Active biomonitoring (ABM) of the Rietvlei Wetland System using antioxidant enzymes, non-enzymatic antioxidants and histopathology as biomarkers.

by

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List of figures and tables

Figure 3.1.	Map of Rietvlei Wetland System
Figure 3.2.	Biomarker responses in *Oreochromis mossambicus*
Figure 3.3.	Biomarker responses in *Melanoides tuberculata* during the high-flow period.
Figure 3.4.	Biomarker responses in *Melanoides tuberculata* during the high-flow and low-flow periods.
Figure 3.5.	Histopathological alterations in *O. mossambicus* gill tissue.
Table 3.1.	Physico-chemical parameters of water at the three sites

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Abstract

The main objective of this study was to perform active biomonitoring in the Rietlvlei Wetland System. Active biomonitoring, which can be defined as "the translocation of organisms from one place to another and quantifying their biochemical, physiological and/or organismal responses for the purpose of water quality monitoring" (De Kock and Kramer, 1994), has several advantages over the chemical monitoring system. Effluents often are complex and poorly characterized mixtures of a large number of chemicals. A combination of many chemicals being present in very small amounts (even below detection limits) can have a substantial impact on organisms, and a chemical-based approach may not identify the source of pollution nor will effect of synergism or antagonism be taken into account (Smolders *et al.,* 2003).

A suite of biomarkers of oxidative stress and histopathology were investigated in the fish *Oreochromis mossambicus* and the mollusk *Melanoides tuberculata.* The organisms were bred under laboratory conditions. They were deployed during the high-flow and low -flow periods, in cages at three sites down the flow gradient of the Rietvlei wetland system, to determine spatial and temporal variations in biomarker responses and general water chemistry in the system. The oxidative stress biomarkers analyzed were catalase (CAT), superoxide dismutase (SOD), peroxidase (GPx), as well as levels of reduced glutathione (GSH) and lipid peroxidation as malondialdehyde (MDA). There was evidence of presence of chemicals inducing oxidative stress in the organisms as indicated by the high levels of MDA, GSH and GPx. Induction of CAT and SOD was not substantial due to possible inhibitory

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factors. This study established that organism transplantation is a feasible strategy for biomonitoring. Overall, no distinct variations were observed in the spatial and temporal comparisons in all the biomarker responses. The findings of this investigation also provide a basis for further investigation into the application of these biomarkers in ecological risk assessment.

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CONTENTS **PAGE**

CHAPTER 1

1. Introduction 7

CHAPTER 2

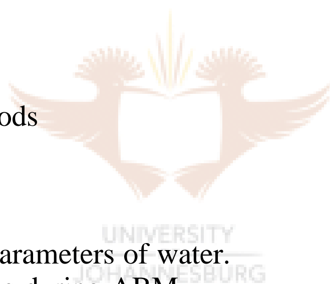
2. Literature Survey 11
2.1. Active Biomonitoring 11
2.2. Bioindicator organisms 12
2.3. Biomarkers of oxidative stress 13
2.3.1. Catalase 14
2.3.2. Peroxidase 15
2.3.3. Glutathione 16
2.3.4. Malondialdehyde 17
2.3.5. Superoxide dismutase 19
2.4. Histopathology 20

CHAPTER 3

3. Research Article 22
3.1. Introduction 22
3.2. Materials and Methods 24
3.2.1. Study site 24
3.2.2. Study design 36
3.3. Results 31
3.3.1. Physico-chemical parameters of water. 30
3.3.2. Biomarker responses during ABM 31
3.3.3. Histopathology 37
3.4. Discussion 38
3.5. Conclusion 44

CHAPTER 4

4. References 45



CHAPTER 1

INTRODUCTION

The aquatic environment is the ultimate sink of toxic chemicals generated by man's industrial, agricultural and domestic activities. Pollutants discharged into streams and rivers are transported over long distances affecting ecosystems miles from the point of discharge. The importance of producing these chemicals is well appreciated because, for example, one third of the world agricultural produce is destroyed during growth, harvest and storage, so pesticides play a crucial role in increasing food production. However the importance of monitoring and preserving the aquatic environment cannot be overemphasized, because water provides the life support system for aquatic life and all life forms. Hence protection of aquatic resources is essential in protecting the entire ecosystem, but how can the degree of harm be measured? How much damage are we prepared to tolerate as the price of progress (Smolders *et al.*, 2003)?

In recent years pollution of freshwaters in third world countries has reached an alarming point where it might extend to a point of irreparable damage with implicating consequences (Jamil, 2001). For example, the massive death of fish experienced in Lake Chivero, Harare's main water supply in March 1996 (Moyo, 1997) is an indication of a serious potential health hazard to both the human population and the aquatic ecosystem dependent on that water source.

Southern Africa is mostly semi-arid and experiences variation in rainfall, both over time and between countries, which means freshwater is a scarce resource. The sub-region is also expected to experience further variability in rainfall, reduced precipitation and increased evaporation, as a result of climate change. With a rapidly growing population, and demands from the domestic, agricultural and industrial sectors for water, freshwater availability is a priority concern for southern Africa. An additional concern is the declining water quality due to domestic and industrial pollution, and eutrophication and salinization due to agricultural pollution. The most common sources of freshwater pollution in the sub-region are sewage, industrial effluents and agricultural run-off (UNEP, 2002).

They are due, in large part, to inadequate wastewater treatment facilities, as well as to ineffective pollution control. Agricultural run-off, which contains fertilizers, pesticides, and salts from farmland, together with eroded soils, are the major non-point sources of pollution. Increases in demand for freshwater are anticipated in all African countries, in all sectors (domestic, agricultural and industrial), over the next 10 years. In some countries, demand is projected to double within the next 30 years (UNEP 2002). Water remains the prime requirement of man for the future in terms of both quality and quantity. Therefore there is immediate need to find freshwater resources of good quality to meet the augmenting needs of the population, as well as to apply rigorous measures to evaluate the quality of the existing resources and protect them for further degradation.

In this project we used caged fish and mussels as ABM tools to assess water quality, pollution levels and ecological risk to biological systems in the Rietvlei–

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Blesbokspruit wetland system. Chemical and physical water parameters were measured, to complement the biological protocols. We used two classes of endpoints at different levels of biological organisation, in order to obtain a holistic and integrative overview of how instream effluent exposure has an impact on mussel and fish organization. The ultimate idea behind this reasoning is that within every increase in hierarchic level of biological organization a unit with higher ecological relevance is reached (Smolders *et al.*, 2003) The first class of endpoints were the biomarkers malondialdehyde (MDA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and peroxidase (GPx), which gave an indication of response to pollutants at cellular levels. The second endpoint was the assessment of histopathological response to or the effect of pollutants that occurred at cellular level.

Rationale

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The Rietvlei Dam on the Sesmyl Spruit provides a wealth of open water and wetland habitats, and some of the smaller inlet streams create spongy marshes. The Rietvlei Dam provides 27% of Pretoria's drinking water, and it is also a major roosting and breeding locality and feeding area for Pretoria's waterbirds. The system receives effluent from industries, agricultural activities, informal settlements and municipal sewage treatment plants. It is in the interest of conserving water resources for the benefit of humans and wildlife populations that this study was carried out. The results should enable the selection of a suitable suite of biomarkers for use in instream health assessment using ABM protocols in water resource management and should contribute to the objectives of the River Health Programme (RHP) of South Africa.

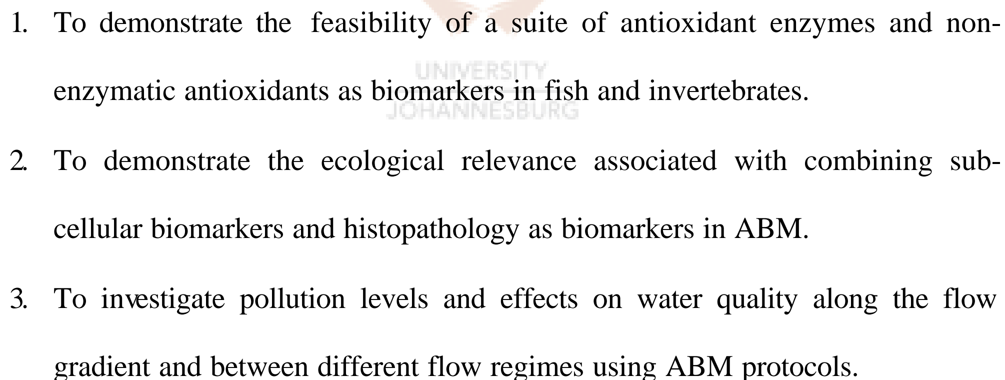
Aim

To test the utility of a suite of biomarkers of oxidative stress and histopathology for application in instream health assessment using active biomonitoring (ABM) protocols.

