THE USE OF GENOTOXIC AND STRESS PROTEINS IN THE ACTIVE BIOMONITORING OF THE RIETVLEI SYSTEM, SOUTH AFRICA

by

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MINI-DISSERTATION

Submitted in partial fulfilment
of the requirements for the degree

MAGISTER SCIENTIAE

in

AQUATIC HEALTH

in the

FACULTY OF SCIENCE

at the

RAND AFRIKAANS UNIVERSITY

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DECEMBER 2003

ABSTRACT

The use of biomarkers in assessing the health of aquatic ecosystems is widely being used. Biomarkers have been proposed as sensitive early warning tools for biological measurement in environmental quality assessment. In this study, DNA damage, HSP 70 expression, Lactate dehydrogenase and alkaline phosphatase were evaluated for their usefulness and applicability in the active biomonitoring of the Rietvlei system using *Melanoides tuberculata* and *Oreochromis mossambicuss* as test organisms. Levels of DNA strand breakage were measured in the exposed test organisms and the references that were kept in the laboratory under unpolluted environment. Different levels of DNA damage were recorded in *M tuberculata* that were exposed during high-flow and those from the low-flow exposure period. There was no difference in DNA between the control and high-flow exposure *M. tuberculata* but the low-flow exposure duration had significantly higher DNA damage for all the three sites when compared to the high-flow and the controls. There was no difference in the amount of DNA damage in both exposed and control *O. mossambicus*. Fish samples were only available for the high-flow four weeks exposure because of the test organisms dying during the low-flow exposure. Heat shock protein 70 expression was determined in *O. mossambious* only and significant differences in expression between sites were demonstrated with highest expression at site 1 decreasing down stream at sites 3 and 5. The activity of LDH in *M. tuberculata* was not different in both control and exposed fish at all the sites. There was no difference in the activity of LDH between the high-flow and low-flow exposure groups in *M. tuberculata*. In *O. mossambicus*, the activity of LDH was significantly inhibited at site 1 with the effects of the contaminants decreasing downstream at sites 3 and 5 where LDH activity did not differ from the controls. There was no alkaline phosphatase activity in both exposed and control *M. tuberculata* but in *O. mossambicus* alkaline phosphatase was

significantly inhibited at the three sites although inhibition did not differ from site to site. This suggests that LDH and alkaline phosphate are not sensitive biomarkers in *M. tuberculata* in the Rietvlei system but are sensitive in *O. mossambicus*. DNA damage was shown to be a sensitive biomarker using *M. tuberculata* but it was difficult to assess this in *O. mossambicus* since fish were only available during the 4 weeks high-flow exposure, which did not show any differences from the control. The lack of DNA damage in *O. mossambicus* maybe a result of low concentration of contaminants during high-flow or because this biomarker is not sensitive in fish.

ACKNOWLEDGEMENTS

I want to thank the following people and institutions:

- My supervisor, Prof J. H. J. van Vuren for his support throughout this study.
- My co-supervisor Dr V. Wepener for his guidance and support during the fieldwork and writing of this mini-dissertation.
- The Rand Afrikaans University and the Water Research Fund for Southern Africa (WARFSA) for financial support during this study.
- My husband, Obert, who had to endure the whole year without me and gave me all the pressure to finish my study.
- My mentors (Byron and Liesel) who helped me with my protocols and use of laboratory equipment.

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CHAPTER 1

GENERAL INTRODUCTION

The release of natural or synthetic substances is one of the important factors in the degradation of the biosphere by human activities (Amiard *et al.*, 1998). Presently, there are no ecosystems free from traces of human activity because even areas unknown to human habitation are contaminated by pollutants carried by movements of air masses or by marine and oceanic currents. It has become clear that in the long term the health of human populations depends to a larger extent on the quality of their environment (Amiard *et al.*, 1998).

Ecotoxicology aims to assess the impacts of chemicals on ecological systems. Assessment in the past used to rely heavily on mortality-based acute tests but it has been shown that a chemical that does not cause death within the duration time may still have long- term deleterious impacts on the test organisms (Wo *et al.*, 1999). Risk assessment cannot be solely based on chemical analysis of environmental samples because this approach does not provide any indication of deleterious effects of contaminants on the biota (De Coen, 1999) hence the use of organisms in the biomonitoring of aquatic ecosystems complements the interpretation of the physical and chemical measurement of water quality. Chemical monitoring of environmental health is based towards the momentary conditions that exist at the time of sample collection. This procedure often misses the short-term events that may be critical to ecosystem health (Roux, 1993). The use of biomarkers has been recently proposed to evaluate the effects of pollutants, such as heavy metals and organic xenobiotics compounds (Variengo *et al.*, 1997). These contaminants can produce alterations of biochemical and physiological processes that can be quantified by estimating

biological parameters (often reported as biomarkers). The biological approach is now being used to complement chemical methods. Biological methods are based on qualitative and quantitative observations of living organisms in their natural environment (Smolders *et al*, 2003). The most popular biological method used is biomonitoring, which can be either active or passive. Active biomonitoring is defined by Smolders *et al.* (2003) 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. Passive biomonitoring is the collection of organisms from their natural environment or habitat at sites where a natural population exists (Smolders *et al.*, 2003)

Advantages of using active biomonitoring (Smolders *et al.*, 2003)

1. Limited impact of field exposure.
2. Experiments can be performed for a known exposure period
3. Easy to compare different sites even if the organism are normally not present at the exposure locations
4. Comparison between transplanted and indigenous organisms can indicate to what extend the indigenous organisms are adapted to the location.
5. Resolution power is optimised by employing statistically similar groups of organisms for defining stress at different locations.

Biochemical, physiological and histological changes have the potential to act as integrative measures at suborganismal level to indicate adverse conditions antecedent to population level effects (Amiard *et al.*, 1998). The use of biomarkers measured at molecular or cellular level have been proposed as sensitive early warning tools for

biological measurement in environmental quality assessment (McCarthy and Shugart, 1990). A biomarker is a biological change, which can be observed or measured at molecular, biochemical, cellular, physiological or behavioural level that reveal its present or past exposure to at least one polluting chemical substance. Experiments under controlled conditions have widely demonstrated that biomarkers can be used to evaluate the biological responses following exposure of individuals to xenobiotics (Amiard *et al.*, 1998). Biomarkers present an integrated evaluation in time and in space of bioavailable pollutants, not only in terms of presence, but also in relation to the effects of these products on animal, plant and microbial populations.

Motivation

Increasing concern has arisen about the presence of genotoxic and potentially carcinogenic xenobiotics in the environment (De Coen, 1999). Although monitoring programmes have been set up to screen for the presence and effects of pollution in the environment, genotoxic effects are generally overlooked. Risk assessment cannot be solely based on chemical analysis of environmental samples because this approach does not provide any indication of deleterious effects of contaminants on the biota. The Rietvlei system is an important source of water for the Rietvlei Nature Reserve game and provides 15% of Pretoria's water requirements. Water in the Reserve receives effluent from industries, agricultural activities, informal settlements and municipal sewage treatment plants (Barnhoorn *et al.*, 2003). Information on the presence of genotoxic contaminants from these sources is important for the health of the ecosystem and the subsequent users of the water. The lack of this information led to the development of an active biomonitoring programme for the system using genotoxic and stress proteins as biomarkers. The quantification of DNA damage

provides the most useful method for measuring genotoxic effects in aquatic organisms. The induction of HSP 70 has been shown to provide cells with stress tolerance to subsequent insults, thus the expression of HSP 70 is indicative of cellular changes, in particular the effects of the stressor on the protein related machinery (Vijayan *et al.*, 1998). HSP 70 expression was used as a biomarker for environmental monitoring since handling stress does not induce them.

Hypothesis

The research hypothesis for this study was, genotoxic and stress proteins could be used effectively as biomarkers in the active biomonitoring of the Rietvlei system using *Melanoides tuberculata* and *Oreochromis mossambicus*.

Main objective

The main objective of this study was to determine if genotoxic and stress proteins could be used as biomarkers in the active biomonitoring of the Rietvlei system

Specific objectives

1. To use DNA damage as a biomarker in the Rietvlei system.
2. To use HSP 70 expression as a biomarker in the active biomonitoring of the Rietvlei system.
3. To use metabolic enzymes (lactate dehydrogenase and alkaline phosphatase) as biomarkers in the active biomonitoring of the Rietvlei system.

CHAPTER 2

LITERATURE REVIEW

Biochemical, physiological and histological changes have the potential to act as integrative measures at suborganismal level to indicate adverse conditions antecedent to population level effects (Amiard *et al.*, 1998). The use of biomarkers measured at molecular or cellular level has been proposed as sensitive early warning tools for biological measurement in environmental quality assessment (McCarthy and Shugart, 1990).

Advantages of using biomarkers in pollution monitoring (Livingstone, 1993)

1. A temporary and spatial integrated measure of bioavailable pollutants are provided by biomarkers.
2. Some biomarkers show very specific responses and through this attribute exposure and risk to environmental pollutants.
3. By applying different biomarkers to species from different habitats and different trophic habitats, they help establishing the importance of different routes of exposure.
4. They can provide information on the relative toxicities of specific chemicals and effluents.
5. Biomarkers are applicable in the laboratory as well as in the field.

Biomarkers can detect effects over the entire continuum therefore they are good measures for earlier and more sensitive detection of toxicity in animal bioassays.

