because the different species are sensitive to different xenobiotics and can therefore aid in the detection of a wide range of pollutants. The incorporation of different taxa, such as invertebrates, diatoms, and macrophytes would strengthen the index. However, laboratory and field studies would need to be conducted to evaluate the weighting of each taxon in the overall index in terms of sensitivities to xenobiotics, durations of exposure, extent of the effect on biota, etc. O'Connor *et al.* (2000) used birds, fish, invertebrates, zooplankton and diatoms to evaluate the condition of 19 lakes in New England and found that each taxonomic group was differentially sensitive to different environmental factors. Therefore the need to evaluate the weighting of each taxonomic group is important if a multi-taxa index is to be used. Time and budget constraints would also dictate the number of species that could be included in an assessment.

There are a number of other useful biomarkers that could be included in the overall index. These include some parallel antioxidant assays which could give an indication of the interaction between LPx and antioxidant systems, and could give a better indication of the manner in which an organism is coping with the toxicant. Examples of these types of assays include the glutathione peroxidase assay, the superoxide dimutases (SODs) assay, catalase (CAT) assay and metallothionein measurements (Kelly *et al.* 1998, Fatima *et al.* 2000, Pena-Llopis *et al.* 2003).

Other important biomarkers that were not included during the present study include biomarkers of fecundity, egg and larval survival success, endocrine disruption biomarkers, stress protein formation and tissue metal burdens. These biomarkers can provide insight into other aspects of the health of estuarine biota and would provide a more detailed indication of biotic integrity. It is important to note that any biomonitoring program will have certain time and budget constraints, and these constraints will inevitably influence which biomarkers will be included in the monitoring package.

Korsloot *et al.* (2004) explain that the cellular defence systems in any organism are integrated and co-operative in order to effectively combat stress. The way in which an

organism reacts to a stressor rests on four mechanisms of stress identification and subsequent stress response initiation. These are:

1. Damage to proteins and enzymes
2. Disturbance to the redox state
3. Changes in free zinc levels
4. Effects on signal transduction systems

If any of these four events occur, the stress is recognised and a response is initiated (Korsloot *et al.* 2004). The response is coordinated by the signal transduction system, which alerts the organelles to the stressor. All four aspects of the cellular defence system will be involved. An example of this would be if a cellular mass underwent oxidative stress; the oxidative stress response system would become activated, and at the same time the normal protein synthesis of the cell would cease and stress protein formation would begin. Stress proteins are formed in order to protect the protein structures already existing in the cell and to limit any damage to DNA material. Additionally, metallothioneins and antioxidant systems (part of the metal stress response system and regulated by free zinc levels in the cell) would be deployed to the affected area in order to scavenge any free radicals generated by the oxidative damage. Korsloot *et al.* (2004) therefore propose that biomonitoring programs should include investigations into all four of the above mentioned mechanisms of stress identification.

The present study only examined two of the four reactions outlined above. The LPx assay measures the redox balance in the cell. This is important as Korsloot *et al.* (2004) state that the redox state is pivotal in integrating and activating the stress response of the cell. The AChE assay is a measure of the damage to proteins and enzymes of the cell.

The signal transduction system is a network of reactions which together ensure the harmonisation of a number of mechanisms to generate the appropriate response to a stressor (e.g. generating a large amount of antioxidants during an oxidative stress event) (Korsloot *et al.* 2004). This was not included in the biomonitoring protocol of the present study because it yields no information on the initial stressor that activated the stress response.

The free zinc levels were also not addressed in this project. However, heavy metals have been detected in the sediments of certain Eastern Cape estuaries and are thought originate from inadequate waste disposal (particularly discarded metal food cans) in informal settlements which fringe the banks of many systems (W. Froneman, *pers comm.*). Heavy metal pollution may therefore be an issue in some estuaries and needs to be investigated. AChE inhibition has been linked to heavy metal exposure (Guilhermino *et al.* 1998, Guilhermino *et al.* 2000, Corsi *et al.* 2003) but additional water and sediment samples would be needed to confirm that the inhibition was a result of heavy metals rather than other anticholinesterase substances.

Uncertainty regarding which xenobiotic(s) is producing the recorded effect is a negative aspect of the biomonitoring approach. AChE activity, LPx levels and histological changes can be influenced by a range of chemicals. Biomarkers can indicate that an organ or cells are being affected by xenobiotics, but they cannot usually identify exactly what those chemicals are. It is therefore proposed that if a problem becomes apparent using a suite of biomarkers, chemical and sediment analyses should be undertaken to establish the cause(s) of these problems. In this way, biomarkers are useful early warning systems; if a stressor is causing a measurable biological effect, further studies would need to be undertaken in that system to ensure the implementation of appropriate management interventions. If no biological effects are measured, no further resources are needed to investigate the system (Peakall & Walker 1994).

Forbes *et al.* (2006) describe in detail many shortcomings of biomarkers because of the lack of clearly definable relationships between exposure and response in field conditions under mixed effluent situations. The biomarker strategy for evaluating estuarine health is limited in terms of what it can indicate with regards to identifying the pollutant, because further tests are normally required to confirm the responses recorded in the field. However, where information on contaminants is lacking (as is generally the case in most South African estuaries) biomarkers are a useful assessment method when compared to conventional chemical or sedimentary analyses. The reason for this is that it is almost impossible to test for every possible contaminant in an estuary using either sedimentary or chemical analyses. In the case

of the present study, an educated guess was made to determine which chemicals should be tested for based on the catchment activities of the three estuaries and, despite this, none of the chemicals tested for were detected. This may have been due to the fact that the multi-residue analyses performed were not sensitive enough to detect any chemicals in the water samples. It may also have been that other types of pollutants for were present in the water and were responsible for the biological responses recorded. However, had the biomarker approach not been used, and only a community based approach been applied, none of the problems which have come to light as a result of this study would have been detected. Therefore despite certain shortcomings of biomarkers in terms of their ability to identify the exact chemical which is causing the problem, this approach is considered suitable for assessing estuarine health.