Hypothesis

Antioxidant enzymes, non-enzymatic antioxidants and histopathology as biomarkers in active biomonitoring are reliable procedures for instream health assessment.

Objectives



1. To demonstrate the feasibility of a suite of antioxidant enzymes and non-enzymatic antioxidants as biomarkers in fish and invertebrates.
2. To demonstrate the ecological relevance associated with combining sub-cellular biomarkers and histopathology as biomarkers in ABM.
3. To investigate pollution levels and effects on water quality along the flow gradient and between different flow regimes using ABM protocols.

CHAPTER 2

LITERATURE SURVEY

2.1 Active Biomonitoring

Active biomonitoring can be defined as the translocation of organisms from one place to another and quantifying their biochemical, physiological and/or organismal responses for the purpose of water quality monitoring (De Kock and Kramer, 1994). This approach employs the collection of organisms from unstressed populations and their translocation in cages to polluted sites e.g. along a pollution gradient. The ecotoxicological consequences of this translocation can then be followed in time and space to estimate the effect of translocation on different selected endpoints (Salazar and Salazar, 1999). A second approach is called passive biomontoring (PBM) and comprises the collection of organisms from their natural habitat at sites where a natural population exists (De Kock and Kramer, 1994). Therefore PBM uses indigenous organisms for biomonitoring purposes.

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Biological methods have several advantages over the chemical monitoring system:

- Biological communities integrate the effects of multiple stresses and demonstrate cumulative impact.
- They serve as an early warning system because they can detect intermittent pollution and subtle disruption. A pollution event at a particular source may be

short-lived, but with considerable consequences. The pollutant is quickly swept away and it cannot be detected by chemical methods.

- Chemical methods tell us nothing about the effect of pollutants on living organisms.
- They are inclusive of many types of pollutants. Chemical systems are menu driven and therefore may miss pollutants. In addition not all pollutants are chemical.

Active biomonitoring has further advantages over PBM. One is that, in the former, the exposure time of the organisms is known and they are not given time to adapt to the environment. In PBM the organisms sampled may only be the tolerant ones, the more sensitive having already suffered mortality, predation, and reproductive impairment (Lovett Doust *et al*., 1994).

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2.2 Bioindicator organisms

According to Ramade (1993), biological indicators of pollution are species particularly sensitive to a contaminant, that serve as some sort of sentinel organisms and reveal degradation of a medium by a given pollutant by their scarcity in the community even before the effects of the pollutants have attained large proportions. Bioindicator organisms represent various types of toxic effects, which correspond to the response of biomarkers of effect in individuals, or represent ecological data i.e., effects on populations and variations in the structure and function of communities. Marine and freshwater molluscs are excellent indicators of contamination because of their strong capacity to bio-concentrate xenobiotics. According to Van der Oost *et al*.

(2003) fish are generally considered the most feasible organisms for pollution monitoring in aquatic systems.

2.3 Biomarkers of oxidative stress

A biomarker is defined as a change that can be observed and/or measured at the molecular, biochemical, cellular, physiological or behavioural level and that reveals present or past environmental exposure of an individual to at least one chemical (Lagadic *et al.*, 2000). Many parameters fall within this definition, so there is a wide range of biomarkers to assess nearly every type of chemical pollution occurring in the environment.

Oxidative stress may ensue when the ability to buffer against reactive oxygen species (ROS) is exceeded either by excessive production of ROS or by depletion of antioxidant. This can alter cellular redox-poise and initiate a variety of responses via intracellular pathways (Sun and Oberley, 1996).

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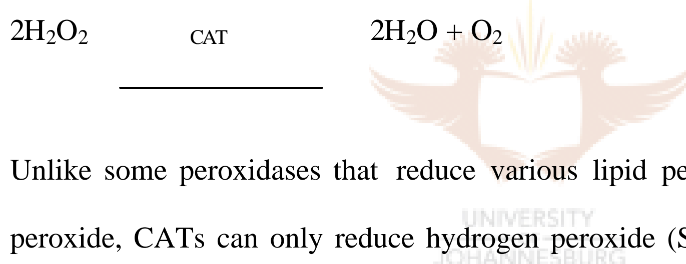
Oxygen toxicity is defined as injurious effects due to cytotoxic ROS, also referred to as reactive oxygen intermediates (ROI), oxygen free radicals or oxyradicals (Di Giuloi *et al.,* 1989; Halliwell and Gutteridge 1999, Winzer 2001). These reduction products of molecular oxygen (O_2) are the superoxide anion radical ($O_2^{-\circ}$), hydrogen peroxide and the hydroxyl radical (OH°), an extremely potent oxidant capable of reacting with critical cellular macromolecules, possibly leading to enzyme inactivation, lipid peroxidation (LPOX), DNA damage and, ultimately, cell death (Winston and Di Giulio, 1991). Defence systems that tend to inhibit oxyradical

formation include the antioxidant enzymes SOD, CAT, GPx and GSH. Superoxide dismutase, CAT and GPx are critically important in the detoxification of radicals to non-reactive molecules (Stegeman *et al.,* 1992; Lopez-Torres *et al.,* 1993). Oxidative stress typifies the toxicity induced by xenobiotics (Lemaire and Livingstone, 1993).

2.3.1. Catalase (CAT)

Catalase belongs to the cellular antioxidant system that counteracts the toxicity of ROS. Catalases are haem-containing enzymes that facilitate the removal of hydrogen peroxide (H_2O_2), which is metabolized to molecular oxygen (O_2) and water.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$



Unlike some peroxidases that reduce various lipid peroxides as well as hydrogen peroxide, CATs can only reduce hydrogen peroxide (Stegeman *et al*., 1992; Filho, 1996). Pandey *et al.* (2003) have suggested that since both induction and inhibition are observed in fish after exposure to environmental pollutants CAT activity cannot be considered a valid biomarker for environmental risk assessment.

2.3.2. Glutathione Peroxidase (GPx)

Peroxidases are enzymes that reduce a variety of peroxides to their corresponding alcohols. While CAT employs one molecule of H_2O_2 as donor in the reduction of another H_2O_2 molecule, peroxidases employ other reductants. The principal peroxidase in fish is a selenimun-dependent tetrameric cytosolic enzyme that employs

GSH as cofactor. Glutathione peroxidase catalyses the metabolism of hydrogen peroxide to water involving a concomitant oxidation of reduced GSH to its oxidised form (GSSG) (Van der Oost *et al.*, 2003).

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

Glutathione peroxidase is considered to play an especially important role in protecting membranes from damage due to LPOX. This observation led to the view that the major detoxification function of GPOX is the termination of radical chain propagation by quick reduction to yield further radicals (Lauterburg *et al.*, 1983). Numerous studies have demonstrated enhancements of LPOX in various tissues from fish species exposed *in vivo* to a variety of chemicals e.g. paraquat-exposed carp (Gabryelak and Klekot, 1985), channel catfish and brown bullhead exposed to t-butyl hydroperoxide (Ploch *et al.,* 1999), sea bass exposed to heavy metals and bluegill sunfish exposed to anthracene and UV-light (Choi and Oris, 2000). Van der Oost *et al.* (2003) suggest that more research is required to determine the potential utility of GPx activity in fish a biomarker for Ecological Risk Assessment purposes.

2.3.3. Glutathione (GSH)

Glutathione (g-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. Glutathione is ubiquitous in animals, plants and microorganisms, and being water-soluble is found mainly in the cell cytosol and other aqueous phases of the living system. Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants. In

addition to being necessary cofactor for GPX and GST activity, GSH is itself an effective protectant capable of quenching oxyradicals (Ross, 1988).

Cells of both simple and complex aerobic organisms contain components which protect them against damage caused by free radicals. Many small molecules such as a-tocopherol and glutathione are part of the intracellular defense apparatus. The tripeptide thiol GSH has facile electron-donating capacity, linked to its -SH group (Slater *et al.*, 1995). Glutathione is an important water-phase antioxidant and essential cofactor for antioxidant enzymes; it provides protection also for the mitochondria against endogenous oxygen radicals. Its high electron-donating capacity combined with its high intracellular concentration endows GSH with great reducing power, which is used to regulate a complex thiol-exchange system (-SH <…> -S-S-) (Ondarza , 1989). This functions at all levels of cell activity, from the relatively simple (circulating cysteine/-SH thiols, ascorbate, other small molecules) to the most complex (cellular –SH proteins).