DNA damage

Initial studies suggesting genotoxic impact of pollution on biota originated from analysis of neoplasms and tumors in fish (Stich and Acton, 1976) and increasing number of studies have indicated a growing occurrence of tumors and neoplas diseases in fish as a result of chemical exposure (Couch and Harshbarger, 1985). Black (1983) gave clear evidence of the genotoxic nature of certain types of environmental contaminants through experiments in which organisms were exposed to polluted sediments resulting in neoplasm and tumor formation. Because of the ubiquity of genotoxic agents in the environment, biomarker assays to detect genotoxicity are being developed (Black *et al.*, 1996). The quantification of DNA damage provides the most useful method for measuring genotoxic effects in aquatic organisms. Induction of DNA damage leads to the onset of DNA repair systems but despite the activation of these repair mechanisms, the DNA interaction and damage can persist as a result of various physical interactions and chemical reactions (De Coen, 1999). Interaction between a genotoxic compound and the DNA molecule is highly complex and in general leads to a cascade of effects (Shugart, 1990). Some compounds react directly while others cause damage indirectly or only after metabolism and these interactions give different types of damage to the DNA molecule such as adduct formation, strand breaks, intra/inter strand links and calation (De Coen, 1999). Radionuclides can damage DNA directly and hydrogen sulphides react spontaneously with oxygen to produce free radicals, which in turn is capable of inflicting damage on DNA (Hartwig, 1998). Reactive oxygen species (ROS) have been shown to have a central role in the creation of DNA damage and these species can be produced in large quantities during toxicant induced interactions (De Coen, 1999).

Various methodologies have been established to measure contaminant induced direct and indirect genetic damage in aquatic organisms and these types of methods can be classified depending on the type of DNA damage, which is measured (De Coen, 1999). At cellular level, the micronucleus test is used to monitor nucleus fragmentation as a result of genotoxic insult. Ploidy shifts, changes in genome size and chromosomal aberrations have been used as endpoints to detect cytogenetic damage for mammalian cell cultures. Other sub-cellular methods have focused on assessing covalent interactions of toxicants with the DNA molecule and the most frequently used technique to assess DNA adducts is the 32P - post labelling technique which has been successfully applied to detect contaminant-induced adducts (De Coen, 1999). The majority of available methods focus on detecting DNA damage by measuring the overall DNA integrity. Strand breaks are expected to increase as result of toxic interaction as an indirect consequence of the excision repair of formed adducts (Shugart and Theodorakis, 1994). There are three general approaches to measure DNA strand breaks and these are the alkali unwinding technique, the DNA precipitation technique and agarose gel electrophoresis. Of the three methods, agarose gel electrophoresis is the best and DNA damage in small quantities can be measured. This method is based on the migration of cellular or tissue extracted DNA using electrophoretic separation and the migration distance is inversely proportional to the length of the molecule (De Coen, 1999). This is used to quantify the distribution of the different length classes, as highly fragmented, low molecular weight DNA strands will migrate farther than non-damaged high molecular weight DNA (Black *et al.*, 1996). The DNA can be quantified by densitometry.

Stress proteins

Stress proteins have been proposed as sensitive indicators of sub-lethal exposure to contaminants in the environment. They are synthesized at higher levels when cells are challenged with certain environmental stimuli such as high temperature and toxic chemicals (Cruz-Rodriguez and Chu, 2002). Cells undergo alterations in gene expression in response to environmental stressors (Huggett *et al.*, 1992). The stress protein response is a potentially useful marker in combination with a suite of biomarkers in toxicological studies. Heat shock proteins (HSPs), particularly HSP 60 and HSP 70, have been suggested as suitable biomarkers of the exposure to and effects to environmental contaminants (Sanders, 1990). HSPs are a group of highly conserved, abundantly expressed proteins found in prokaryotes and eukaryotes (Morimoto *et al.*, 1994). Their increased expression has been called a stress response because they can be induced after exposure to some environmentally relevant stressors, including some contaminants such as heavy metals, tributyltin, organophosphate and organochlorine pesticides (Clayton *et al*., 2000). HSP's bind transiently to newly damaged chains combating the tendency of chains to aggregate under intra-cellular conditions thus folding can take place to correct conformations (Ellis, 1998) hence they act as molecular chaperones, assisting in the transport and folding of newly synthesised proteins. Clayton *et al.* (2000) found increasing concentrations of HSP 60 and HSP 70 as a result of increasing tissue concentrations of copper and tributylin in zebra mussels in the laboratory. HSP 60 and 70 are involved in protein homeostasis under normal conditions taking on protective and repair roles upon exposure to adverse environmental conditions (Huggett *et al*., 1992). In cells responding to environmental stress, the HSP 70 family is preferentially expressed over other HSPs accounting for much of the translational activity. Since the

HSP 70 family accounts for much of the translational activity in cells responding to environmental perturbation and since it is one of the most highly conserved proteins, it is an excellent candidate as a biomarker for chemical contamination (Huggett *et al.*, 1992). The HSP 70 family has essential roles in protein metabolism under both stress and non-stress conditions (Hartl, 1996) and is mainly found in the endoplasmic reticulum and mitochondria. Cruz-Rodriguez and Chu (2002) showed that the increased HSP 70 response in *Crassotrea virginica* was due to uptake of polynuclear aromatic hydrocarbons sorbed to the suspended clay particles and contaminants present in the suspended field contaminated sediments. The use of HSP 70 as a biomarker of pollution, especially in non-lethal instances should be a sensitive biomarker, as these proteins are not induced by handling (Vijayan *et al*., 1998).

A major advantage of using stress proteins as biomarkers is that because they are involved in protecting the cell from environmentally induced damage, they reflect the cellular physiological state (Huggett *et al.*, 1992). Two main factors to consider in evaluating stress protein based assays as biomarkers are: (1) the response should be sensitive enough to be induced by contaminant concentration found in the environment and (2) the response being measured must be sustained over time (Huggett *et al.*, 1992).

Enzymes

Biochemical and physiological indicators such as enzymes can be used to identify possible environmental problems before the health of aquatic systems is seriously altered (Jiminez and Stegeman, 1990). The measurement of enzyme concentrations is a classical means by which the health of fish populations is assessed in different

water sources (Landis and Yu, 1995) and has been exploited in assessing the effects of pollution on fish (Gaudet *et al.*, 1975). Living organisms have developed enzymes to enhance their chemical reaction rates to adapt to sudden changes in the environment. The alterations in metabolic rates by the functioning of enzymes at the molecular level form the basis for an organism to self regulate. Enzyme inhibition is a common mechanism of toxicity (Westlake *et al.*, 1983) although toxicity is dependent on the magnitude of inhibition and the concentration of the enzyme present. The altering of enzyme concentrations provide a basis for early detection of stress and give appropriate warning signals of impending damage to organisms, populations and communities before irreversible damage occurs (Landis and Yu, 1995), thus changes in enzyme concentrations may provide indirect evidence of cellular damage and can indicate the toxic mechanism of the pollutant involved. Specific enzymes regulating a variety of metabolic pathways can be altered as a result of stress related homeostatic adjustments induced by toxicant exposure (Head and Gabbott, 1979). For most enzymes, accessory factors (diet, season, temperature, sex and reproductive condition) have been shown to have considerable influence on enzyme activity (Head and Gabbott, 1979) thus the measurement of a battery of enzymes can provide insight into the metabolic status of the organism. Since both increases and decreases in enzyme activity are possible and not always predictable, a battery of enzymes necessarily enhances interpretation.

Lactate dehydrogenase (LDH) catalyses the interconversion of lactate and pyruvate in the presence of NAD/NADH. The main functions of are listed below:

1. It forms the centre for a delicately balanced equilibrium between the catabolism and anabolism of carbohydrates,

2. is the key enzyme in the sequence of reactions that promote breakdown of glucose to lactate under aerobic conditions and in anaerobic glycolysis and is thus essential for ATP production,
3. it also plays a role during glucogenesis in tissues when lactate is converted to glycogen, and
4. it helps generate DPNH and ATP in aerobic tissues such as the heart when lactate is the fuel that is oxidized through the citric acid cycle.

Different metals have been shown to influence the activity of LDH. Mercury inhibits LDH activity in the rosy barb (Gill *et al*., 1990) and other metals listed as inhibitors of LDH are cadmium, copper and zinc (Christensen *et al.*, 1986). LDH activity in fish occurs at greater concentrations in muscle tissue than in other tissues, making total serum LDH levels a potential biomarker of muscle damage (Huggett *et al.*, 1992).

Phosphatases catalyse the hydrolytic cleavage of phosphoric acid esters. They are designated either “acid” or “alkaline” phosphatases according to the pH optima (Linhardt and Walter, 1965). Alkaline phosphatase (ALP) occurs practically in all animal tissue and its inhibition has been used as an indicator of environmental pollution (Linhardt and Walter, 1965).

CHAPTER 3

THE USE OF GENOTOXIC AND STRESS PROTEINS IN THE ACTIVE BIOMONITORING OF THE RIETVLEI SYSTEM

3.1 INTRODUCTION

DNA damage has been proposed as a useful variable for assessing genotoxic properties of environmental pollutants (Huggett *et al*., 1992). Interaction with DNA molecule is manifested primarily by structural alterations to the DNA molecule and can take the form of adducts, strand breakage or chemically altered bases (De Coen, 1999). The consequences of these structural perturbations can be innocuous due to the repair or death of the damaged cells but those lesions that are not properly repaired may result in alterations that are fixed and eventually transmitted into daughter cells (Huggett *et al.*, 1992). DNA is present in cells as a functionally stable, double stranded entity without strand breaks or abnormal structural modifications thereby having high integrity. The rigid maintenance of this integrity is important for survival and is reflected in the low mutational rate observed in living organisms, estimated to be on the order of one mutation per average gene per 200 000 years (Huggett *et al.*, 1992; Steinert, 1999). Because of the ubiquity of genotoxic agents in the environment, biomarker assays to detect genotoxicity have been developed (Black *et al.*, 1996). Techniques employing gel electrophoresis allow the direct quantification of DNA fragment lengths. Highly fragmented, low molecular weight DNA strands migrate farther than nondamaged high molecular weight DNA strands.