Another problem encountered during the present study using biomarkers is the large variability in individual fish responses. Due to this, large samples from each estuary were required in order to determine whether significant differences existed. A power analysis (Barker Bausell & Li 2002) indicated that a sample size of 100 fish per estuary would result in an 80% chance of obtaining a significant result between the estuaries investigated. The bimodal response seen in the LPx data from the OW might not have been detected had a smaller sample size been collected. This sample size would be unsustainable if continuous sampling were required in a small estuary. However, once a large enough dataset has been collected (as was the case during the present study) smaller subsequent sample sizes might be sufficient and could be compared to the larger dataset. The initial large sample size could provide a good initial indication of the range of possible values. An integrated biomarker response index for small samples sizes has been developed in other studies (Beliaeff & Burgeot 2002, Leinio & Lehtonen 2005). This method was not used in the present study because large sample sizes were collected.

It should be highlighted that the index developed in this chapter is only a proposed model for future biomonitoring work. It has not been validated over time and space in either these or other estuaries and this will require future work to determine the sensitivity of such a monitoring tool. However, data from biomonitoring studies such as this needs to be presented in a manner that can be understood by non-scientist

audiences. An index is easily understood and can portray data in a simple, clear manner which is accessible to all.

Rhabdosargus holubi as an indicator species for Eastern Cape estuaries

In a recent review of pollution biomarkers in estuarine fish were found to be more sensitive to ChE inhibitors than invertebrates (Monserrat *et al.* 2006). A popular argument for using invertebrates in biomonitoring is that many species are sessile and can therefore indicate localised conditions in an estuary. On the other hand, it is difficult to select an appropriate invertebrate species in South African estuaries because:

1. Most invertebrates species that utilise estuaries do not necessarily occur in all estuary types (e.g. permanently open versus temporarily open/closed systems)(Hastie & Smith 2006).
2. Invertebrates generally tend to be specific to sediment types (e.g. sand versus mud) and these substrata do not occur in all the reaches of an estuary (Kalejta & Hockey 1991, Teske & Wooldridge 2003).
3. Many invertebrate species have a more limited tolerance to extreme salinity conditions when compared to fishes and may not be present along the entire salinity range found within an estuary (Teske & Wooldridge 2003).

In view of the above, it is suggested that fish are more suitable for estuarine biomonitoring. A suite of invertebrates could be used; however, this would require laboratory work to determine basal levels of the biomarkers being measured for each species. Diatoms or macrophytes could also be used in monitoring programs; however sub-organism level biomarkers such as histological analyses, LPx or AChE assays cannot be performed on plant material.

There are two main categories of fish that can be used in biomonitoring studies in South African estuaries, namely estuarine resident and marine migrant species. Estuarine resident species would be ideal candidates for biomonitoring studies because they spend their entire lifecycle within estuaries and therefore representative samples from all the life stages can be obtained. The problem with this category of estuarine fish is that all the species which fall within this category are small (< 50 mm

SL). This means that the amount of biological material available per specimen for analysis is greatly reduced. Thus, one specimen could not be used to undertake both histological and biochemical analyses on the liver, and other analyses would also be compromised due to limited tissue availability, e.g multiple analyses on kidney or gonad tissues.

The other possibility is to use a species from the marine migrant category. The problem with these types of estuary-associated fishes is that they usually spend only a portion of their life cycle within estuaries and that they tend to migrate between the estuarine and marine environment, especially when adult. This means that these animals are only suitable for studies which analyse short-term exposure to estuarine waters. Another problem associated with this group of fish is that the duration that one particular fish has spent in an estuary is difficult to determine, thus rendering any exposure period determinations difficult.

From the above, it is proposed that the most appropriate group of estuary-associated fishes for biomonitoring would come from the estuary dependent marine migrant group. These fish are entirely dependent on estuaries during their juvenile phases, with most species entering estuaries as postlarvae and remaining within this environment until just prior to maturation. These species often occur in large numbers in estuaries and are therefore easy to catch; they are also large enough for researchers to be able to conduct a range of histological and biochemical analyses.

The use of *R. holubi* as an indicator species proved successful in this study. It is an abundant and readily available fish which is easy to catch in large numbers in estuaries. Originally the study was going to use *Mugil cephalus*; however these fish were difficult to catch in large enough numbers and it was found to be unsuitable for laboratory work, as has been reported in other studies (De Kock & Lord 1988). The biology and ecology of *R. holubi* has been well studied (Whitfield 1984, Cyrus & Blaber 1987a, Cyrus & Blaber 1987b, Cowley *et al.* 2001, Lukey *et al.* 2006). This species is a primary and secondary consumer and feeds mainly on aquatic plants such as filamentous algae, submerged macrophytes but also on epibenthic vertebrates such as polychaetes and crustaceans (Whitfield 1998). The varied diet means that *R. holubi*

is exposed to a range of food types and possible toxicants, thereby increasing its value as an indicator species.

The mean standard length (SL) for individuals caught during the present study was 9.3 cm (± 1.39 SD) and according to Whitfield (1998) juveniles attain this length following approximately one year of residency within an estuary. Blaber (1974) undertook a three year study of the *R. holubi* population in the West Kleinemonde estuary and found no mature individuals (>14 cm SL) within this system. A 6 cm growth rate over summer months for *R. holubi* occurred during two consecutive years, and the fish generally left the estuary once they had reached a size of 13-14 cm SL. None of the fish examined had developed gonads and from this Blaber (1974) concluded that estuaries are important areas for juvenile *R. holubi* but not for adults. Hormones are documented to affect levels of some of the biomarkers used in this study and also introduce differences between males and females. Therefore it is important that the specimens collected during the present study were all sexually immature.

A recent study conducted in Argentina assessed the sensitivity of two different freshwater fish species to the same pollutant using the AChE assay (De la Torre *et al.* 2002). An indigenous fish, *Cnesterodon decemmaculatus*, and an exotic fish, *Cyprinus carpio*, were placed in cages in a polluted river for a month and their responses assessed. The authors found that the indigenous fish was a more sensitive indicator of exposure than the exotic fish, and suggested that endemic species be considered for biomonitoring programs.