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Since induction of antioxidants represents a cellular defence mechanism to counteract toxicity of (ROS), they have been extensively used in several field studies to assess the extent of pollution in rivers, lakes and coastal waters (Goksoyr, 1995). Glutathione exists in two forms. The antioxidant "reduced glutathione" tripeptide is conventionally called glutathione and abbreviated GSH; the oxidized form is a sulfur-sulfur linked compound known as glutathione disulfide or GSSG. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress. Redox phenomena are intrinsic to life processes, and GSH is a major pro-homeostatic modulator of intracellular sulfhydryl (-SH) groups of proteins. Many important enzymes (e.g., adenylate

cyclase, glucose-6-phospahetase, pyruvate kinase, the transport Ca-ATPases), and at least eight participating in glucose metabolism are regulatable by redox balance as largely defined by the balance of (2 –SH <…..> -S-S) (Ondarza , 1989).Other proteins (thioredoxins, metallothioneins) have –SH groups at or near the active sites, or are otherwise regulated by the ambient redox state (Hidalgo *et al.*, 1990).

2.3.4. Malondialdehyde (MDA)

Lipid perodixation leads to destruction of membrane lipids and production of lipid peroxides and their by-products such as aldehydes. Malondialdehyde is formed from the breakdown of polyunsaturated fatty acids (PUFA) and it serves as a convenient index for determining the extent of lipid peroxidation (Jamil, 2001). It is a biomarker of effect representing the state of membrane lipid peroxidation.

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Lipid peroxidation is induced by organics and by transition metals, namely iron and copper (Fenton reaction), by acting as catalysts in the formation of oxygen radicals. Mercury, which can be present under different oxidation states in aqueous medium, may generate oxygen radicals (Roméo and Gnassia-Barelli, 1998). Malondialdehyde levels were shown to be increased by copper and mercury and not by cadmium (which does not undergo redox cycling) in the Mediterranean clam, *Ruditapes decussates*, experimentally exposed to these metals (Roméo and Gnassia-Barelli, 1998). In the marine phanerogam *Posidonia oceanica*, collected in the field, differences in MDA levels were found in samples from Villefranchesur-mer from the Bay of Cannes (Lérins island, considered as a very clean area) (Hamoutène *et al.*, 1996).

Since the typical reaction during oxidative stress is peroxidative damage to unsaturated fatty acids, the oxidative stress response could conveniently be used as a biomarker of effect of oxidative stress-inducing chemical pollutants (Pandey *et al.,* 2003). Lipid peroxidation appears to have considerable potential as a biomarker for environmental risk assessment (Stegeman *et al.,* 1992; Hai *et al*., 1995), although it can occur as a consequence of cellular damage due to a variety of insults other than exposure to xenobiotics causing oxidative stress (Kappus, 1987).

Malondialdehyde level is determined by measuring the thiobarbituric acid reactive substances in the microsomal fractions (Livingstone *et al.,* 1990). Generally the ecological relevance of the biochemical events remain low. Although MDA level is considered as a biomarker of damage, an equilibrium between damage and adaptation is often noted by different authors when several markers of damage or of adaptation are measured in the same organisms. When phanerogams, *P. oceanica,* were exposed to cadmium, MDA levels were decreased by cadmium whereas GST activities were increased. This phenomenon may be interpreted as a protective role of GST against lipid peroxidation (Hamoutène *et al.,* 1996). Khessiba *et al.* (2001) measured MDA levels as well as GST and CAT activities in the mussels *Mytilus galloprovincialis* collected from two areas of the lagoon of Bizerta. The authors hypothesized that both the presence of pollutants but also the origin of the mussels may be the reasons for the differences found in the biomarker response of the animals collected either in the channel of Bizerta (wild mussels) or Menzel Jemil (cultured mussels). In this paper, too, the delicate equilibrium between damage (MDA) and protection or "adaptation" (CAT, GST) was underlined.

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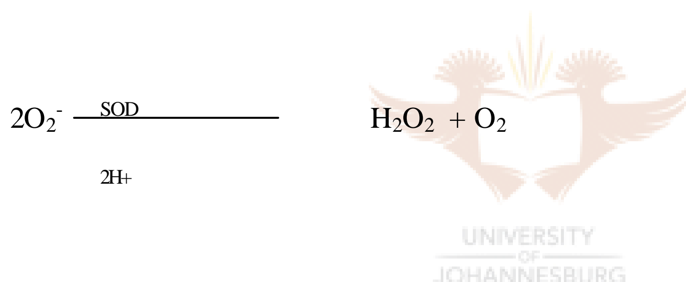
2.3.5. Superoxide dismutase (SOD)

The SODs are a group of metalloenzymes that catalyze the conversion of reactive superoxide anions ($O_2^{-\circ}$) to yield H_2O_2, which is in itself an important ROS as well. Hydrogen peroxide is subsequently detoxified by two types of enzymes; CATs and glutathione-dependent peroxidases (GPOXs). Superoxide dismutases are considered to play a pivotal antioxidant role. Their importance is indicated by their presence in all aerobic organisms examined (Stegeman *et al.*, 1992). The SOD-CAT system provides the first defence against oxygen toxicity. Superoxide dismutase cata lyses the dismutation of the $O_2^{-\circ}$ to water and hydrogen peroxide, which is detoxified by the CAT activity.



$$2O_2^- \xrightarrow[2H+]{SOD} H_2O_2 + O_2$$

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Usually a simultaneous induction response in the activities of SOD and CAT is observed when exposed to pollutants (Dimtrova *et al.*, 1994), even though Pandey *et al.* (2003) state that they observed no such relationship in CAT and SOD in fish in their own study.

2.4. Histopathology

Indicators of stress at several levels of biological organization have been used to evaluate effects of contaminants at organizational level. These approaches vary from measures of genetic integrity to growth and reproductive competence of the individual

(Teh *et al.*, 1996). As far as the individual organism is concerned, manifestations of stress at the tissue level represent an intermediate effect between the biochemical and reproductive levels (Hinton, 1990). Histopathological characteristics of specific organs express condition and represent time-integrated endogenous and exogenous impacts on the organism stemming from alterations at lower levels of biological organization (Chavin, 1973). According to Segner and Braunbeck (1988), histological changes occur earlier than reproductive changes and are more sensitive than growth or reproductive parameters and, as an integrative parameter, provide a better evaluation of organism health than a single biochemical parameter.

Many contaminants can damage specific cell types or organ systems. The feasibility of using histopathological parameters in fish as a biomarker for aquatic pollution has been reviewed by Hinton *et al.* (1992). Epidemiological studies on the occurrence of fish diseases in relation to their usefulness in monitoring marine pollution have been reviewed by Vethaak and Rheinallt (1992). They concluded that on the most convincing examples of a causal relationship between fish disease and pollution was provided by intensive and detailed studies carried out in North America, particularly on liver pathology. It is generally assumed that histopathological biomarkers are valuable as indicators of the general health of fish and mirror the effects of exposure to a variety of anthropogenic pollutants (Hinton *et al.*, 1992). According to Hinton (1994), sufficient information is at hand to assemble cellular and histopathological biomarker approaches and to apply them in integrated field studies. Histopathology is only one component of several measures of fish health, so the changes observed at this level will be related to effects seen at the sub-cellular level.

CHAPTER 3

ACTIVE BIOMONITORING OF THE RIETVLEI WETLAND SYSTEM USING ANTIOXIDANT ENZYMES, NON-ENZYMATIC ANTIOXIDANTS AND HISTOPATHOLOGY AS BIOMARKERS.

3.1 Introduction

The response of biomarkers can be regarded as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (Van der Oost et al., 2003). The most compelling reason for using biomarkers is that they can give information on biological effects to pollutants rather than a mere quantification of their environmental levels. Various biochemical parameters in fish have been tested for their response to toxic substances and tested for their use as biomarkers of exposure and effect (Van der Oost *et al.*, 2003).