Heat shock proteins belong to a family of proteins, which play a major role in cellular stress response (Morimoto *et al.*, 1994). They are known to be induced by a variety of

environmental perturbations including heavy metals, xenobiotics, oxidative conditions, anoxia, salinity stress, teratogens and hepatocarcinogens (Huggett *et al.*, 1992). HSP 70 was shown by Vijiyan *et al.* (1997) to be also induced in rainbow trout by β- naphthoflavone, a potent inducer of P450 enzymes. HSP 70 is present in constitutive levels in cells, and has been shown to be important in the normal functioning of cells. However, when cells are exposed to a stressor, the rapid activation of this gene and the subsequent synthesis of the protein have been shown to protect the cells form the harmful effects of the stressor (Morimoto *et al.*, 1994). HSP 70 assist in the correct folding, assembly and compartmentalisation of a wide number of proteins (Dietz and Somero, 1993). The induction of HSP 70 has been shown to provide cells with stress tolerance to subsequent insults, thus the expression of HSP 70 is indicative of cellular changes, in particular the effects of the stressor on the protein related machinery (Vijayan *et al.*, 1998). HSP 70 expression can be used as a biomarker for environmental monitoring since handling stress does not induce them.

The catalytic function of enzymes in biochemical reactions renders them essential for the normal function of all organisms. The inhibition of ALP has been used as an indicator of environmental pollution (Linhardt and Walter, 1965). Increased LDH activity in blood of brown trout (*Salmo trutta fario*) exposed to river and sewage plant effluent has been used as sensitive biomarker (Escher *et al.*, 1999). For the purpose of this study, LDH and ALP were used as biomarkers.

This study aimed at assessing the usefulness of DNA damage and stress proteins (HSP 70 and metabolic enzymes LDH and ALP) as biomarkers in the Rietvlei system.

The Rietvlei system was selected as the study area because of the lack of information on genotoxic contaminants yet it is an important source of drinking water for Pretoria and the Rietvlei Nature Reserve game. The system is also known to have endocrine disrupting contaminants that induce feminization in feral *Clarias gariepinus* (Barnhoorn *et al.*, 2003).

3.2 MATERIALS AND METHODS

3.2.1 *Study Area*

Three sampling sites were selected in a 10km stretch along the Sesmlyspruit River in the Rietvlei Nature Reserve (Figure 1). Site 1 was located just above Marais Dam while site 3 was located between the two dams within a wetland and site 5 was downstream from Rietvlei Dam just outside the Nature Reserve. Site 1 receives effluent from industries, agricultural activities, informal settlements and municipal sewage treatment plants. The Marais Dam acts as a sludge dam and is theoretically more polluted than the Rietvlei Dam downstream and the river drains through a wetland between these dams. It is therefore expected that a pollution gradient should occur with increased concentrations of contaminants at site 1, the furthest most upstream site and decreasing downstream as it moves from Marais Dam to Rietvlei Dam past site 3 and finally passes through site 5 as water flows out of Rietvlei Dam. Reference organisms were kept under laboratory conditions in the aquarium of the Department of Zoology, RAU.

3.2.2. *Sampling and transplant of test organisms*

Two bioindicators were used in this study i.e. a mollusk, *Melanoid es tuberculata* and a fish species, *Oreochromis mossambicus*. Two surveys (during high-flow conditions - April 2003 and during low-flow conditions – August 2003) were undertaken to the three selected sites in the Rietvlei wetland system. The selected bio indicator organisms were deployed in specially designed active biomonitoring cages at the selected sites for at least six weeks prior to sampling. The bioindicator organisms were bred and reared in the aquarium at the Zoology Department, RAU and were three months old when they were deployed. Samples were collected four weeks and six weeks after deployment. Fish were sampled after 4 weeks exposure during the high-flow period. No fish were collected after the low-flow exposure period since all fish were killed within one week of exposure to the sampling sites. The deployment was repeated, with the same results. The snails were collected after 4 and 6 weeks exposure to the study sites during the high-flow exposure period. However they could only be sampled after the 4 weeks exposure during the low-flow period since the 6 weeks exposure were also all killed.

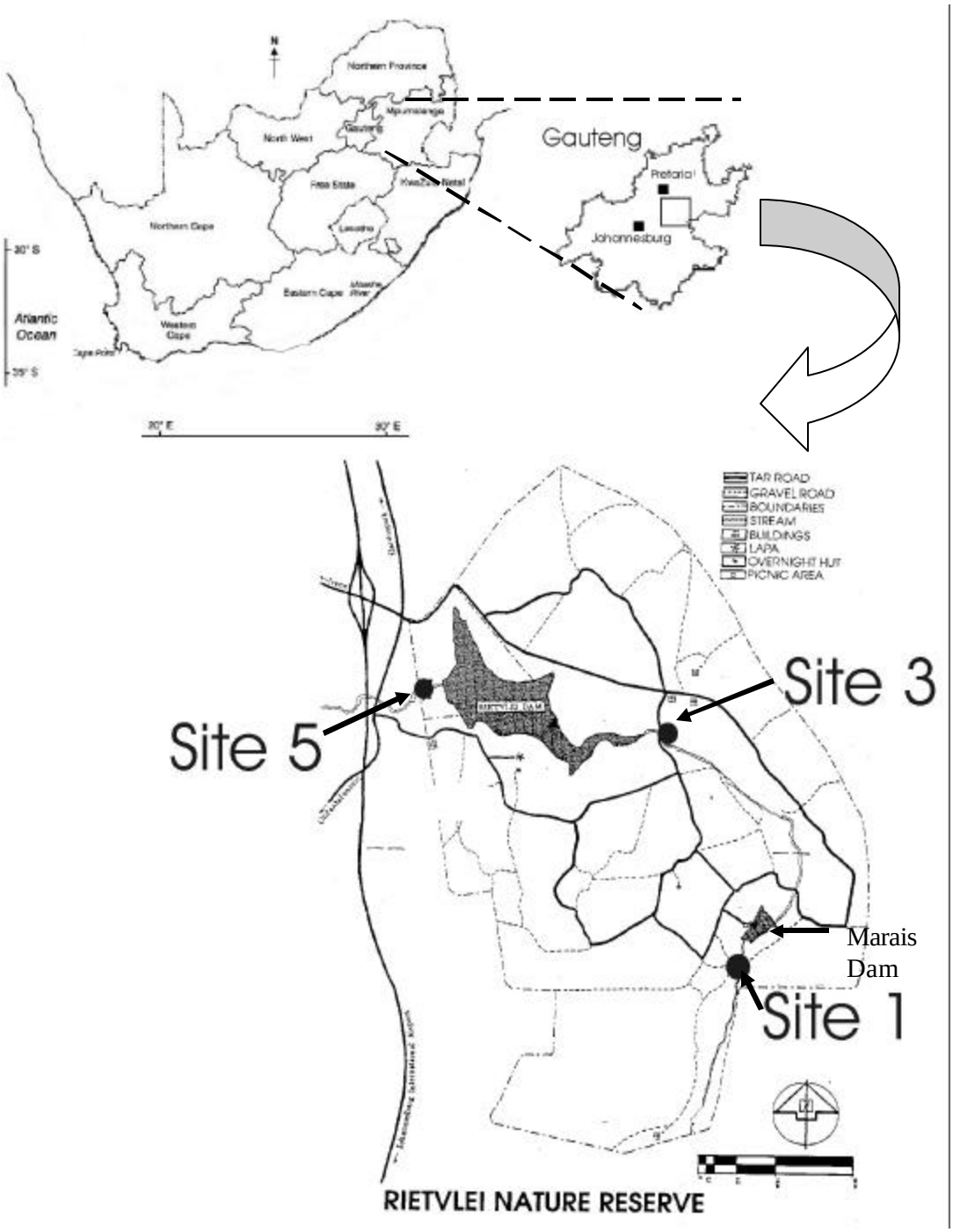

Figure 1: Position of the sampling sites in the Rietvlei Nature Reserve.

3.2.3. *In situ water quality analyses*

Physicochemical parameters were measured during deployment and sampling. The following water quality parameters were measured: temperature, pH, redox potential, conductivity, dissolved oxygen and percentage oxygen saturation using Eutech CyberScan pH, Conductivity and Dissolved Oxygen meters.

3.2.4. *Biomarker determination*

DNA damage

DNA damage was determined using the method described by De Coen (1999). Tissue samples were gently homogenised using a glass rod in DNA extraction buffer containing 250mM NaCl, 100mM Tris base and 100mM ethylenediaminetetraacetic acid (EDTA). Cells in the homogenate were then lysed by adding 10% sarcosyl solution. Ribonuclease (3µl) was added followed by incubation on ice for 10 minutes. Protein kinase (5µl) was added and then incubated in a warm water bath for 30 minutes. Adding PCI and centrifuging for 7 minutes at 3000rpm twice and repeating the procedure using chloroform purified the extracts. Purified extracts were loaded on a horizontal electrophoresis unit along with a DNA molecular weight marker Hind II (0.12-23.1kbp) as a standard on a non-denaturing gel containing 0.5% agarose in TBE buffer (65mM Tris HCL, 22.5mM boric acid and 0.25mM EDTA) at 70V for ± 3½ hours. Bromophenol blue was loaded in the first well of each gel to follow the migration of the samples. The gels were stained for at least 40 minutes in the dark in 200 ml TBE buffer with 20µl SYBR Green I. The stained gels were analysed using Ultraviolet transilluminator (UVP). Photos were taken using the annotated image

capture system, Grab-IT (version 2.5) and by using SigmaScan. The image was quantified using OptiQuant image analyse software.

HSP 70 analysis

HSP 70 expression was determined in the gills of *O. mossambicus* only because there were no suitable antibodies sensitive enough to detect the HSP 70 in *M. tuberculata* . The gills were chosen for HSP 70 determination because they are the first organs to be affected by contaminants via water exposure. The gills were homogenised in sucrose buffer (0.25M sucrose; 50mM Tris base; pH 7.4) and centrifuged at 15 000 rpm for fifteen minutes. The HSP 70 concentration was determined in the supernatant, which was kept at -80^{o}C until analysis. The protein concentration for the samples was determined using the Bradford method (Bradford, 1976) to get the amount of sample needed to obtain 15µg protein per well. The proteins were separated using sodium dodecysulphate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane by western blotting at 30V for 16 hours at 4^{o}C. The transferred proteins were stained in Ponceau S for 15 minutes. The nitrocellulose membrane was blocked for 3½hours in non-fat dried milk containing 0.02% sodium azide then incubated in HSP 70 primary antibody for 20 hours. The membrane was washed 3 times in phosphate buffer saline and once in Tris buffer saline for 10 minutes each then incubated in non-fat azide free blocking solution with secondary HSP 70 antibody for 3 hours. The membrane was then washed in four changes of Tris buffer saline before Enhanced Chemiluminescence (ECL) detection. ECL-Western blotting reagents (Amersham Pharmacia Biotech) were used to detect the HSP 70 proteins. The HSP 70 bands were quantified as relative intensities using UVP GRAB IT (image analyser) and Gene Tools from

Sygene both from the same hyperfilm and were expressed as arbitrary units (Grant, pers. com[1].).