R. holubi is an ideal candidate for biomonitoring programs in South African estuaries for several reasons:

- It is endemic to southern Africa.
- It occurs in estuaries from the Berg Estuary in the Western Cape to Inhaca Island in Mozambique.
- It is common in estuaries and it is therefore easy to obtain large samples quickly.
- Its biology and ecology is well understood.

- It can tolerate wide salinity and temperature fluctuations and is found throughout most estuaries.
- It is representative of a number of commercially important marine fish which also use estuaries during their juvenile stages.
- The liver and brain are of sufficient size for biochemical analyses to be performed.
- It is readily adaptable to laboratory conditions and is suitable for toxicity testing.

Negative aspects about *R. holubi* include the fact that it is not an estuarine resident species; therefore no long-term data regarding the condition of an estuary can be obtained from this species. Similarly, impacts of pollution exposure on reproductive capacity or lifetime exposure studies cannot be carried out using this species. The movements of *R. holubi* within the estuarine environment are unclear at present; therefore delimiting areas within an estuary according to specific point sources of pollution would be difficult to assess using this species.

Carignan & Villard (2002) highlight several attributes which are desirable in indicator species. Specifically these authors suggest using multiple species with different habitats requirements (and over different time scales) and that these species should be differentially sensitive to changes in ecological processes (for example different salinity and temperature tolerances). In terms of the present study, only one species was used because this was a pilot study. Future investigations should investigate the use of other species, for example species with small home ranges or sessile organisms because they are more sensitive to localised changes in water quality (Carignan & Villard 2002). For larger scale assessments, a bird population could be used as they have been shown to be good indicators of large scale changes to ecosystems (Carignan & Villard 2002).

7.2 Conclusions

This study has shown that the biomonitoring techniques applied to investigate the health status of three Eastern Cape TOCEs were sufficiently sensitive to detect differences between the three systems. It also showed that under stressed conditions,

biomarkers of exposure can be traced from lower (biochemical) to higher (organ level) biological organisation levels. An appropriate monitoring candidate, *R. holubi*, was identified for monitoring in South African estuaries.

As mentioned in the introductory chapter, the three estuaries investigated during this study were chosen because they are representative of a number of other similar systems in the Eastern Cape Province. The results from the present pilot study have important implications for other estuaries of this type. Most estuaries in South Africa were studied by Harrison *et al.* (2000) using a composite index that included the fish community. The latest version of this index (EFCI) was used to determine estuarine health but information on the organ and cellular health of fish in South African estuaries was lacking. However, as can be seen from the results from the MTN and OW estuaries, the EFCI does not necessarily give a true indication of the health status of an estuary. The OW was found to be in need of immediate management attention based on the parameters measured for this study, despite scoring well in terms of the EFCI. The MTN was found to be in need of immediate management attention despite scoring 'moderately' in terms of the EFCI. Thus organism health is an important additional consideration for effective estuarine management.

Future research should include the use of other biomonitoring techniques. Antioxidant biomarkers are required to complement the LPx findings and to aid in the interpretation of results. Other important areas that were not investigated in the present study relate to fecundity, egg and larval survival and possible effects of endocrine disruption. Finally the extension of this work to similar estuaries in other southern African biogeographical areas would reinforce the applicability of the Eastern Cape work to the wider region.

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Appendix 1

List of definitions for histological terms (Chapter 3).

- Hyalinization: Hyaline droplet formation. This is a translucent substance which collects in cells as a result of pathological degeneration of tissues (web based dictionary).
- Hydropic change: A type of cellular swelling and is characterised by a cloudy cytoplasm and enlarged hepatocytes without a change in the diameter of the nucleus (Vermulen, pers comm.). It is a result of a prolonged insult to hepatocytes and is generally reversible (Tuskegee University web page).
- Hyperplasia: An abnormal multiplication of cells, usually the regeneration of cells following a loss of cells. In the case of the liver, this usually refers to hyperplasia of the hepatocytes (Hinton & Lauren 1990).
- Hypertrophy: Abnormal enlargement of an organ or part of it. It is a result of hyperplasia and is a net gain in overall numbers of cells or tissues within an organ but is not a result of cellular swelling (Hinton & Lauren 1990).
- Megalocytic hepatosis: A type of cellular swelling and is characterised by a cloudy cytoplasm and enlarged hepatocytes and contrary to hydropic change, the diameter of the nucleus increases (Vermulen, pers comm.).
- Melanomacrophage centres: These are pigment containing cells which increase in range and size in the presence of disease or as a result of environmental stress. These cells engulf degenerated cells and toxic tissue materials (Aigus & Roberts 2003).
- Mono-nuclear leukocytes: This is also known as lymphocyte infiltration. Phagocytotic lymphocytes engulf dead or dying cellular material following necrosis in a particular area (Hibiya 1982).
- Necrosis: The death of cells or tissues as a result of external damage (Lawrence 1996).
- Neoplasm: An uncontrolled growth of cells, sometimes self-limiting as in benign tumours, sometimes malignant (Lawrence 1996).
- Neoplastic: cells or tissues arising as a result of uncontrolled growth, e.g. malignant or tumorous (Lawrence 1996).

- Preneoplastic: preceding the formation of a benign or malignant neoplasm (Lawrence 1996).
- Vacuolation: The formation of vacuoles. In the liver it is a result of an inability of the hepatocytes to remove lipids from inside the cell. This may be as a result of protein synthesis inhibition, energy depletion, disruption of pathways for the removal of fats from the cell, and a change in the substrate that usually catalyses enzymatic reactions (Hinton & Lauren 1990).

Appendix 2

List of figures which show the histological characteristics described in the results and discussion section.