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Previous papers have shown that transplanting molluscs from a reference site to a polluted area can be a feasible strategy for biomonitoring the effects of environmental changes in an aquatic system (Romeo *et al.,* 2003). Active biomonitoring is based on the comparison of chemical and/or biological properties of samples which have been collected from one population and which have, after randomisation and translocation, been exposed to different environmental conditions at monitoring sites. Lagadic *et al.* (2000) underlined the interest in measuring several biomarkers at the same time in the same animals, which allows a pertinent approach to evaluate the effects of pollutants on individuals.

Numerous endogenous sources of oxyradical production exist, but of more immediate interest with respect to environmental biomarkers is the ability of a number of structurally diverse compounds to enhance intracellular oxyradical production through the process of redox cycling. Redox active compounds include aromatic diols and quinines, nitroaromatics, aromatic hydroxylamines, bipyridyls and certain transition metal chelates (Winston and Di Guilio, 1991). In the redox cycle, the parent compound is typically first enzymatically reduced by a NADPH-dependent reductase to yield a xenobiotic radical. This radical donates its unshared electron to molecular O_2, yielding $O_2^{-\cdot}$ as well as the parent compound. Thus, at each turn of the cycle, two potentially deleterious events have occurred. First a reductant has been oxidised and, secondly, an oxyradical produced (Winston and Di Giulio, 1991). Oxidant-mediated effects with a potential suitability as biomarkers include either adaptive responses, such as increased activities of antioxidant enzymes and concentrations of non-enzymatic compounds, or manifestations of oxidant-mediated toxicity such as oxidations of proteins, lipids and nucleic acids, as well as perturbed tissue redox status (Filho, 1996).

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Defence systems that tend to inhibit oxyradical formation include antioxidant enzymes such as SOD, CAT and GPx, investigated in this research, which are of critical importance in detoxification of radicals to non-reactive molecules, and the low-molecular weight antioxidant GSH. MDA levels are investigated to determine the extent of (LPOX) due to effect of pollutants. Lipid peroxidation or the oxidation of polyunsaturated fatty acids (PUFA) is a very important consequence of oxidative stress and has been investigated extensively (Stegeman *et al.*, 1992). The process of LPOX proceeds by a chain reaction and as in the case of redox cycling, demonstrates

the ability of a single radical species to propagate a number of deleterious biochemical reactions.

Many contaminants can damage specific cell types or organ systems. The feasibility of using histopathological parameters in fish as a biomarker for aquatic pollution has been reviewed by Hinton *et al*., (1992). Histopathological characteristics of specific organs express condition and represent time-integrated endogenous and exogenous alterations at lower levels of biological organization (Chavin, 1973).

AIMS OF THIS CHAPTER

- To demonstrate the feasibility of a suite of antioxidant enzymes, non-enzymatic antioxidants and histopathology as biomarkers in fish and invertebrates using ABM protocols.
- To use biomarker response to assess anthropogenic stress in the Rietvlei System along the flow gradient and during the high flow and low flow periods.

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3.1. Material and Methods

3.2.1. Study Site

Figure 3.1 shows the sampling points at which cages containing *O. mossambicus* and *M. tuberculata* were transplanted during the high-flow and low-flow periods in the wetland system.

3.2.2 Study Design

a. Sampling

The surveys were carried out during the high-flow period (March, 2003) and low-flow period (August, 2003). The bioindicator species used were the fish *Oreochromis mossambicus* and the mollusk *Melanoides tuberculata*, bred under laboratory conditions. These were transplanted in 20 x 20 x 30 cm cages at three locations down the flow gradient. Within each survey period the organisms were collected after four weeks and six weeks. The fish were only collected once, after four weeks. Control samples were collected from the laboratory in which the transplanted organisms were bred. Physico-chemical water parameters were also measured during each sampling period. The organisms were killed on the day of collection and stored in Henrikson buffer at -80°C.

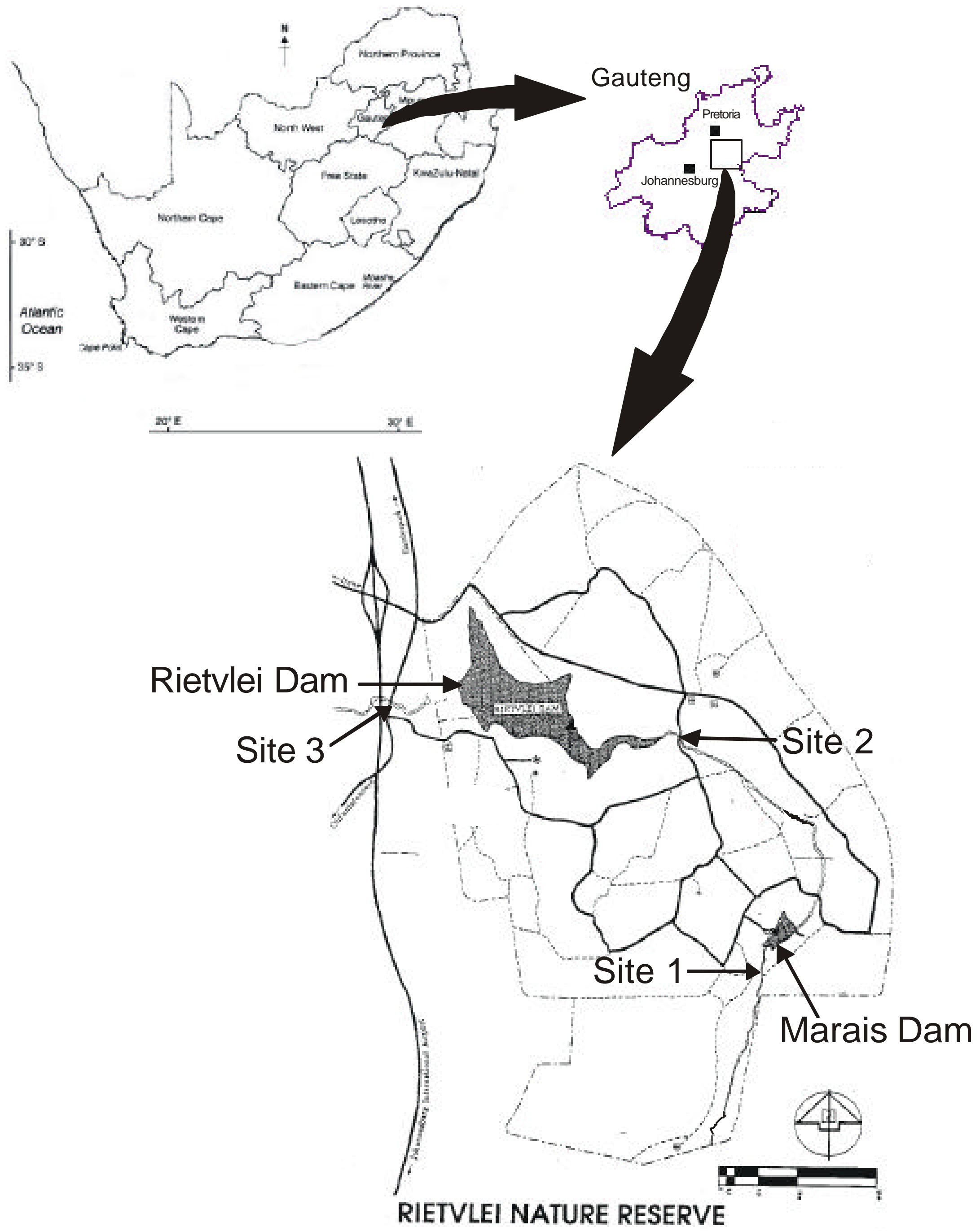

Fig. 3.1. Map of Rietvlei Wetland system indicating sampling sites.

b. Sample preparation

Biomarkers

Biomarkers were tested on the whole organism. Homogenates of fish and de-shelled snails were be prepared in 50 mM Tris buffer containing 0.25 M sucrose at pH 7.4. The homogenate was centrifuged at 500 x g to obtain a cytosolic fraction.

Histopathology

The four gill arches from both sides were removed using a clean blade and placed in individual glass bottles containing 10% phosphate-buffered formalin fixative.

c. Biochemical assays

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Catalase

Catalase activity was determined by the method of (Cohen *et al.*, 1970) using an ELX 800 Universal microplate reader. The tissue homogenate was centrifuged at 10 000 rpm, for 10 minutes at 4°C. An aliquot of cold 4 mM H_2O_2 was added to a volume of supernatant. The reaction was stopped rapidly after 3 minutes using 6 N H_2SO4. An aliquot of 0.01 N $KMnO_4$ was added and absorbance read at 492 nm within 30-60 seconds. Protein content was determined using method of Bradford (1976), and catalase activity was expressed as µmol H_2O_2 mg protein^{-1} .min.