Metabolic enzymes

M. tuberculata and *O. mossambicus* samples were homogenised in phosphate buffer and centrifuged at 3000rpm for 10 minutes at 4°C. The activities of LDH and ALP were determined in the supernatant. The activity of LDH was determined using the UV assay with pyruvate and NADH (Bergmeyer and Bernt, 1974) by determining of the amount of NADH oxidation at 340 nm. Phosphate/pyruvate solution (3 ml) (50 mM phosphate, pH 7.5, 0.63 mM pyruvate) was pipetted into cuvettes and 50 µl NADH solution (11.3 mM β-NADH) added then 100 µl of sample homogenate was added and mixed immediately and the extinction was read after every minutes for 4 minutes. The LDH activity was calculated using the following formula:

$$\text{Volume of activity} = 5056 \times \Delta E/\text{min [U/l]}$$

The activity of ALP was calculated by determining the amount of 4-nitrophenol liberated at 410 nm (Walter and Schutt, 1974). Buffer substrate solution (2 ml) (0.1M diethanolamine, pH 9.8; 1.25 mM 4-nitrophenylphosphate) was pipetted into a test tube and 50 µl of sample was then added followed by incubation for exactly 30 minutes at 25 °C. The reaction was stopped by adding 10 ml 0.05 N NaOH and the extinction was then read at 410 nm. The increase in extinction above the blank in which the sample was added after adding 0.05N NaOH was used for calculating the volume of activity using the following formula:

$$\text{Volume of activity} = \Delta E \times 28.8 \text{ [U/l]}$$

[1] Mr. B. Grant, Department of Zoology, RAU.

3.2.5. *Statistical Analysis*

Analysis of variance (ANOVA), with a post-hoc Scheffe's test was used to analyse the data. All data were tested for homogeneity of variance by Levene's test. ANOVA was used to test for differences in the biomarker responses between sites and exposure durations. When ANOVA indicated that significant differences existed, Scheffe's multiple comparison-test was then used. All data were analysed at a 0.05 level of significance.

3.3 RESULTS

3.3.1. *Water quality*

Physicochemical parameters measured during deployment and sampling are shown in Tables 1 and 2. Conductivity generally decreased from site 1 down the pollution gradient.

Table 1: Physicochemical parameters measured at three sites in the Rietvlei System during the high-flow, sampling period.

Parameter	Site 1			Site 3			Site 5		
	Deployment	4 weeks	6 weeks	Deployment	4 weeks	6 weeks	Deployment	4 weeks	6 weeks
Temperature (^{o}C)	19.6	19.6	12.6	21.5	21.5	12.7	24.5	24	15.5
DO (mg/l)	3.9	3.9	8.58	9.95	9.95	8.32	6.68	3.84	5.37
DO (% saturation)	53.8	53.8	83.2	138	138	65.5	96.5	55.1	58.3
PH	7.9	7.9	7.5	7.9	7.9	7.5	8.8	7.8	7.6
Redox	-74.5	-74.5	-61.3	-90.4	-90.4	-68.8	-120.8	-75.8	-70.3
Conductivity (µS/cm)	601	600	345	515	546	671	450	450	535
TDS (mg/l)	300	300	153	252	333	341	221	234	263

DO = Dissolved oxygen, TDS = Total Dissolved Solids

Table 2: Physicochemical parameters at three sites in the Rietvlei System during the low-flow sampling period

Parameter	Site 1		Site 3		Site 5	
	Deployment	4 weeks	Deployment	4 weeks	Deployment	4 weeks
Temperature (°C)	13.37	14.3	13.5	17.4	12.6	19.4
DO (mg/l)	12.38	7.75	11.05	4.91	12.0	6.20
DO (% saturation)	116.0	78.5	111.3	58.0	111.0	74.7
pH	8.2	7.5	8.6	8.0	8.7	8.1
Redox	-91.5	-69.9	-121.4	-90.3	-121.8	-102.7
Conductivity (µS/cm)	742	546	652	695	556	586

3.3.2. *DNA damage*

Melanoides tuberculata

The DNA strand lengths were different ($P<0.05$) from the controls at all the sites following 4 weeks and 6 weeks high-flow (significantly longer) and 4-week low-flow exposures (significantly smaller) (Figure 2). Although all sites and exposure periods were significantly different from the control, the low-flow week 4 exposure had significantly smaller DNA strand lengths ($P=0.001$) from all the high-flow exposures (weeks 4 and 6) for all the sites. The three sites in the Rietvlei system did not show any differences in the DNA strand lengths between sites following 4 weeks low-flow exposure. The proportions of DNA extracts found in the different length classes were shown to be different from the controls for all the sites during all exposure durations. The proportion of DNA extracts in the different size classes at 4 weeks, 6 weeks high-flow and 4 weeks low-flow are shown in Figures 4 to 6.

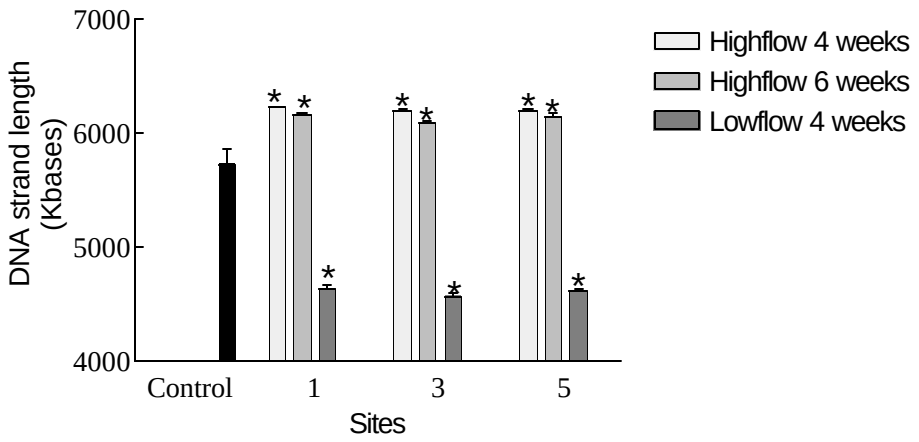

Figure 2: Mean DNA strand length (± SE) in control and exposed *M. tuberculata* at the three sites in the Rietvlei system. * = significantly different from the controls (P< 0.05).

Oreochromis mossambicus.

There were no differences in the DNA strand lengths between the control fish and fish from all three sites in the Rietvlei system (Figure 3). The proportion of control DNA extract in the D class (3319 bp) differed significantly (P= 0.023) from site 1 and between site 1 and site 3 (Figure 7) but there was no difference between site 5 and the control.

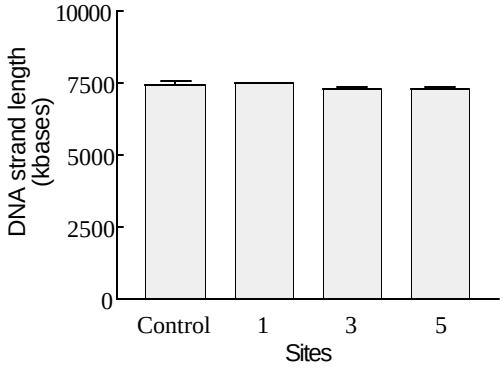

Figure 3: Mean DNA strand length (± SE) in control and 4 week exposed fish at the three sites in the Rietvlei System.