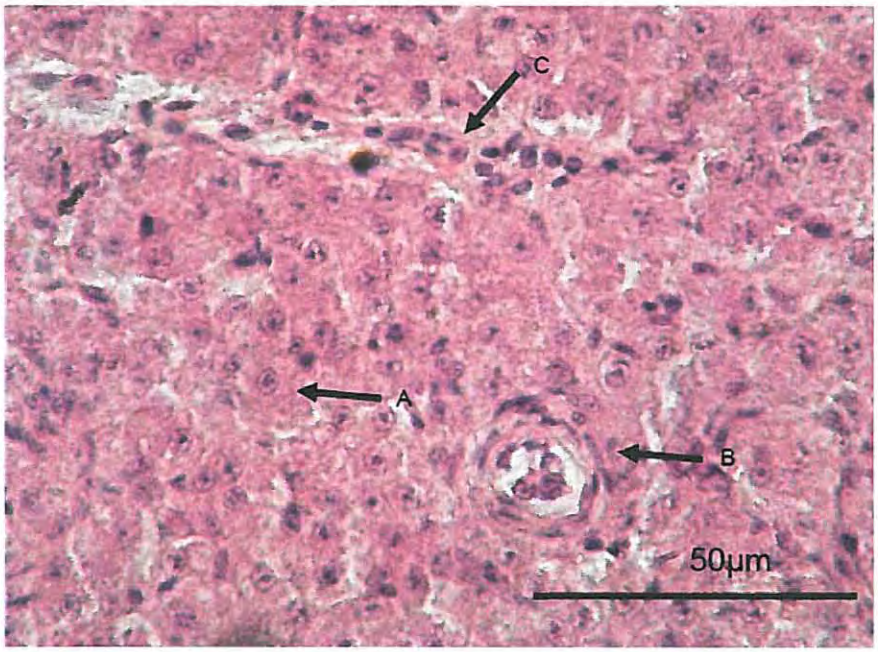

Figure 3.3.1 Normal liver parenchyma. A: hepatocytes, B: Bile duct, C: sinusoid. H&E stain, x100 magnification.

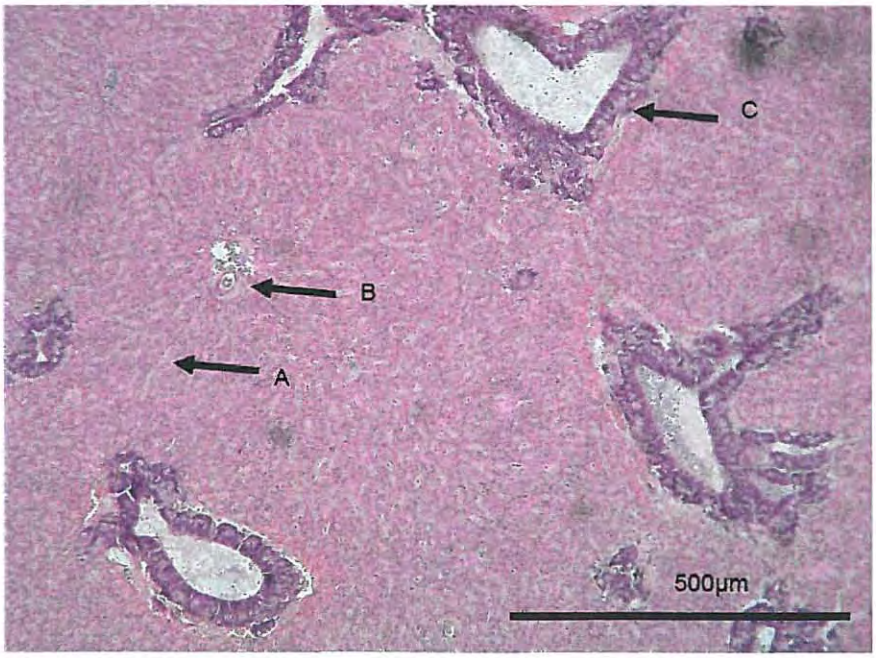

Figure 3.3.2 Normal liver parenchyma. A: sinusoids, B: Bile duct, C: hepatopancreatic tissue. H&E stain, x 10

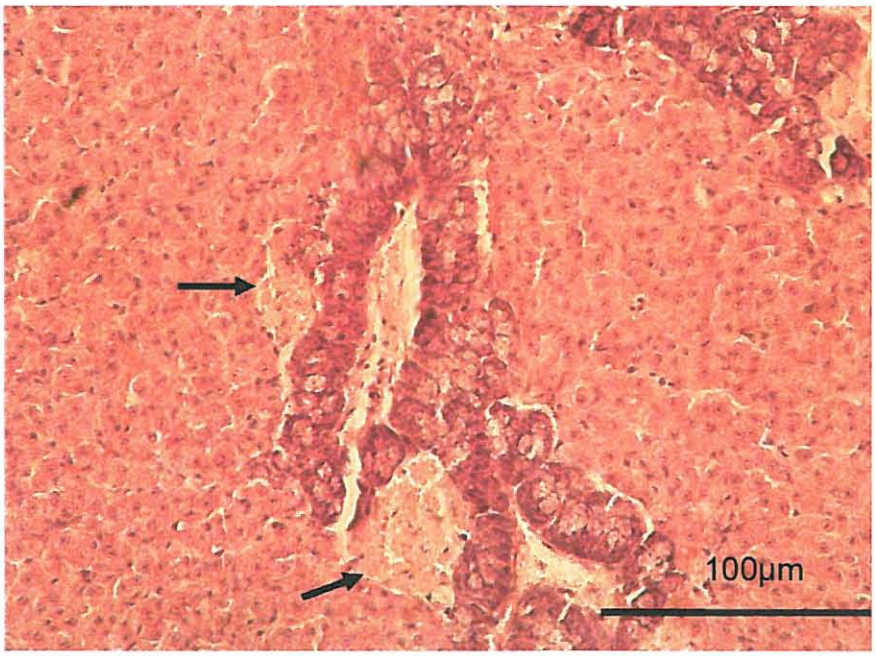

Figure 3.3.3 Normal melanomacrophage centres (MMCs) around hepatopancreatic tissue. H&E stain, x 40 magnification.