Glutathione peroxidase

Glutahione peroxidase activity was determined based on the indirect coupled procedure by Flohe and Gunzler (1984). The assay mixture containing test sample, 2.4 Units ml^{-1} glutathione reductase, 10 mM GSH, 100 mM Azide and 0.1 M potassium phosphate buffer with 1Mm EDTA was preincubated at 37℃ for 10 minutes. To determine hydroperoxide independent oxidation, 1.5 mM NADPH was added and the decrease in absorbance at 340nm measured using a Hitachi 150-20 Spectrophotometer. The overall reaction was determined after addition of 4 mM hydrogen peroxide and following the H_2O_2 dependent oxidation at 340 nm for 5minutes. The interfering effect of NADPH consuming factors different from glutathione peroxidase in the enzyme was subtracted from the overall reaction. The results were expressed as nmol NADPH oxidized min^{-1} g^{-1} of proteins, using the Molar extinction coefficient of 6.22 x 10^3 M cm^{-1}.

Glutathione

Activity was determined by the fluorimetric method of Cohn and Lyle (1966). The tissue was homogenized in ice-cold versene, with 25% phosphoric acid to precipitate proteins, and left to stand for 10 minutes. It was centrifuged at 3000rpm for 10 minutes. The reaction mixture consisted of supernatant, 0.1M sodium-phosphate buffer and 0.1% O-pthalaldehyde, and was left to stand in the dark at room temperature for 15 minutes. Fluorescence was measured at 460 nm resulting from excitation at 355 nm. The results were expressed as µg GSH of consumed g^{-1} wet tissue.

Malondialdehyde

The tissue was homogenized in 5% trichloroacetic acid and methanolic butylated hydroxytoluene and heated for 30 minutes in a capped tube at 90°C. The tube was cooled to room temperature and centrifuged at 3000rpm for 10 minute. The supernatant was added to thiobarbituric acid and heated at 90°C for 10 minutes. Absorbance was measured at 533 nm using a Hitachi 150-20 Spectrophotometer. Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS). These were considered as "MDA-like" products and were quantified by reference to MDA absorbance Molar extinction coefficient of 156 x 10^3 $M^{-1}cm^{-1}$. The results were expressed as nmoles of MDA per gram of wet tissue.

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Superoxide dismutase

Activity was assayed according to the method described by Misra (1989) using a Hitachi 150-20 Spectrophotometer. The analysis of SOD activity is complicated by the ephemeral nature of the substrate (O_2^-), which even in the absence of SOD will rapidly react with itself and dismutate. Hence an indirect technique was used whereby a source of O_2^- and an indicating scavenger of O_2^- are added to the tissue being assayed for SOD activity. First a rate of 0.025 absorbance units (AU) min^{-1} at 480 nm due to epinephrine autoxidation was obtained by adjusting the volumes of 0.01M epinephrine and 200 µM adrenochrome in 50 mM $NaHCO_3$-Na_2CO_3 buffer with 0.1 mM EDTA. Different volumes of sample to be assayed for SOD activity were substituted for equal volumes of buffer to determine the amount that caused 50%

inhibition of epinephrine autoxidation at 480 nm. The SOD activity was expressed as μg/ml of SOD protein required to cause 50% inhibition of epinephrine autoxidation.

Protein

Protein concentration in samples was determined using the Bradford Method (Bradford, 1976) on the ELX 800 Universal microplate reader.

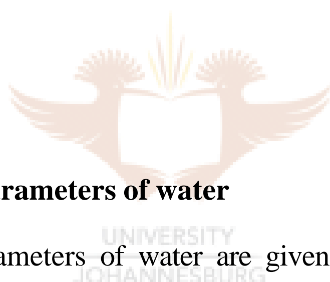
d. Histopathology

The fixed gill tissues were dehydrated through ascending grades of alcohol. Before infiltrating with wax all water was removed from the fixed tissue, starting with 30% ethanol, to prevent distortion caused by direct transfer to absolute alcohol. The final transfer to absolute alcohol was for complete dehydration. Clearing was done in xylene to render the tissue transparent. The tissue was then impregnated in wax at 60°C. Blocking was done by pouring molten wax into metal moulds smeared with glycerine. The tissue was transferred into the wax using heated forceps and embedded in a transverse orientation. The blocks were kept in the cold overnight to harden then cut into 5μm thick sections. The sections were mounted on glass slides. Before staining the sections were completely de-waxed by placing in two changes of xylene each for 5-10 minutes durations. They were then transferred to water by first removing the xylene in absolute alcohol for 2-5 minutes and passing through the series of descending grades of alcohol. The sections were then washed with tap water and stained with Mayer's haematoxylin and eosin (H & E).

e. Statistical analyses

The graphical presentations were performed using the GraphPad Prism Programme and the data reported as mean +S.E. The variations in each biomarker were tested by one-way analysis of variance (ANOVA), considering site and flow regime as variables. Data were tested for normality and homogeneity of variance using Kolmogorov-Smirnoff and Levene's tests, respectively. When the ANOVA revealed significant diferences, post-hoc multiple comparisons between sites, and between flow periods, were made using Scheffé test to determine which values differed significantly. The significance of results was ascertained at $P<0.05$.

3.3 Results



3.3.1. Physico-chemical parameters of water

Some physico-chemical parameters of water are given in Table 3.1. The low-flow period was generally characterized by higher temperatures than the high-flow, and in an increase in total dissolved solids, conductivity and oxygen saturation.

Table 3.1. Physico-chemical analysis of water at the three sampling sites in the Rietvlei Wetland system.

Site	Flow regime	% Oxygen saturation	pH	Redox Potential (Mv)	Conductivity (MS)	TDS (ppm)	Temperature (^{o}C)
1	High flow	83.2	7.46	-61.3	345	153	12.6
1	Low flow	86.7	7.74	-77.9	729	351	15.2
2	High flow	65.5	7.54	-68.8	671	341	12.7
2	Low flow	72.7	7.94	-89	681	345	17.9
3	High flow	58.3	7.62	-70.3	535	263	15.5
3	Low flow	88	8.16	-100.9	580	279	19.2

3.3.2. Biomarker response during ABM

In Figure 3.2A, CAT activity recorded at sites 2 and 3 was significantly lower than the control. At site 1 the activity recorded was significantly higher than the control. The graph shows that the CAT activities between site 2 and 3 did not differ significantly. The NADPH activities at all three sites, in Figure 3.2B, were significantly higher than the control, but did not differ significantly from one other at all three sites. In Figure 3.2C, high levels of GSH were recorded, but were not significantly higher than the control, except for site 3. There was also no significant variation between sites 1 and 2. Figure 3.2D shows MDA levels significantly higher than the control from all three sites, but they did not differ significantly from each other. In Figure 3.2E, there was no statistically significant variation in SOD activity between sites 1 and 2, but the activity in the control was significantly higher than that at all three sites. Site 3 recorded a higher activity than site 1 and 2.

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In Figure 3.3A, no CAT results were recorded for site 2 and for site 1 week six due to sample shortage, as many had suffered mortality. During week four at site 1 and site 3 CAT activities recorded were significantly lower than the control. CAT activity at site 3 during week six was significantly higher than the control.

Figure 3.3B shows that the GSH levels in week four and week six exposures were significantly higher than the control at all three sites. During week four GSH levels numerically increased down the flow gradient from site 1 to site 3, but the difference was not statistically significant. There was no significant difference in the week six levels at sites 1 and 2. There are no results for site 3 week six because the molluscs died.

The MDA levels, shown in figure 3.3C, in week four and week six exposures were significantly higher than the control at all three sites. There were no significant variations among the exposed molluscs at the different sites, except for the levels at site 1, during week four, which were significantly lower than the rest.

In Figure 3.4A CAT activities recorded during the high flow periods at sites 1 and 3 were significantly lower than the control but did not differ significantly from one another. The low flow values at sites 1 and 3 did not differ significantly from each other or the control. At site 1 and site 3, CAT activities during the low flow period were significantly higher than the high flow activities. In Figure 3.4B, the GSH levels recorded at all sites during the low flow period did not differ significantly from each other or the control. The levels at the three sites during the high flow period were significantly higher than the control. At all three sites, high flow GSH levels were significantly higher than low-flow levels. In Figure 3.4C, MDA levels in both flow regimes were significantly higher than control. At site 1 and 3, low flow levels were higher than high flow.