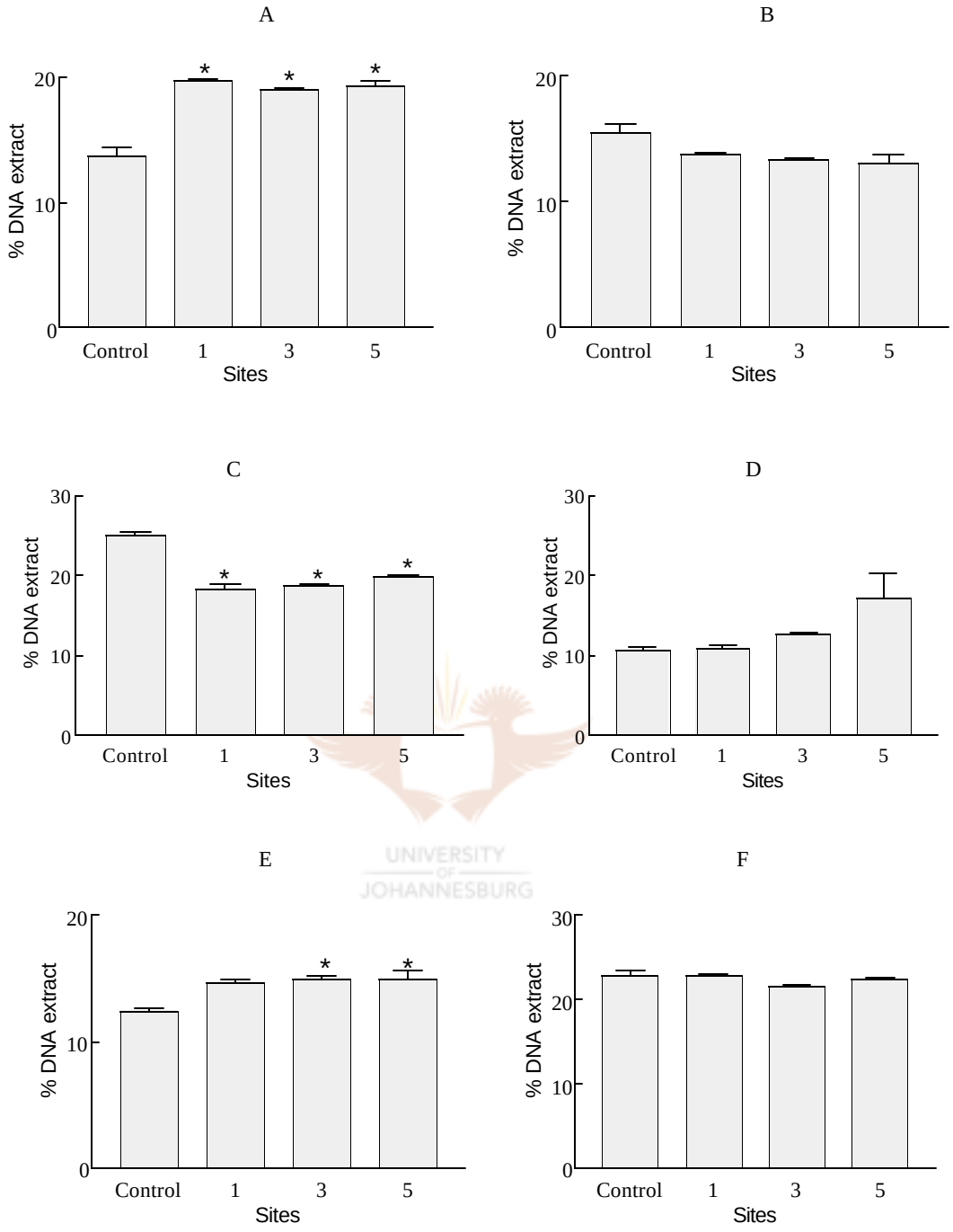

Figure 4: Proportional fractions of *M. tuberculata* DNA extracts belonging to different length classes after four weeks exposure in the Rietvlei System during highflow. Class A= 16273 bp, B= 7987 bp, C= 5459 bp, D= 3319 bp, E= 2175 bp and F= 1296 bp. * = significantly different from the control (P<0.05). bp=base-pairs.

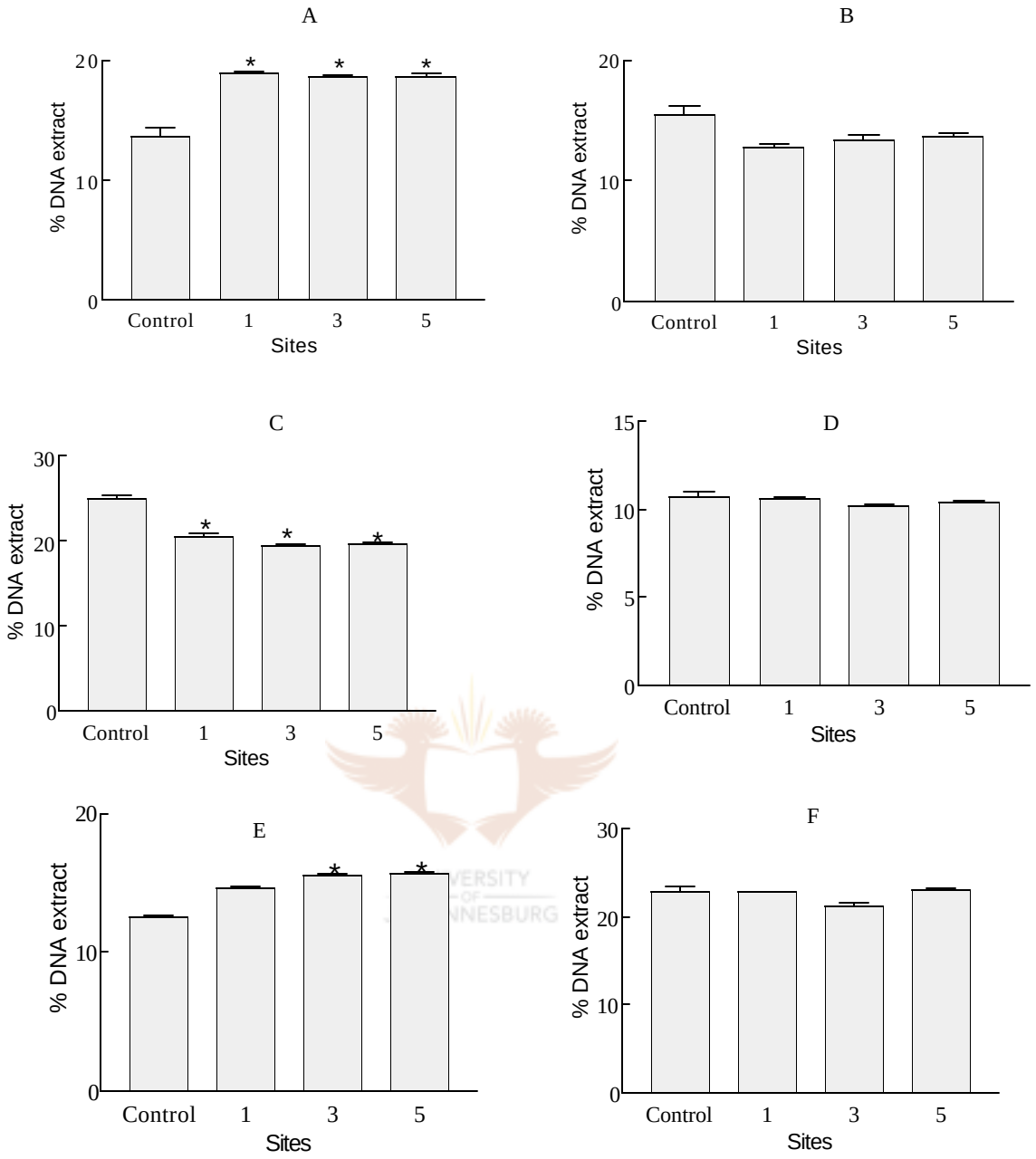

Figure 5: Proportional fractions of *M. tuberculata* DNA extracts belonging to different length classes after six weeks exposure in the Rietvlei System during highflow. Class A= 16273 bp, B= 7987 bp, C= 5459 bp, D= 3319 bp, E= 2175 bp and F= 1296 bp. * = significantly different from the control (P<0.05). bp=base-pairs.

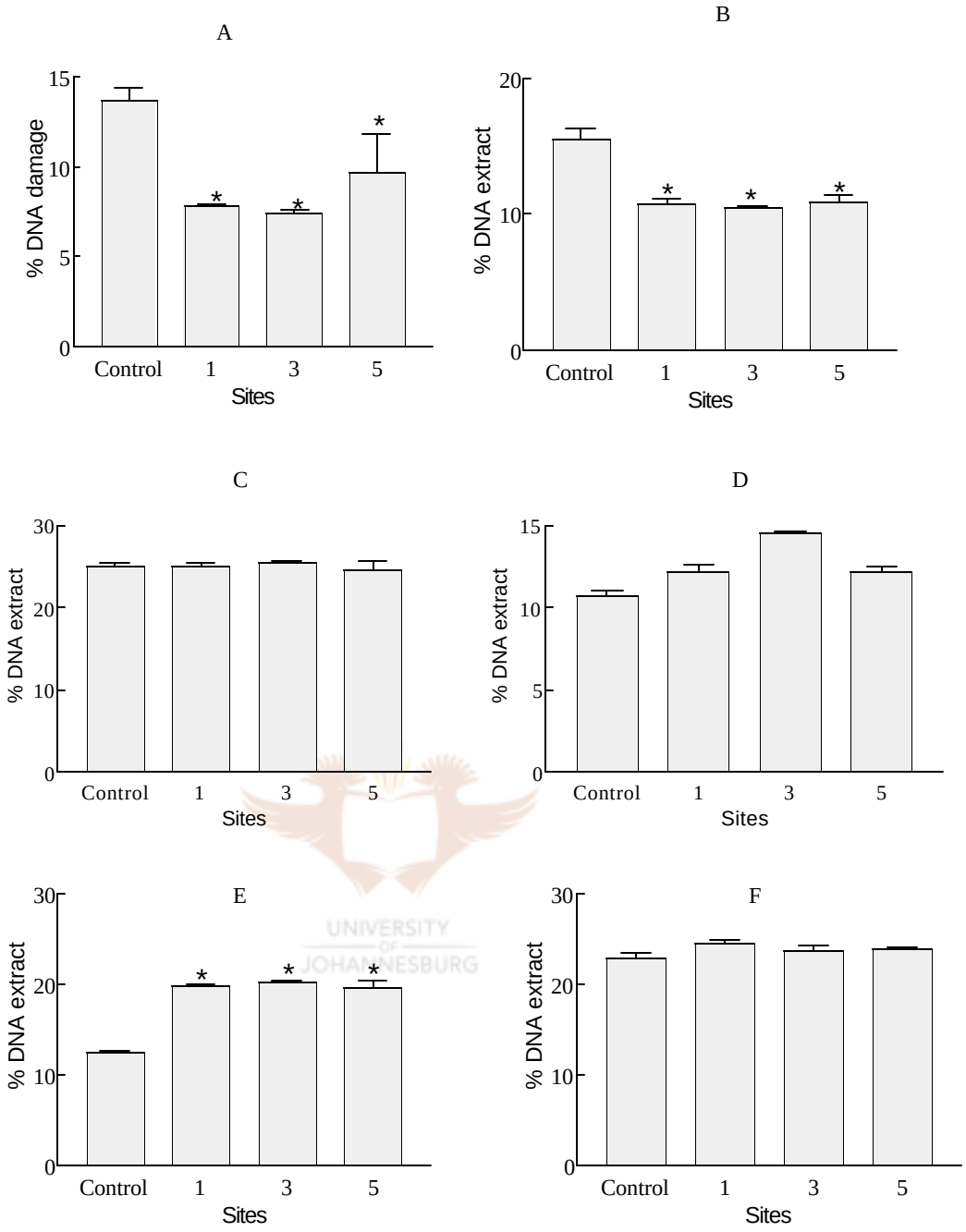

Figure 6: Proportional fractions of *M. tuberculata* DNA extracts belonging to different length classes after four weeks exposure in the Rietvlei System during lowflow. Class A= 16273 bp, B= 7987 bp, C= 5459 bp, D= 3319 bp, E= 2175 bp and F= 1296 bp. * = significantly different from the control (P<0.05).