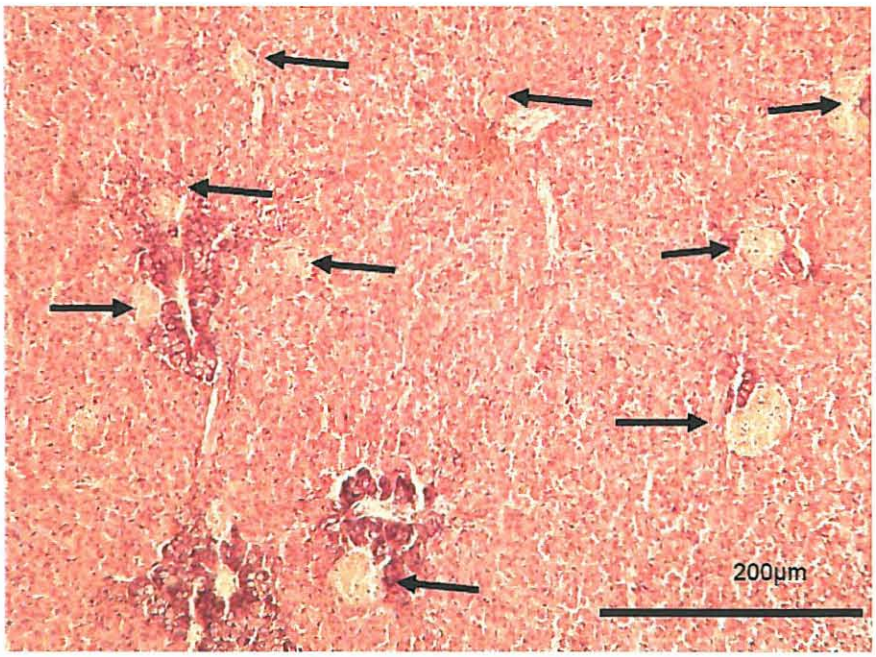

Figure 3.3.4 Increase in MMCs. Arrows point to individual MMCs. H&E stain, x 20 magnification

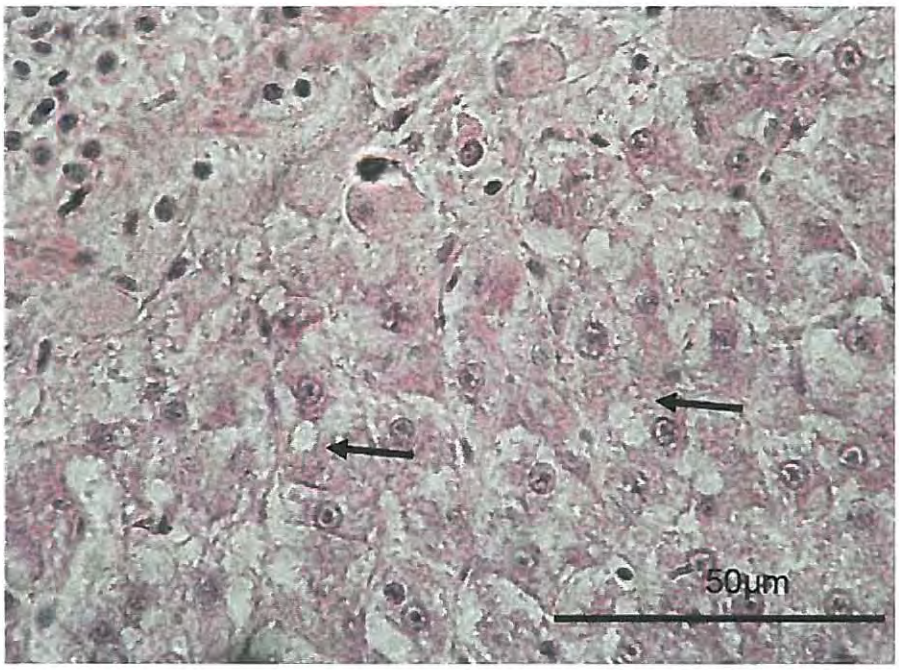

Figure 3.3.5 Vacuolation of hepatocytes. Arrows point to vacuoles. H&E stain x 100 magnification

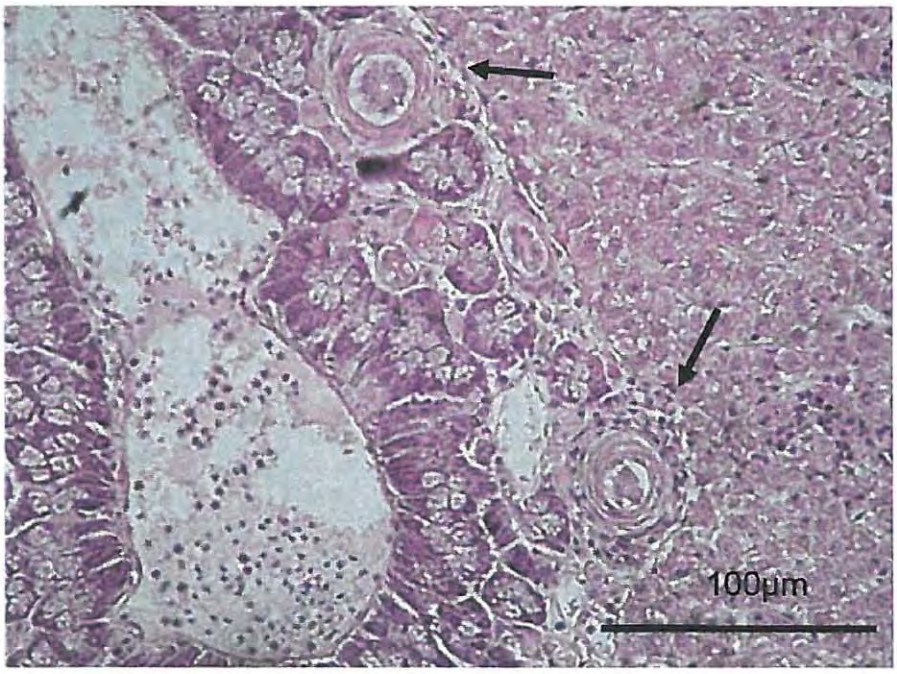

Figure 3.3.6 Increase in perivascular tissue. Arrows point to the perivascular tissue around the bile ducts. H&E stain, x 40 magnification.