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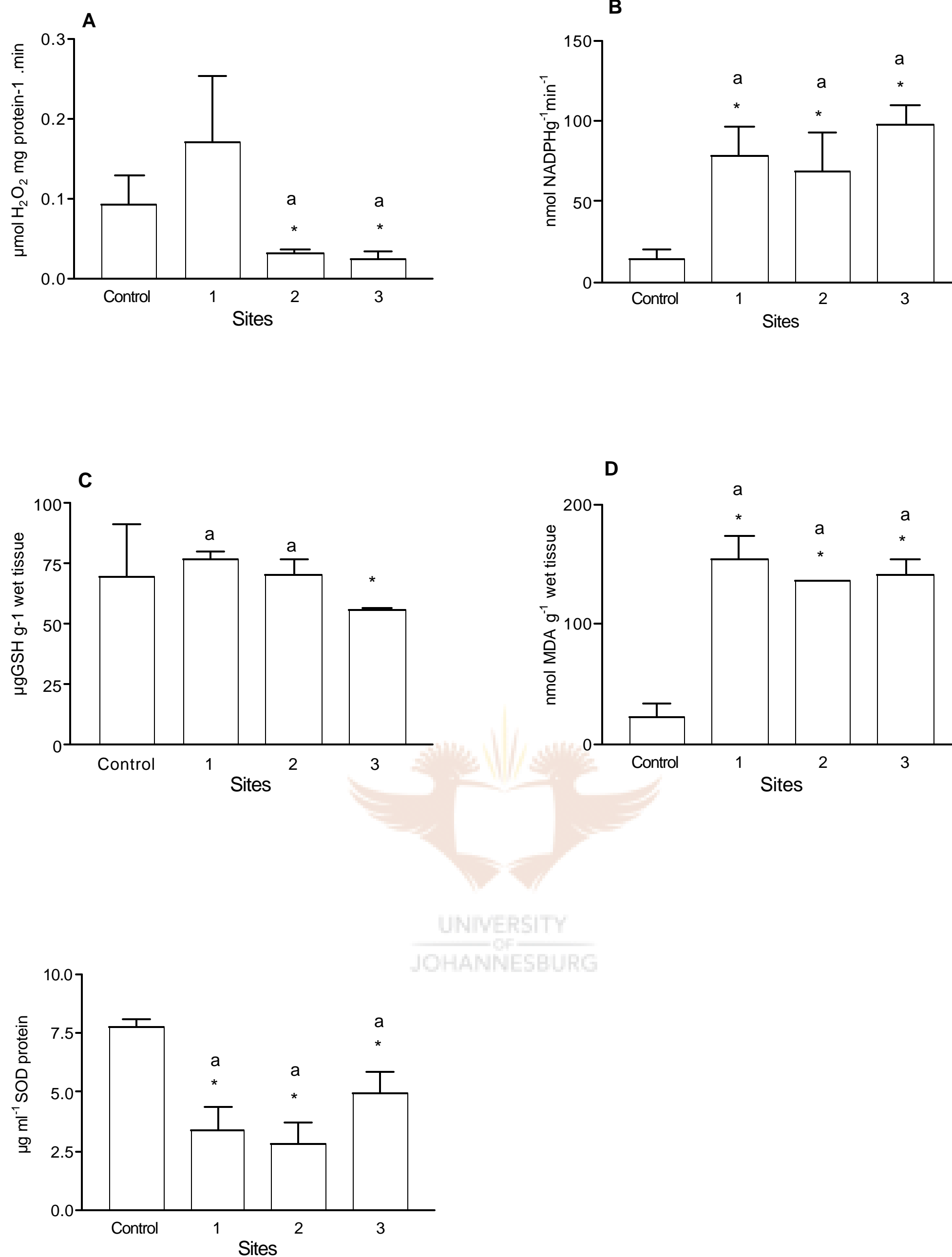

Figure 3.2. The responses of *O. mossammbicus* at three different sites in the Rietvlei Wetland system following a four-week exposure period during high flow conditions (March 2003). Data for A: CAT, B: GPx, C: GSH, D: MDA and E: SOD, are presented as means+ SE. Asterisks indicate exposure groups that differ significantly from the laboratory control groups ($P<0.05$). Sites with common letters do not differ significantly from each other.

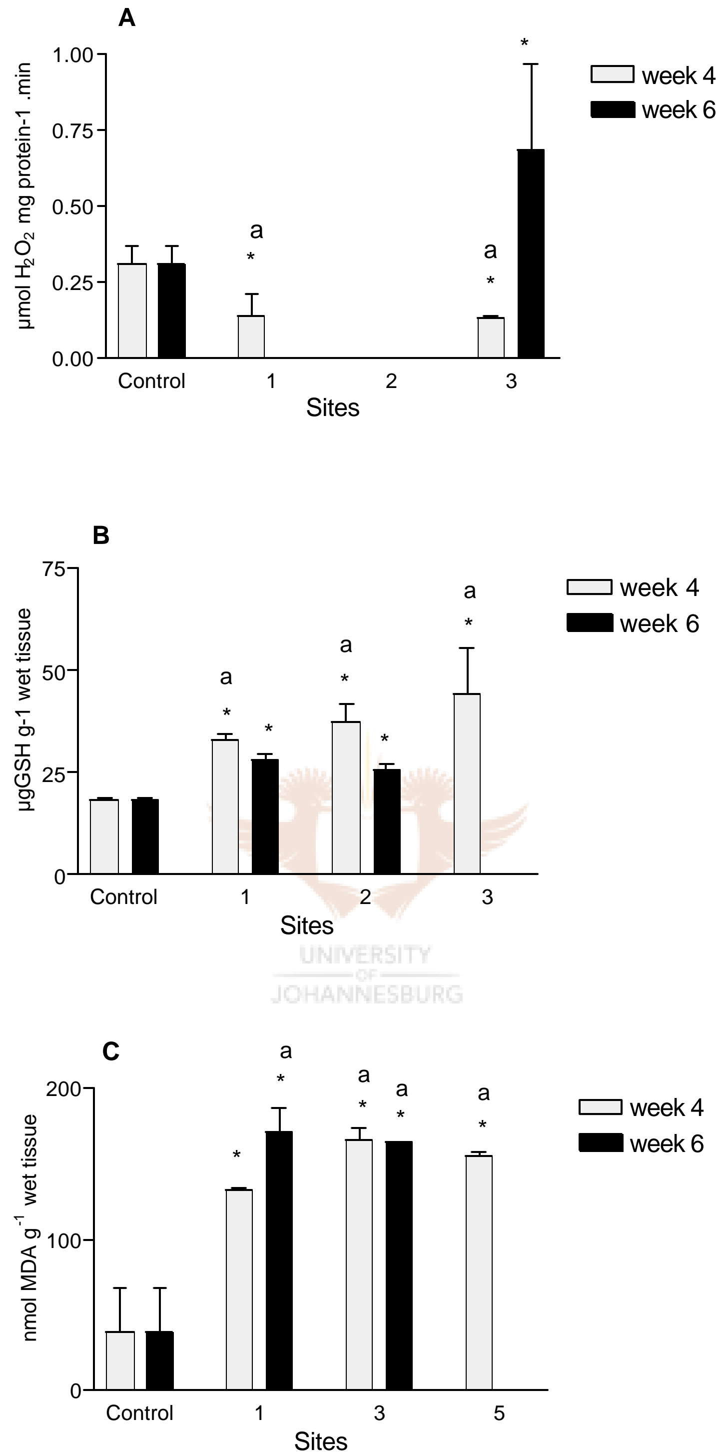

Figure 3.3. Biomarker responses of *M. tuberculata* at three different sites in the Rietvlei Wetland System following a four and six-week exposure period during high-flow conditions (March 2003). Data from A CAT, B: GSH, C: MDA are represented as means +SE. Asterisks indicate exposure groups that differ significantly from the laboratory control groups ($P<0.05$). Within exposure periods, sites with common letters do not differ significantly.