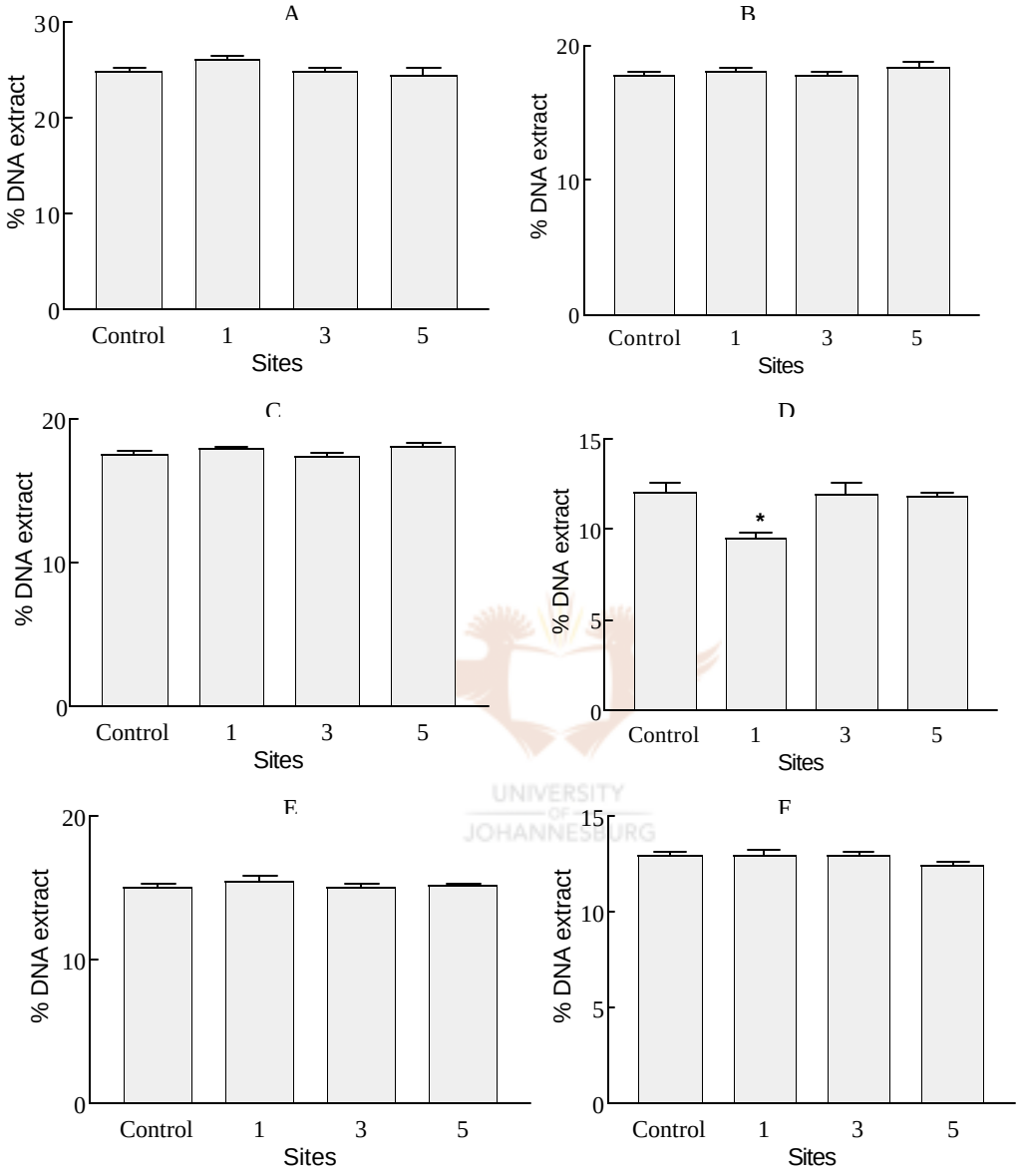

Figure 7: Proportional fractions of *O. mossambicuss* DNA extracts belonging to different length classes after four weeks exposure in the Rietvlei System during high-flow. Class A= 16273 bp, B= 7987 bp, C= 5459 bp, D= 3319 bp, E= 2175 bp and F= 1296 bp. * = significantly different from the control ($P<0.05$). bp=pase-pairs.

3.3.3. *HSP 70*

An example of Enhanced Chemiluminescense (ECL) exposed hyperfilm showing HSP 70 in *O. mossambicus* is presented in Figure 8. Only *O. mossambicus* HSP 70 from the high-flow four weeks were available because of the death of the deployed organisms during the low-flow period. HSP 70 expression in *O. mossambicus* during the high-flow exposure regime was highest at site 1 decreasing downstream at site 3 and with no difference from the control at site 5 (Figure 9). Fish exposed at Sites 1 and 3 showed significant differences from the control fish ($P<0.05$). Although HSP 70 expression at Site 3 was reduced when compared to site 1, this was still significantly different from the control fish ($P=0.04$) and fish exposed at site 5 ($P=0.04$).

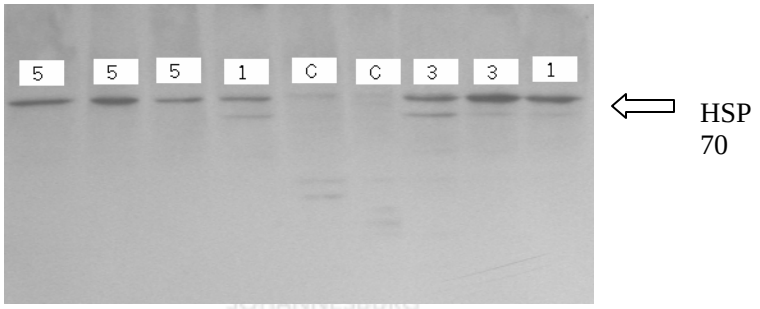

Figure 8: An example of Enhanced Chemiluminescense (ECL) exposed hyperfilm showing HSP 70 in *O. mossambicus* for the different sites (1, 3 and 5) in the Rietvlei System and the controls (C).

3.3.4. *Metabolic enzymes*

LDH activity in *M. tuberculata* at all the three sites and between the sites did not differ from the controls (Figure 10). There was no difference between the high-flow and low-flow exposures after both 4 and 6 weeks. No ALP activity was detected in both control and exposed *M. tuberculata*.

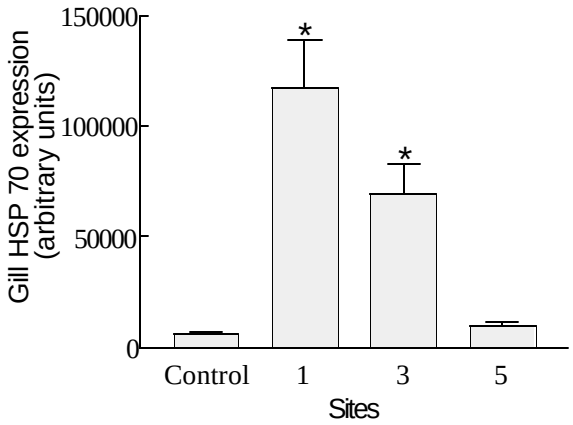

Figure 9: Relative HSP 70 expression in *O. mossambicus* controls and at the three sites in the Rietvlei System (* = significantly different from the control); mean + SE; n = 10 at each site).

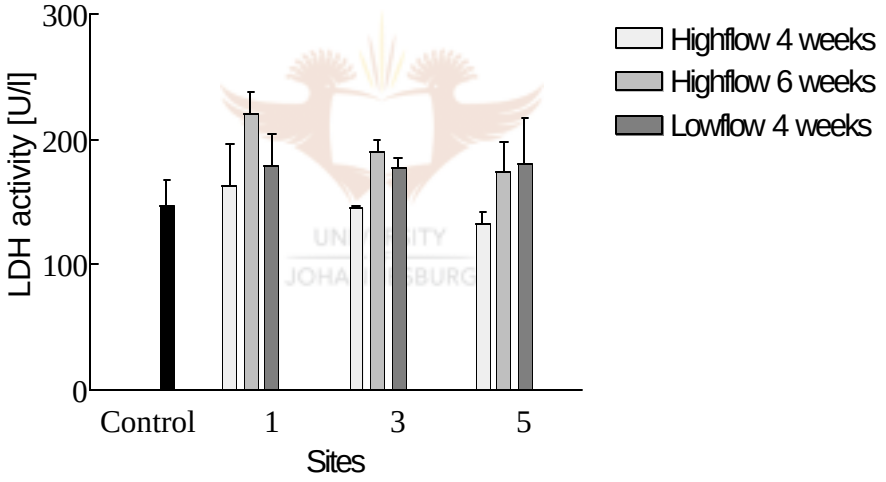

Figure 10: The mean (+ SE) LDH activity at the different sites in *M. tuberculata* exposed in the Rietvlei System, N = 6 at each site.

The activity of LDH in *O. mossambicus* was lower ($P < 0.05$) at site 1 compared to sites 3, 5 and the controls (Figure 11). Activity in fish exposed at sites 3 and 5 were not different from the control fish or between the two sites. ALP activity in *O. mossambicus* was lower at site 1 than the controls and at sites 3 and 5increasing

downstream but this was not statistically significant (P=0.053). All sites were different from the control (Figure 12) although the three sites were not different from each other. The ALP activity was highest in the controls but the enzyme was inhibited in fish exposed in the Rietvlei system.