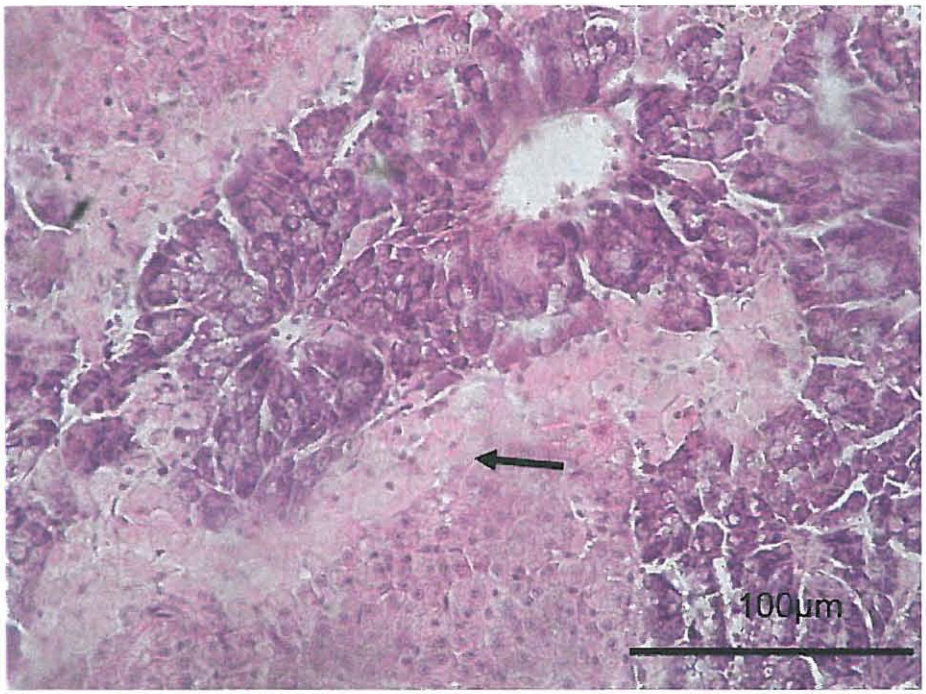

Figure 3.3.7 Hypertrophy (clear pink areas around the hepatopancreatic tissue) of hepatocytes around hepatopancreatic tissue (arrow). H&E stain, x 40 magnification.

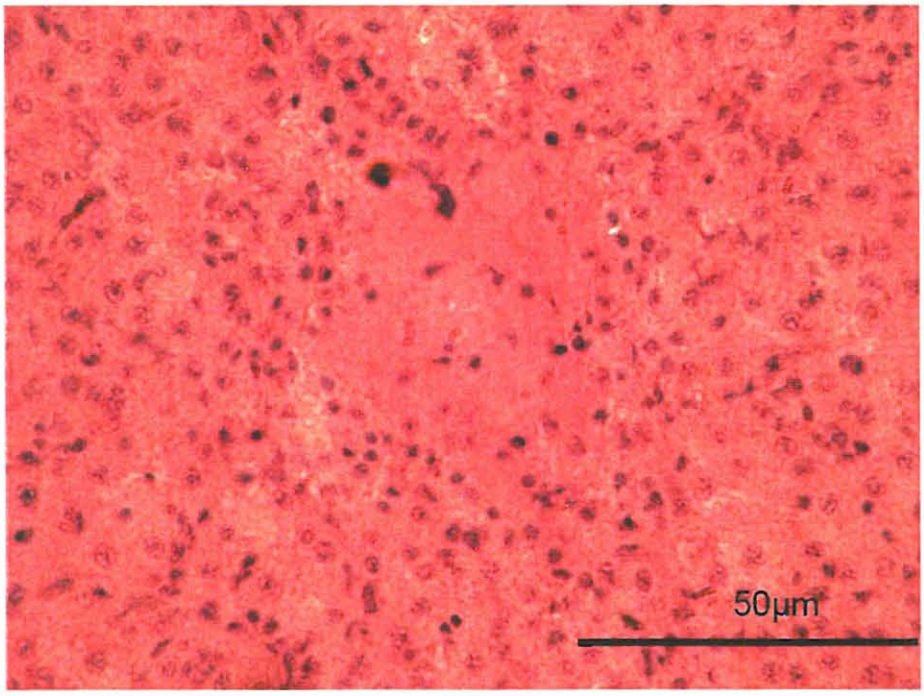

Figure 3.3.8 Focal area of necrosis in the middle of the photograph. H&E stain, x 100 magnification

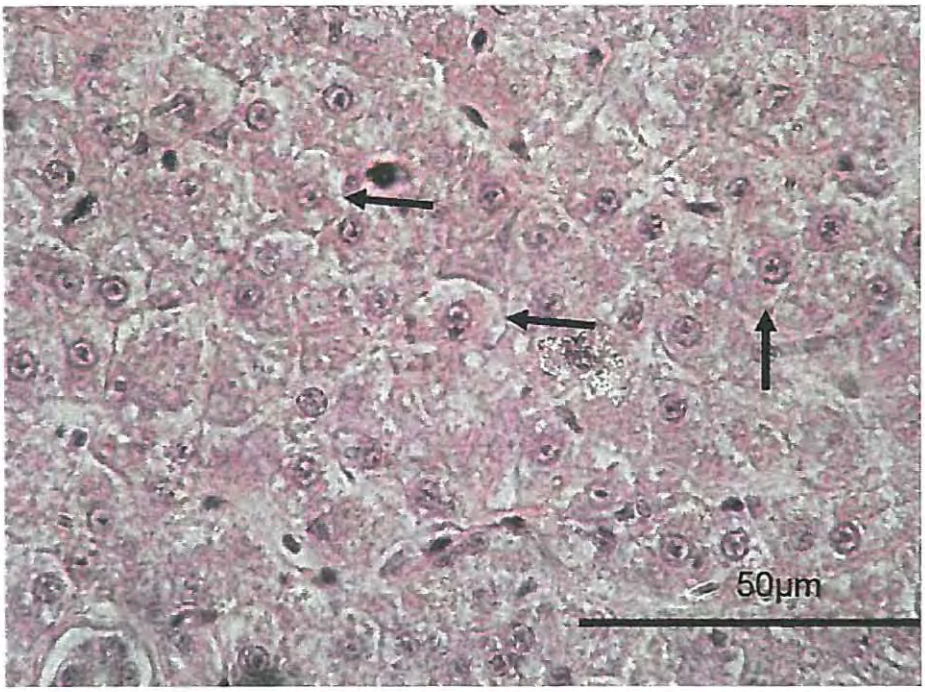

Figure 3.3.9 Hydropic change in the hepatocytes. Arrows point to examples. H&E stain, x 100 magnification

