

**Physiological and Cytological Biomarker Studies using  
*Perna perna* for Marine Pollution Monitoring**

By

Anisha Rajkumar

Submitted in fulfillment of the academic requirements for the degree of Master of Science in the  
School of Conservation and Biological Sciences,  
University of KwaZulu-Natal  
Durban

December 2008

## **PREFACE**

The experimental work described in this dissertation was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from January 2003 to December 2005, under the supervision of Associate Professor D. J. Marshall. Doctor A. J. Smit supervised the write-up, statistical analysis and compilation of the thesis.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

Anisha Rajkumar

February 2009

## **ACKNOWLEDGEMENTS**

First and foremost, I have to thank my supervisor, Doctor A. J. Smit for all his guidance, patience and invaluable advice. Your input has been a key contribution to this study. I am especially grateful to you for teaching me the key principles of being a good scientist.

I would like to thank Professor D. J. Marshall for his initial contributions to this study, as well as the Zoology Department technical staff members from the former University of Durban Westville for their help during my experimental work.

I would also like to thank the staff of the Natal Sharks Board (NSB) for all their assistance during the transplant study in Durban harbour.

This study was funded by the University of KwaZulu-Natal (UKZN) Research Grants division and the National Research Foundation (NRF). Thank you for making this study possible.

On a special note, I would like to thank my closest friends for their help, encouragement and support throughout this study. Your friendship has meant the world to me.

Thank you to my brother Rajeev Rajkumar, my partner in crime, for keeping me company on those early morning boat outings.

To Anban Velayudan, I will be forever grateful for your loyal support which gave me the motivation to persevere. Thank you for being my light during the bleakest of times.

This thesis is dedicated to my parents, whose love and encouragement carried me through the completion of this study. Thank you for giving me something to inspire to.

## ABSTRACT

Urbanised and industrial coastal areas in South Africa are most vulnerable to the effects of marine pollution, and the Kwa-Zulu Natal coastline is particularly at risk. The mussels *Perna perna*, from a polluted (Isipingo Beach) and unpolluted (Park Rynie) site, and *Brachidontes semistriatus* were evaluated for their use as potential bioindicator organisms. The mussels were subjected to increasing copper concentration treatments to assess the following biomarker responses: cardiac activity, lysosomal membrane stability, malate dehydrogenase enzyme (MDH) activity and body condition index. *Brachidontes semistriatus* exhibited significant variations in biomarker responses only when exposed to higher Cu dosages, whereas *P. perna* from Park Rynie displayed distinct changes in heart rate, lysosomal membrane stability and MDH activity with increasing contaminant exposure. *Perna perna* from Isipingo Beach displayed significant biomarker variation in cardiac activity and lysosomal membrane stability, however differences in MDH activity were only evident at the highest Cu concentration of 100  $\mu\text{g.L}^{-1}$ . Both species from the different Cu treatments failed to show any significant changes in body condition indices due to the limited time of contaminant exposure. The mussel *P. perna* was therefore selected as a suitable biomonitoring species, and cardiac activity, lysosomal membrane stability and body condition index were chosen as reliable biomarkers for the study. Native *P. perna* from KZN responded to a distinct pollution gradient along the coastline by displaying significant bradycardia, reduced lysosomal membrane stability, poor condition indices and high heavy metal tissue concentrations. Durban, Isipingo and Umkomaas were singled out as the most contaminated sites along the coast, and Zinkwazi and Park Rynie as the least polluted. In addition, significant correlations between tissue and sediment metal concentrations suggest that the species is an effective heavy metal bioaccumulator of Cd, Pb, Zn, Cr and Fe. Similar spatial trends in seawater and mussel tissue concentrations in Durban Harbour were identified. Stations in the port located nearest to the freshwater inputs and stormwater drains displayed the highest metal concentrations in tissue and seawater, as well as adverse biomarker responses from transplanted *P. perna*. These results suggest that Durban Harbour is strongly influenced by tidal exchange and contaminated freshwater inflow entering the harbour. It was also found that reproduction imposes a considerable effect on *P. perna* body condition as spawning events in winter months result in pronounced body mass loss. The study concluded that *P. perna* is a highly effective bioindicator species, and cardiac activity, lysosomal membrane stability and body condition index can successfully be employed in marine pollution monitoring programmes.