Scheffe's multiple comparison probability matrices for all the biomarkers assessed are given in Tables 3 and 4 for *M. tuberculata* and *O. mossambicus*, respectively.

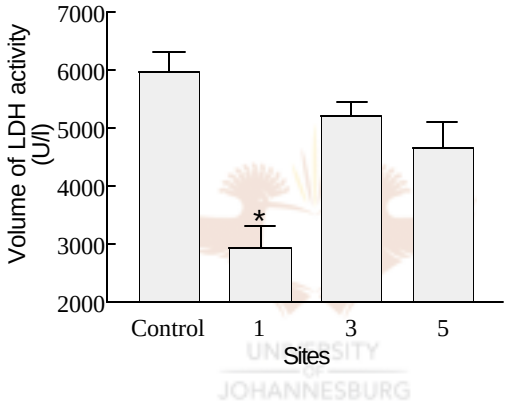

Figure 11: The mean (+ SE) LDH activity at the different sites in *O. mossambicus* exposed for 4 weeks during highflow in the Rietvlei System (* = significantly different from the control), n= 8 at each site.

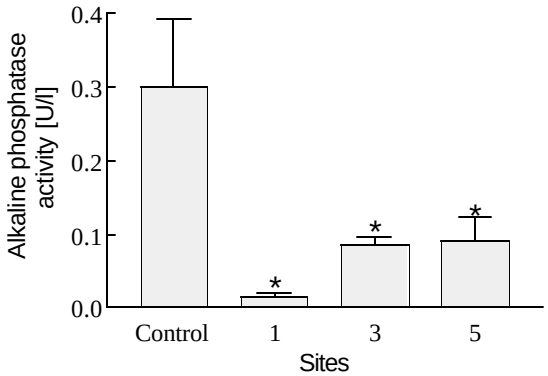

Figure 12: Mean (+ SE) alkaline phosphatase activity in *O. mossambicus* (n = 8 at each site) exposed for 4 weeks during highflow in the Rietvlei System (* = significantly different from the control).

Table 3: Scheffe's multiple comparison probability matrix for *M. tuberculatta* at three sites and the control
(H6=High flow 6 weeks, H4 = High flow 4 weeks, L4 = Low flow 4 weeks

DNA = average base pair length
A-F = different size classes referred to in Figures 4 - 6.

Site 1 H4	LDH = 1.000								
	DNA = 0.000								
	A = 0.000								
	B = 0.989								
	C = 0.000								
	D = 0.999								
	E = 0.097								
	F = 0.022								
Site 1 H6	LDH = 0.687	LDH = 0.886							
	DNA = 0.000	DNA = 0.924							
	A = 0.000	A = 0.560							
	B = 1.000	B = 0.934							
	C = 0.000	C = 0.052							
	D = 0.991	D = 1.000							
	E = 0.052	E = 1.000							
	F = 0.003	F = 1.000							
Site 1 L4	LDH = 1.000	LDH = 1.000	LDH = 0.996						
	DNA = 0.000	DNA = 0.000	DNA = 0.000						
	A = 0.000	A = 0.000	A = 0.000						
	B = 0.007	B = 0.002	B = 0.043						
	C = 0.702	C = 0.000	C = 0.000						
	D = 1.000	D = 0.998	D = 0.982						
	E = 0.000	E = 0.000	E = 0.000						
	F = 1.000	F = 0.089	F = 0.019						
Site 3 H4	LDH = 1.000	LDH = 1.000	LDH = 0.665	LDH = 0.999					
	DNA = 0.000	DNA = 1.000	DNA = 0.999	DNA = 0.000					
	A = 0.000	A = 0.813	A = 1.000	A = 0.000					
	B = 1.000	B = 1.000	B = 0.999	B = 0.011					
	C = 0.000	C = 1.000	C = 0.218	C = 0.000					
	D = 1.000	D = 0.994	D = 0.968	D = 1.000					
	E = 0.030	E = 1.000	E = 0.000	E = 0.995					
	F = 0.000	F = 0.407	F = 0.493	F = 0.000					
Site 3 H6	LDH = 0.991	LDH = 1.000	LDH = 0.999	LDH = 1.000	LDH = 0.989				
	DNA = 0.000	DNA = 0.144	DNA = 0.819	DNA = 0.000	DNA = 0.428				
	A = 0.000	A = 0.216	A = 0.999	A = 0.000	A = 0.992				
	B = 1.000	B = 1.000	B = 0.996	B = 0.007	B = 1.000.				
	C = 0.000	C = 0.792	C = 0.898	C= 0.000	C = 0.983				
	D = 0.972	D = 1.000	D = 1.000	D = 0.955	D = 0.933				
	E = 0.002	E = 0.937	E = 0.902	E = 0.000	E = 0.995				
	F = 0.077	F = 1.000	F = 0.999	F = 0.251	F = 0.186				
Site 3 L4	LDH = 1.000	LDH = 1.000	LDH = 0.996	LDH = 1.000	LDH = 1.000	LDH = 1.000			
	DNA = 0.000	DNA = 0.000	DNA = 0.000	DNA = 0.960	DNA = 0.000	DNA = 0.000			
	A = 0.000	A = 0.000	A = 0.987	A = 0.000	A = 0.000	A = 0.000			
	B = 0.090	B = 0.002	B = 0.041	B = 1.000	B = 0.011	B = 0.008			
	C = 0.934	C = 0.000	C = 0.000	C = 1.000	C = 0.000	C = 0.000			
	D = 1.000	D = 0.996	D = 0.977	D = 1.000	D = 1.000	D = 0.949			
	E = 0.000	E = 0.000	E = 0.000	E = 0.999	E = 0.000	E = 0.000			
	F = 0.0501	F = 0.948	F = 0.795	F = 0.843	F = 0.022	F = 0.997			
Site 5 H4	LDH = 1.000	LDH = 0.999	LDH = 0.541	LDH = 0.994	LDH = 1.000	LDH = 0.948	LDH = 0.994		
	DNA = 0.000	DNA = 1.000	DNA = 1.000	DNA = 0.000	DNA = 1.000	DNA = 0.482	DNA = 0.000		
	A = 0.000	A = 0.999	A = 0.958	A = 0.000	A = 0.996	A = 0.663	A = 0.000		
	B = 1.000	B = 0.996	B = 1.000	B = 0.028	B = 1.000	B = 1.000	B = 0.026		
	C = 0.000	C = 0.557	C = 0.985	C = 0.000	C = 0.900	C = 1.000	C = 0.000		
	D = 0.058	D = 0.026	D = 0.008	D = 0.072	D = 0.242	D = 0.008	D = 0.216		
	E = 0.026	E = 1.000	E = 1.000	E = 0.000	E = 1.000	E = 0.996	E = 0.000		
	F = 0.003	F = 1.000	F = 1.000	F = 0.013	F = 0.825	F = 0.984	F = 0.625		
Site 5 H6	LDH = 1.000	LDH = 1.000	LDH = 0.989	LDH = 1.000	LDH = 0.999	LDH = 1.000	LDH = 1.000	LDH = 0.992	
	DNA = 0.000	DNA = 0.683	DNA = 1.000	DNA = 0.000	DNA = 0.967	DNA = 0.949	DNA = 0.000	DNA = 0.980	
	A = 0.000	A = 0.167	A = 1.000	A = 0.000	A = 0.995	A = 1.000	A = 0.000	A = 0.637	
	B = 0.999	B = 1.000	B = 0.978	B = 0.001	B = 0.100	B = 1.000	B = 0.001	B = 1.000	
	C = 0.000	C = 0.463	C = 0.940	C = 0.000	C = 0.871	C = 1.000	C = 0.000	C = 1.000	
	D = 0.976	D = 1.000	D = 1.000	D = 0.958	D = 0.939	D = 1.000	D = 0.955	D = 0.004	
	E = 0.000	E = 0.778	E = 0.673	E = 0.000	E = 0.959	E = 1.000	E = 0.000	E = 0.970	
	F = 0.016	F = 1.000	F = 0.998	F = 0.998	F = 0.105	F = 1.000	F = 0.989	F = 0.976	
Site 5 L4	LDH = 0.999	LDH = 1.000	LDH = 0.995	LDH = 1.000	LDH = 0.995	LDH = 1.000	LDH = 1.000	LDH = 0.985	LDH = 1.000
	DNA = 0.000	DNA = 0.000	DNA = 0.000	DNA = 0.999	DNA = 0.000	DNA = 0.000	DNA = 1.000	DNA = 0.000	DNA = 0.000
	A = 0.000	A = 0.000	A = 0.000	A = 0.995	A = 0.000	A = 0.000	A = 1.000	A = 0.000	A = 0.000
	B = 0.003	B = 0.001	B = 0.020	B = 1.000	B = 0.005	B = 0.003	B = 1.000	B = 0.013	B = 0.000
	C = 0.999	C = 0.000	C = 0.000	C = 0.994	C = 0.000	C = 0.000	C = 1.000	C = 0.000	C = 0.000
	D = 1.000	D = 0.992	D = 0.958	D = 1.000	D = 1.000	D = 0.917	D = 1.000	D = 0.955	D = 0.917
	E = 0.000	E = 0.000	E = 0.000	E = 1.000	E = 0.000	E = 0.000	E = 1.000	E = 0.000	E = 0.000
	F = 0.717	F = 0.699	F = 0.392	F = 0.965	F = 0.003	F = 0.927	F = 1.000	F = 0.262	F = 0.821
	Control	**Site 1 H4**	**Site 1 H6**	**Site 1 L4**	**Site 3 H4**	**Site 3 H6**	**Site 3 L4**	**Site 5 H4**	**Site 5 H6**

Table 4: Scheffe's multiple comparison probability matrix for *O. mossambicus* at the three sites and the control (DNA = average base pair length; A-F = different size classes referred to in Figure 8).