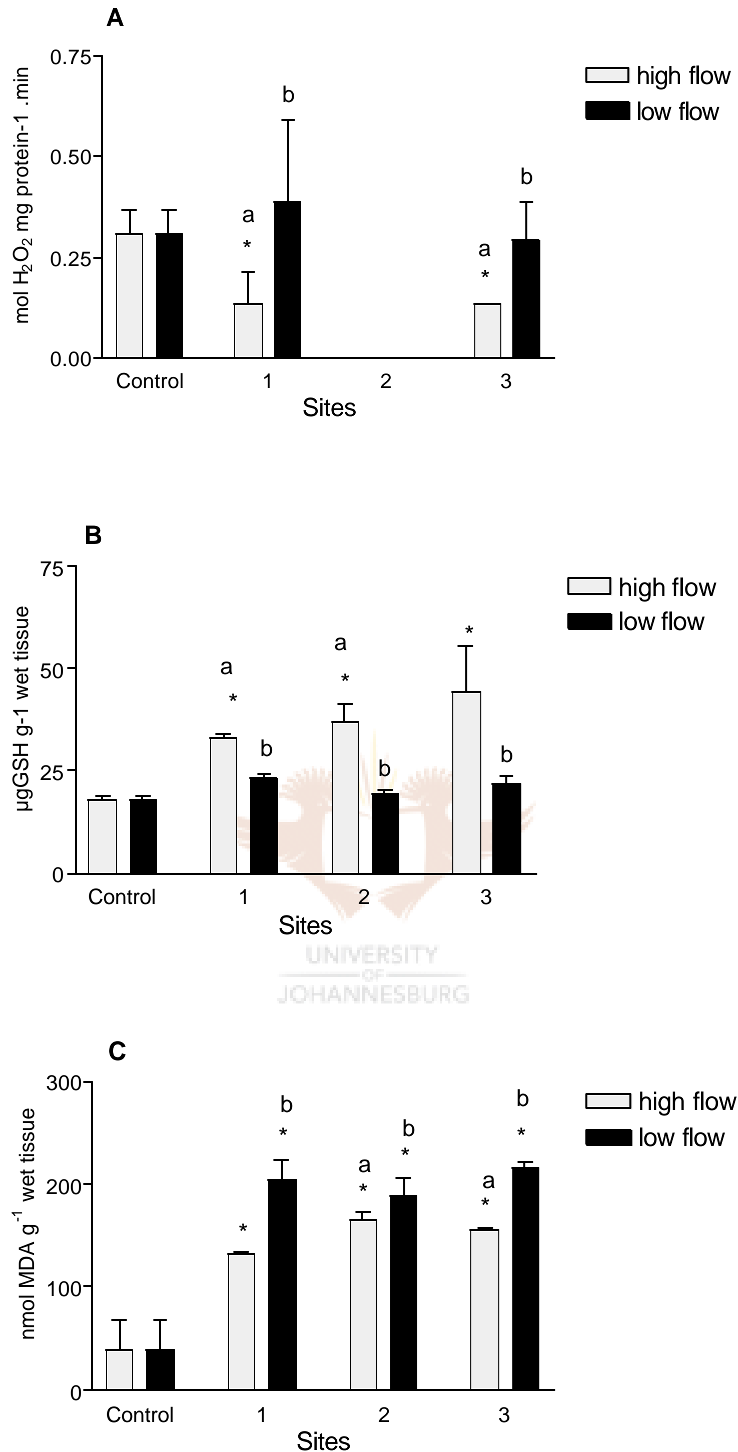

Figure 3.4. Biomarker responses of *M. tuberculata* at three different sites in the Rietvlei wetland system following a four week exposure period during high-flow (March 2003) and low-flow (August 2003) conditions. Data for a: CAT, B: GSH, C: MDA, are presented as means +SE. Asterisks indicate exposure groups that differ significantly from the laboratory control groups ($P<0.05$). Sites within flow conditions with similar letters do not differ significantly from one another.

3.3.3. Histopathology

Figure 3.5B highlights epithelial lifting and curling of the lamella in the fish from site 1. Figure 3.5C and 3.5D show considerable hyperplasia and epithelial lifting compared to the control gill in figure 3.5A.

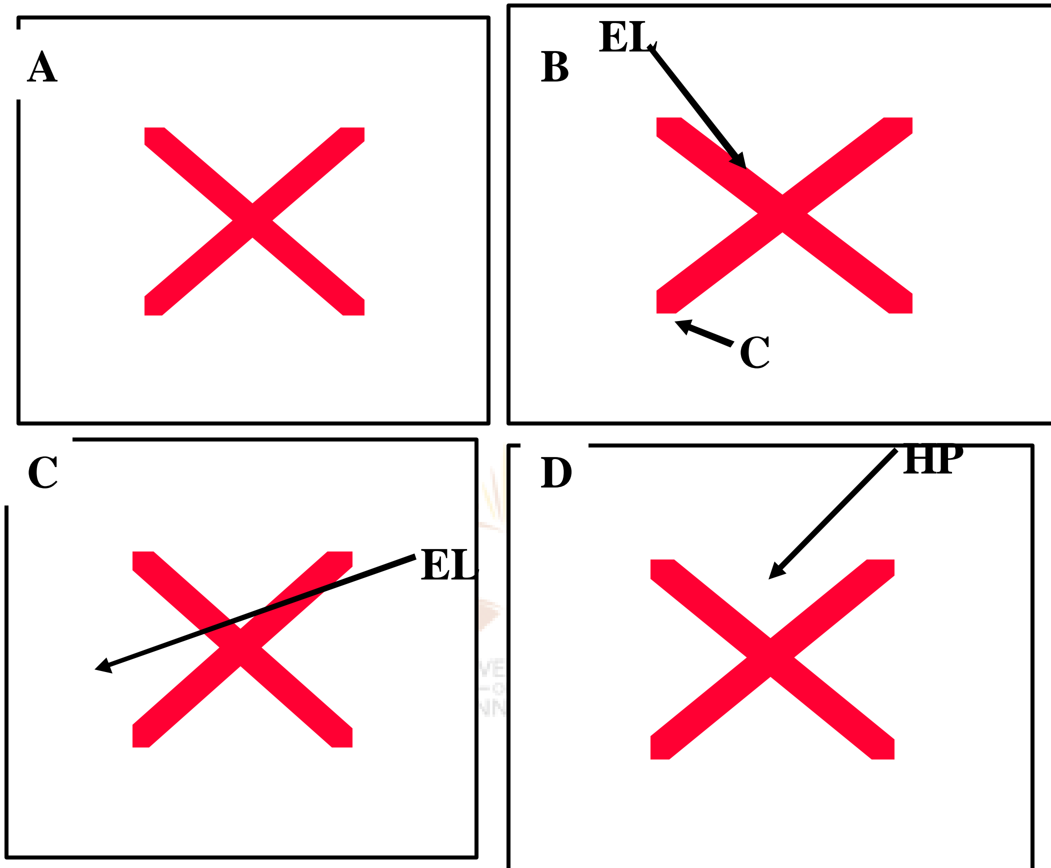

Figure 3.5. Histology of gill filaments. A represents a normal gill filament, sagittal section through venous sinus (Formalin, H&E, 100x). 1. primary lamella; 2. secondary lamella; 3. epithelial cell;4. mucous cell; 5. pillar cell; 6. lacuna (capillary lumen); 7. erythrocyte within capillary lumen; 8. undifferentiated basal cell; 9. central venous sinus
B represents a sagittal section (Formalin H&E, 100x) of gill filaments of *O. mossambicus* following four weeks *in situ* exposure at site 1. C represents site 3, and d represents site 5. EL = epithelial lifting, HP = hyperplasia, C = curling of the lamellae

3.4 Discussion

Effects of pollutants on ecosystems and communities originate in effects on individual organisms and ultimately, all effects of pollutants are the result of the interaction between a foreign chemical and one or several biomolecules in an individual. This interaction may lead to a disturbance in the cell function, which in turn, may be important enough to alter the function of the organ (Hogstrand 2000).

In biological systems, several processes involving environmental pollutants form ROS. Thus organisms inhabiting polluted aquatic environments are exposed to a variety of oxyradicals, leading to oxidative damage of different biomolecules such as lipids or proteins. Oxidative damage reflects an imbalance between the production of oxidants and scavenging or removal of those oxidants. The intensity of oxidative damage suffered by an organism depends on the fine balance among its individual antioxidant enzymes (Lenártová *et al.,* 1997).

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The fish transplanted during the low-flow period suffered 100% mortality on two occasions. There was also 100% mortality in molluscs collected after six weeks during low-flow. This may have been caused by a sudden pollution episode during the exposure period since the Rietvlei system receives effluent discharged from sewage waste, industrial, agricultural and domestic activities and in the surrounding area (Barnhoorn *et al.*, 2003).