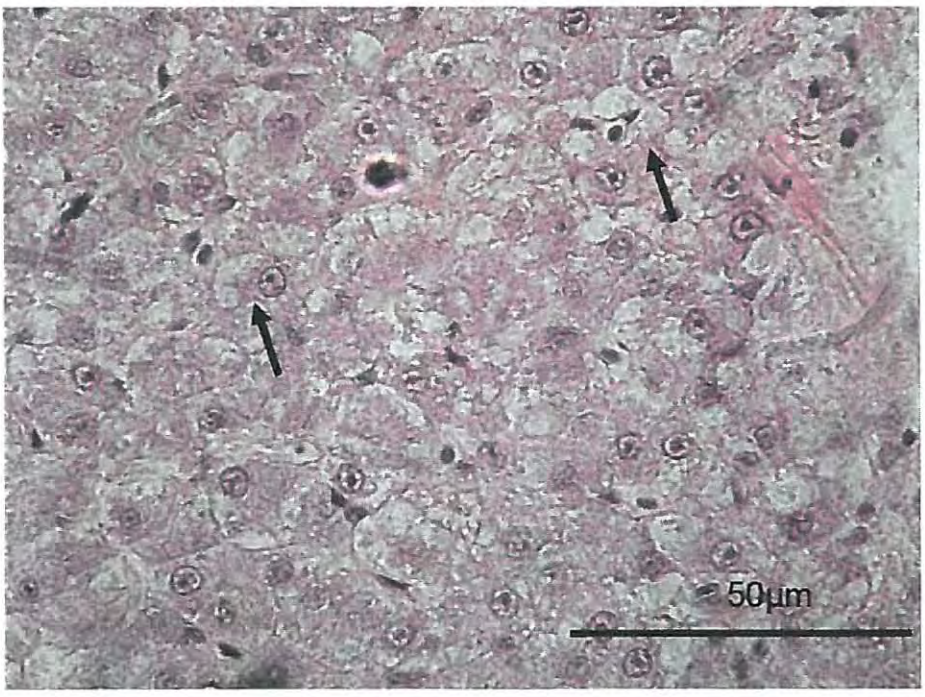

Figure 3.3.10: Glycogen vacuolation of hepatocytes. Arrows point to glycogen accumulation in the cytoplasm of the hepatocytes. H&E stain, x 100 magnification.

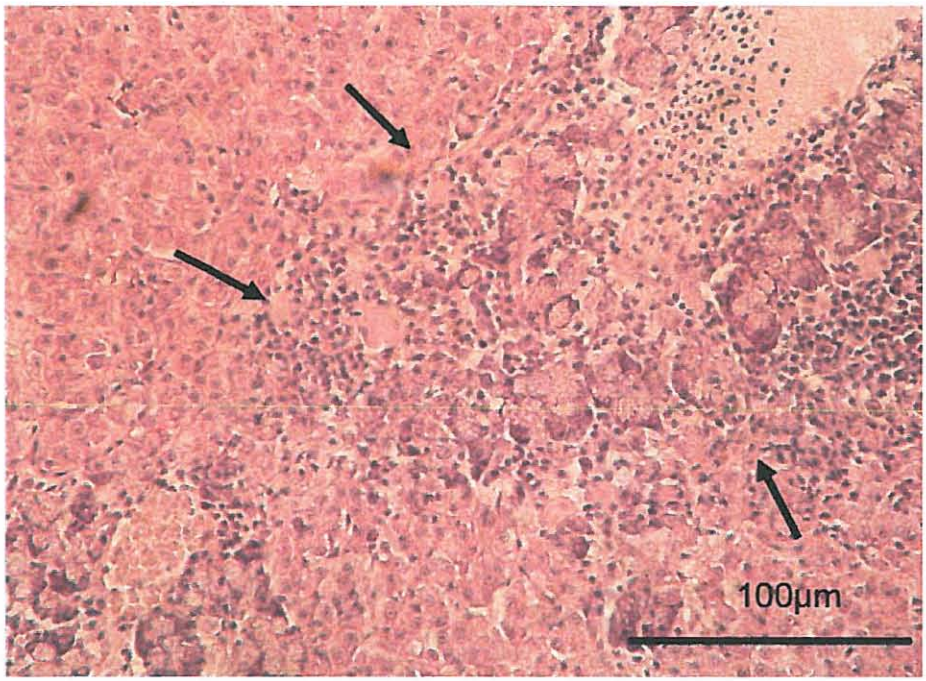

Figure 3.3.11: Increase in Mono-nuclear leukocytes (lymphocyte infiltration). Arrows point to affected areas. H&E stain, x 40 magnification