SITE 3	LDH = 0.001 ALP = 0.802 HSP = 0.146 DNA = 0.610 A = 0.350 B = 0.862 C =- 0.384 D = 0.023 E = 0.838 F =1.000		
SITE 5	LDH = 0.05 ALP = 0.689 HSP = 0.001 DNA = 0.711 A = 0.248 B = 0.983 C = 0.997 D = 0.098 E = 0.951 F = 0.511	LDH = 0.780 ALP = 1.000 HSP = 0.039 DNA = 1.000 A = 0.948 B = 0.726 C = 0.401 D = 0.998 E = 0.998 F = 0.470	
CONTROL	LDH = 0.000 ALP = 0.009 HSP = 0.001 DNA = 0.985 A = 0.273 B = 0.862 C = 0.639 D = 0.022 E = 0.822 F = 1.00	LDH = 0.633 ALP = 0.053 HSP = 0.036 DNA = 0.788 A = 0.998 B = 1.000 C = 0.966 D = 1.000 E = 1.000 F = 1.000	LDH = 0.160 ALP = 0.031 HSP = 0.999 DNA = 0.852 A = 0.978 B = 0.726 C = 0.620 D = 0.997 E = 0.997 F = 0.519
	SITE 1	**SITE 3**	**SITE 5**

3.4. DISCUSSION

3.4.1. *DNA damage*

Highly fragmented low molecular weight DNA strands migrate farther than non-damaged high molecular weight DNA strands. The average DNA strand lengths in *M. tuberculata* in the Rietvlei system were larger than the controls during high-flow and lower during low-flow although there were no differences between the sites at low-flow 4 weeks. This shows that there are genotoxic contaminants in the Rietvlei system that become more concentrated during the low-flow period as the dilution capacity of the river is reduced. Concentration dependent differences in electrophoretic mobility of DNA was also found by Black *et al.* (1996) in mussels exposed to lead where 50µg/l lead showed large zones of smaller DNA fragments which migrated farther than larger fragments. The dilution capacity of the river during the high-flow period can be the reason for a larger DNA strand length as compared with the controls and the low-flow period. The lower levels of the contaminants may have induced repair processes for the double stranded DNA so that DNA damage was not detected thus average strand lengths were actually above the controls. Increased average base pair lengths above the controls due to induction of DNA repair was also found by Hoff *et al.* (2003) in *Cyprinus carpio* exposed for 5 days in 16, 270 and 864 ng/g perfluorooctane sulfonic acid. This is also similar to findings by Black *et al.* (1996) although in their case increased lead concentrations caused no detectable DNA damage after exposure of mussels to different concentrations of lead for 28 days. Pruski and Dixon (2003) found that mussels from the shallowest and less active vents showed greatest amount of DNA damage as compared to two deeper and putatively more toxic sites. Although it was expected that the effects of the contaminants on DNA strand length would differ significantly

from site 1 to site 3 because of the presence of Marais Dam that acts as a sludge dam and a wetland that filters the contaminants, the sites were not different. DNA damage can persist as a result of various physical and chemical reactions resulting in complex interactions (De Coen, 1999) thus the reasons for the lack of differences between site 1 and site 3 are not clear. Metals notably inhibit DNA repair enzymes and enhance the production of highly toxic hydroxyl radicals (Hartwig, 1998). The complex reactions that result in DNA damage makes it difficult to deduce the type of effluent, which is causing DNA damage. Although it is known that the Rietvlei receives effluent from industries, agricultural activities, informal settlements and municipal sewage treatment plants (Barnhoorn *et al.*, 2003) the complexity of the reactions between a genotoxic compound and the DNA molecule makes it difficult to link the type of effluent with DNA damage in the system. Some compounds react directly while others cause damage indirectly or only after metabolism (De Coen, 1999).

There were no differences in the amount of DNA damage in *O. mossambicus* exposed for four weeks in the Rietvlei system at all the sites although the proportion of DNA extract belonging to the D class at site 1 had lower proportions when compared to the control and site 3. This may be as a result of induced DNA repair processes so that DNA damage was not detectable by the end of our exposure period or simply because the contaminants were diluted to such a degree that they did not cause any DNA damage. It has been demonstrated that contaminant exposure does not lead to corresponding increases in DNA damage (Steinert, 1999). Thus it can be deduced that the level of contaminants during high-flow was too low to induce DNA damage.

3.4.2 *Protein damage*

In the unstressed cell, stress proteins have constitutive functions that are essential in protein metabolism. The HSP 70 family is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone and mediate the repair and degradation of altered or denatured proteins (Basu *et al.*, 2002). The increased expression of HSP 70 in *O. mossambicus* at sites 1 and 3 above the control in the Rietvlei system indicates that these proteins are induced by contaminants in the system. The expression of HSP 70 in the control fish shows that HSP 70 is expressed constitutively in *O. mossambicus.* HSPs can be up regulated in cells that are exposed to a variety of stressors, particularly those that denature proteins (Basu *et al.*, 2002). In fish, a correlation between increased levels of heat shock proteins and exposure to stressors within an ecologically relevant range has been demonstrated (Basu *et al.*, 2002) suggesting that the cellular stress response is likely to be playing some role in enhancing the survival and health of the stressed fish. The increased HSP 70 expression at site 1 decreasing downstream at site 3 shows that the contaminants are more concentrated at site 1 and decrease down the pollution gradient. Site 1 receives effluent from industries, agricultural activities, informal settlements and municipal sewage treatment plants that settle in Marais Dam, which acts as a sludge dam. Elevated levels of HSP 70 have been measured in tissues of fish exposed to environmental contaminants such as heavy metals (Boone *et al.*, 2002), industrial effluents (Vijayan *et al.*, 1998), pesticides (Sanders, 1993) and polycyclic aromatic hydrocarbons. Elevated HSP 70 levels have been observed in hepatic, head and kidney tissues of coho salmon (*Oncorhnchus kisutch*) infected with *Reibaterium salmoninarium* the causative agent of a chronic disease of salmonids (Basu *et al.*, 2002) thus providing evidence that a relationship exits between heat shock proteins and diseases in fish. In this study only disease free fish were used hence elevated

HSP 70 levels can only be attributed to contaminants in the system. Studies in fish have shown that the appearance and decay of HSPs share a close temporal relationship with the induction and disappearance of thermotolerance (Mooser *et al.*, 1987). Elevated HSP 70 levels in the Rietvlei system are similar to findings by Williams *et al.* (1996) where HSP 70 levels were significantly higher in juvenile rainbow trout exposed to metals in water and diet. This study indicates that *O. mossambicus* employ HSP 70 in their normal course of physiological adaptation to change in environmental conditions. Fader *et al.* (1994) also found that fish (*Ictalurus natalis*) respond to normal environmental variations in their native streams by producing HSP 70. HSP 70 from tissues of a number of fish species share common epitopes (Abukhalaf *et al.*, 1994) hence increased HSP 70 expression in stressed fish enables the detection of stress proteins in ecologically and commercially important fish species and consequently to establish the conditions that may prove hazardous to fish and other organisms. Although HSP 70 is a non-specific indicator of stress the results clearly show in this study that it is a useful and applicable biomarker in the active biomonitoring of the Rietvlei system using *O. mossambicus* as test organism. This provides early warning that the test organisms experience potential stressful conditions. To quantify the ecological relevance of the stress response it will be necessary to carry out more detailed aquatic community studies to determine whether there are any changes in the population structures. However, data were only available for one exposure period, i.e. 4 weeks high-flow and it remains to be seen whether similar results are obtainable following longer, term exposures. It is also essential that research into the development of anti-bodies for determination of HSPs levels in other aquatic organisms such as freshwater mollusks continue.

3.4.3. *Metabolic enzymes*

Enzyme inhibition is a common mechanism of toxicity (Westlake *et al.*, 1983). In this study LDH activity in *M. tuberculata* did not differ at all from the three sites and from the controls but in *O. mossambicuss* the activity of LDH was significantly reduced at site 1 when compared to the other sites and the controls. This is similar to findings by Gill *et al*.. (1990) where the activity of LDH were inhibited by mercury in the rosy barb. The lack of difference in activity in *M. tuberculata* indicates that LDH is not a sensitive biomarker in the Rietvlei system using *M. tuberculata*.

The inhibition of ALP has been used as an indicator of environmental pollution (Linhardt and Walter, 1965). There was no ALP activity in *M. tuberculata* exposed in the Rietvlei system and in the controls. Information on the use of ALP in invertebrates is limited thus the lack of activity in *M. tuberculata* in the Rietvlei system may be because this method is not applicable in these freshwater mollusks.

The increase in LDH activity down the pollution gradient indicates that there are contaminants that inhibit LDH which are more concentrated at site 1 and are less concentrated downstream. Escher *et al.*. (1999), however found higher LDH activity in blood of brown trout (*Salmo trutta fario*) exposed to river and sewage plant effluent. The inhibition of LDH activity in *O. mossambicus* exposed in the Rietvlei system suggest that there are toxicants in the system that inhibit LDH activity although the nature of the toxicants need to be verified.

3.5. Conclusion

This study demonstrated that the selected biomarkers could be used in the active biomonitoring of the Rietvlei system although some biomarkers are less sensitive when using either *M. tuberculata* or *O. mossambicus*. The research hypothesis for this study was that genotoxic and stress proteins could be used effectively in the active biomonitoring of the Rietvlei system using both *M. tuberculata* and *O. mossambicus*. However, it was shown in this study that DNA damage was not a sensitive biomarker in *O. mossambicus* since there were no differences with the controls. This may be because only fish were obtained for the high-flow four weeks sampling period when the effects of the pollutants in the system were not that evident, whereas the fish died during the low-flow exposure. The lack of ALP activity in *M. tuberculata* indicated that this biomarker couldn't be used effectively in the system using this test organism. The loss of test organisms (fish) together with the unavailability of antibodies for *M. tuberculata* for HSP 70 determination made it difficult to give concrete conclusions although it was demonstrated that these biomarkers are applicable in the system. It was shown in this study that there are genotoxic contaminants that are concentrated during the low-flow period. The stress proteins are non-specific biomarkers that can be induced by a variety of contaminants. Their induction shows that the effluent it receives, contaminates the Rietvlei system.

CHAPTER 4

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