The highest CAT activity in fish was recorded at site 1. Values recorded at sites 2 and 3 were significantly lower than for the control organisms, suggesting that there was

enzyme inhibition. It has been suggested that superoxide anions and nitrites have an inhibitory effect on CAT activity *in-vivo* (Arrillo and Melodia, 1991). The spatial comparisons therefore suggest an increase in the concentration of substances that caused CAT inhibition, down the flow gradient in the high flow period. The activities recorded for *M. tuberculata* also suggests the presence of factors causing enzyme inhibition during the high flow period, and none during the low flow. Between sites, variation in CAT activity did not exhibit any specific trend for *M. tuberculata.* The high activity observed at site 3 during the high flow period represents an anomaly in the pattern shown by the organisms at all other sites, where activity was either inhibited or remained the same as in the control organisms.

There was significant GPx induction in *O. mossambicus* at the three sampling sites, and this supports the utility of GPx as a biomarker of oxidative stress in fish. There was no significant spatial variation in GPx activity between the three sampling sites. Increased GPx activities have been observed in experiments with fish exposed to paraquat, PAH (3MC), PCB's and HCB-contaminated food (van der Oost *et al.,* 2003). Therefore the significant induction of the enzyme in the fish in Rietvlei could be indicative of the presence of chemicals of this nature. However no GPx activity was recorded in *M. tuberculata* and this agrees with the suggestion by Torres *et al.* (2002) that, based on their findings, GPx may not be a suitable biochemical marker of oxidative stress in molluscs.

Increased fluxes of oxyradicals can impose a drain on intracellular reducing equivalents with potentially profound consequences on a variety of metabolic processes. The consumption of GSH due to direct scavenging of oxyradicals or as a

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cofactor for GPX activity may represent such a drain (Di Giulio *et al.,* 1995). Levels of GSH in fish were not significantly higher than the control, and there were no significant variations between the sites, except for site 3 where slightly lower levels were recorded. The highest levels were recorded at site 3 during the high flow period. In *M. tuberculata,* there was evidence of GSH induction in the high flow period as the levels recorded at all sites differed significantly from each other and the control and were significantly higher than the low flow levels. Strong increases (>500% of control) in hepatic GSH levels have been reported in English sole from a site heavily polluted with PAH's and PCB's (Stein *et al.,* 1992).

In *O. mossambicus* MDA levels were significantly higher than the control, from all three sites, but showed no spatial variation. Temporal comparisons were done with *M. tuberculata*, where MDA levels were significantly higher in the low-flow period than the high flow period. The reduction in water volume during the low-flow may have resulted in higher concentration of chemicals that induce oxidative stress leading to lipid peroxidation. A general increase in TBARS has been observed with increase in water temperature (Pellerin-Massicote, 1997), so the increased temperature during the low-flow period (see Table 3.1) may have contributed to the higher MDA levels. In aquatic ecosystems dissolved oxygen and temperature are environmental variables that are likely to influence oxidative processes (Parihar *et al*., 1997). Elevated MDA levels have also previously been reported in mussels after only one week exposure to copper (Lenártová *et a*l., 1997). Hence the extent of lipid peroxidation in this study may also be attributed to possible presence of heavy metals discharged into the system.

There was no statistically significant variation in SOD activity between the three sites, but the activity in the control was significantly higher than that at all three sites. The SODs are highly inducible enzymes, and this feature is the basis for their utility as biomarkers. However, due to the high SOD activity in the control organisms compared to the exposed ones in this study, there is no evidence of SOD induction in the fish. These results agree with studies done by Lenártová *et al.* (1997) who indicated that fish exposed to oxidative stress (due to metals) exhibit higher GPx activities and MDA levels and lower SOD and CAT activities. Maintenance of normal SOD activity has also been reported by Torres *et al.* (2002) from their studies on *Mytella*.

By all indications, the application of ABM using biomarkers clearly showed that the test organisms were subjected to antioxidant stressors in the Rietvlei System. To ascertain the ecological significance of these stress responses, they can be further elucidated using traditional biomonitoring techniques such as invertebrate and/or fish community responses, at the different sites.

Multiple stressors in the freshwater environment can alter structural integrity of critical tissues and organs (Teh *et al*., 1996). There were visible structural alterations and lesions of the secondary lamellae in fish collected from all three sites. Such kinds of alterations have previously been attributed to the presence of high levels of unionised ammonia (NH_3) (Arrillo *et al*., 1984). Sublethal concentrations of nitrite have also been reported to induce gill damage (Arrillo *et al*., 1984). Uptake of nitrite is mediated over the chloride cells in the gill epithelium (Mallatt, 1985) and since the system receives sewage effluent it could mean that the concentrations of nitrogenous

compounds are above the threshold at which the fish are able to withstand. Consideration is also given to such environmental stressors such as pathogens, parasites, varying temperature and flow regimes and altered food and habitat availability. The present results are not conclusively indicative of direct effect of pollutants on fish health. According to Mallatt (1985) toxicant-induced gill alterations do not reflect specific actions of irritants as much as they seem to reflect physiological responses in fish.

The gill is probably the organ that is most sensitive to toxicants in fish (Hogstrand, 2000). Since respiratory gases must pass through the lamellar epithelium by diffusion alone, this surface is quite delicate compared with the rest of the surface of the fish (Mckim and Goeden, 1982). The fish gill is a very complex organ as its function is not limited to oxygen uptake and carbon dioxide excretion. The gills are also important for the water and salt balance in the body and for the excretion of nitrogenous waste, which in most cases is ammonia (Hogstrand, 2000). A typical chronology of gill damage from acute exposure to a test chemical is first a lifting of the outer layer of the lamellar epithelium (desquamation), usually starting in the area of the chloride cells. Edematous spaces are formed between the layers of epithelium and these may become infiltrated with leucocytes. Eventually the whole epithelium sluffs off and the lamella loses rigidity (Heath, 1987). There was evidence of mild morphological alterations attributed to poor water quality, including curling and twisting of the secondary lamellae in fish from all three sites.

Observed in figure 3.5A is one of the most commonly recorded alterations in gill morphology: the lifting of the epithelial cells from the gill lamellae and the inter-

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lamellae zones of the gill filament, representing an infiltration of this epithelium with fluid (Skidmore and Tovell, 1972). Figure 3.5D shows signs of lifting, swelling and hyperplasia of the epithelium. These alterations serve a defence function, as they increase the distance across which waterborne irritants must diffuse to reach the bloodstream. The resultant lamellae fusion, indicated in Figure 3.5D could be protective in that it diminishes the amount of vulnerable gill surface area. Branchial responses that serve to slow entry of toxicant have the undesirable effect of threatening to suffocate the fish (Skidmore, 1964; Burton *et al.*, 1972). This may have been the course of fish deaths in the low flow period if toxicant concentrations had increased significantly. The kind of structural damage observed in the fish exposed at all three sites is more or less a stereotyped physiological response to environmental stressors. Even non-chemical stressors, such as hypoxia (low oxygen in the water) can elicit a similar response (Hogstand, 2000). One potential problem often encountered in studies of histopathology of gill tissue of freshwater fish following exposure to pollutant is susceptibility of the fish to bacterial gill disease. This disease is frequently brought on by environmental stress and is characterized by proliferation of the gill epithelium and clubbing and fusing of the lamellae (Heath, 1987). However since the responses from the biomarkers of oxidative stress suggest the presence of high concentrations of toxic chemicals, the gill alterations observed can also be largely attributed to the toxic effect of these chemicals.

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3.5 Conclusion

Based on the findings of this study, the use of biomarkers of oxidative stress is a good indicator of pollution levels in water bodies. Fish and molluscs can be easily and efficiently used in ABM of water quality and to quantify in-stream responses to pollution. For reproducible results sample size should be high. The exposure period of four weeks also seems the appropriate, as mortalities seem to increase after this period if the organisms are severely stressed. It is possible to correlate pollution levels to induction of antioxidant enzymes. It is also useful to measure several biomarkers in a single organism. There are so many natural variables that can mask the effects of pollutants that it is very difficult to impossible to attribute a change in an ecosystem to a single chemical or even an effluent. (Hogstrand, 2000). The results of this study suggest that the Rietvlei wetland system is under significant anthropogenic influence. The study demonstrated that the level of contamination is enough to inflict oxidative stress a well as cellular damage. Histological alterations observed are suggestive of multiple environmental stressors on the system.

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CHAPTER 4

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