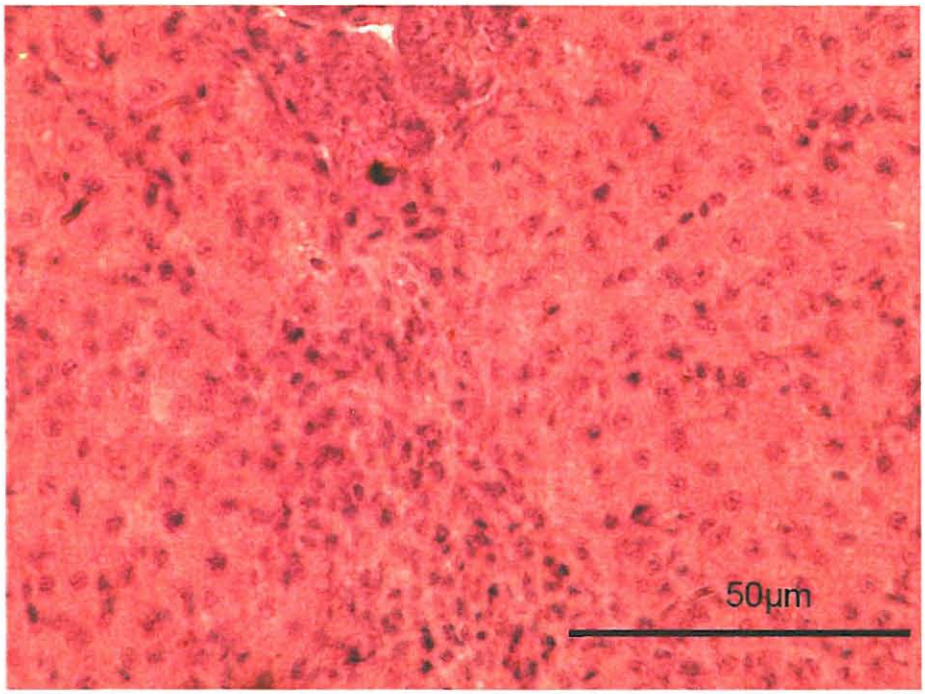

Figure 3.3.12 Overall loss of structure of hepatocytes. H&E stain, x 100 magnification.

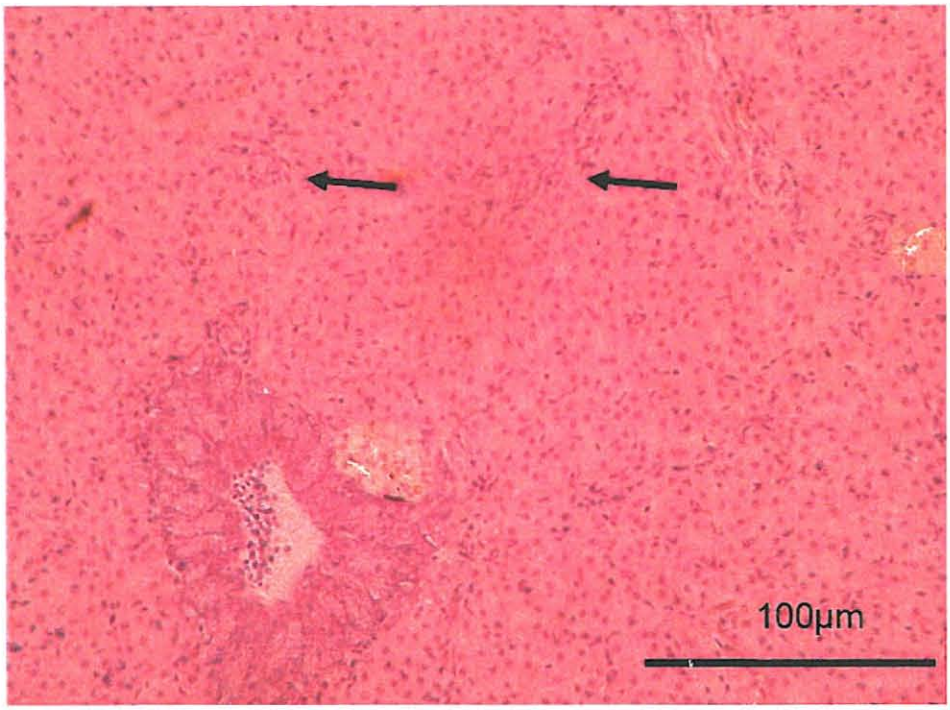

Figure 3.3.13: Congestion of sinusoids (arrows) by blood cells. H&E stain, x 40 magnification.

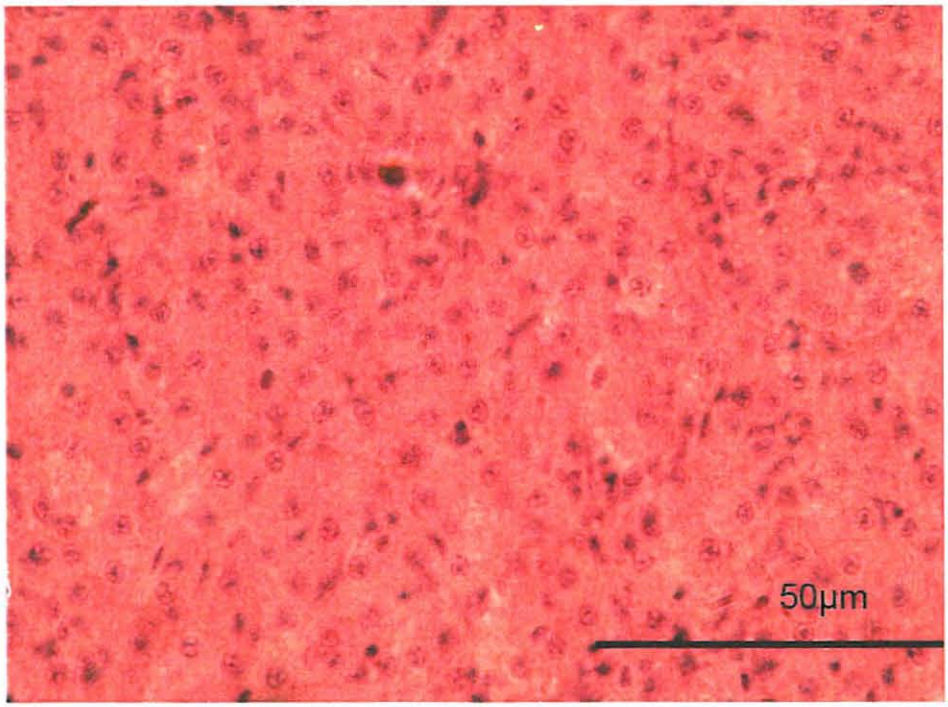

Figure 3.3.14: Hyperplasia of hepatocytes. H&E stain, x 100 magnification.