## TABLE OF CONTENTS

<b>CHAPTER 1: General Introduction .....</b>	<b>1</b>
1.1 Cardiac activity .....	5
1.2 Lysosomal membrane stability .....	6
1.3 Body condition index .....	7
1.4 Malate dehydrogenase enzyme activity .....	8
1.5 Heavy metal bioaccumulation.....	8
1.6 Introduction to the study area.....	10
1.7 Aims and objectives of chapters .....	11
 <b>CHAPTER 2: Physiological, Cytological and Biochemical Responses to Copper</b>	
<b>Exposure of the Mussels <i>Perna perna</i> and <i>Brachidontes semistriatus</i> .....</b>	<b>13</b>
2.1 Introduction.....	13
2.2 Materials and methods .....	17
2.3 Results.....	20
2.4 Discussion .....	24
2.5 Conclusion .....	30
 <b>CHAPTER 3: Biomonitoring of Heavy Metals and Biomarker Responses from</b>	
<b><i>Perna perna</i> in Kwa-Zulu Natal Coastal Waters .....</b>	<b>32</b>
3.1 Introduction.....	32
3.2 Materials and methods .....	37
3.3 Results.....	42
3.4 Discussion .....	50
3.5 Conclusion .....	57

<b>CHAPTER 4:</b>	<b>Spatial variations in physiological and cytological biomarkers from</b>	
	<b><i>Perna perna</i> transplanted in Durban Harbour (KZN)</b>	<b>58</b>
4.1	Introduction.....	58
4.2	Materials and methods .....	60
4.3	Results.....	63
4.4	Discussion .....	72
4.5	Conclusion .....	85
<b>CHAPTER 5:</b>	<b>Conclusion and Recommendations for Future Studies</b>	<b>86</b>
5.1	Biomonitoring.....	86
5.2	<i>Perna perna</i> as a biomonitoring species .....	87
5.3	Cardiac activity as a bioindicator of marine pollution .....	88
5.4	Lysosomal membrane stability as a biomarker .....	88
5.5	Body condition index of <i>P. perna</i> as a biomarker as an indication of polluted environments .....	89
5.6	The use of malate dehydrogenase enzyme activity as a suitable biomarker response .....	90
5.7	Heavy metal bioaccumulation in <i>P. perna</i> .....	91
5.8	Key issues on experimental design .....	92
5.9	Concluding remarks .....	92
<b>REFERENCES</b>		<b>94</b>

## LIST OF FIGURES

<b>Figure 1:</b>	Median heart rates from <i>B. semistriatus</i> and <i>P. perna</i> collected from Park Rynie and Isipingo Beach. ....	21
<b>Figure 2:</b>	Median lysosomal membrane stability from <i>B. semistriatus</i> and <i>P. perna</i> collected from Park Rynie and Isipingo Beach. ....	22
<b>Figure 3:</b>	Body condition indices from <i>B. semistriatus</i> collected at Park Rynie and <i>P. perna</i> collected from Park Rynie and Isipingo Beach. ....	23
<b>Figure 4:</b>	Median MDH enzyme activities from <i>B. semistriatus</i> and <i>P. perna</i> collected from Park Rynie and Isipingo Beach. ..	23
<b>Figure 5:</b>	KZN coastline showing sampling sites and major rivers that drain the province.....	39
<b>Figure 6:</b>	Spatial variations in metal concentration in sediment sampled from sites along the KZN coastline. ....	43
<b>Figure 7:</b>	MDS representation of differentiation of sits by tissue metal concentrations. ....	45
<b>Figure 8:</b>	Spatial variations in soft tissue metal concentration in <i>P. perna</i> retrieved from sites along the KZN coastline..	46
<b>Figure 9:</b>	Correlations between <i>P. perna</i> tissue metal concentrations and sediment metal concentrations. ....	48
<b>Figure 10:</b>	Spatial variations in mean heart rate from <i>P. perna</i> along the KZN coastline. ....	49
<b>Figure 11:</b>	Spatial variations in mean lysosomal membrane stability from <i>P. perna</i> along the KZN coastline. ....	50
<b>Figure 12:</b>	Spatial variations in mean body condition indices from <i>P. perna</i> along the KZN coastline..	51

<b>Figure 13:</b>	Map of Durban Harbour showing sampling stations. ....	61
<b>Figure 14:</b>	Spatial variations of Cu, Cd, Pb, Zn, Cr, Fe and Ni in seawater samples collected at transplant and control stations at the end of the transplant period.....	64
<b>Figure 15:</b>	Between station variations in soft tissue metal concentrations in <i>P. perna</i> retrieved from Durban Harbour and Park Rynie (control).....	66
<b>Figure 16:</b>	Linear regression relationships between log-transformed cardiac activities over time for transplanted <i>P. perna</i> . ....	68
<b>Figure 17:</b>	Linear regression relationships between lysosomal membrane stabilities over time from transplanted <i>P. perna</i> . ....	70
<b>Figure 18:</b>	Linear regression relationships between log-transformed body condition indices over time. ....	71



## LIST OF TABLES

<b>Table 1:</b>	Review of marine biomonitors species and their sources of anthropogenic contaminants.....	3
<b>Table 2:</b>	Natural and anthropogenic sources / uses of metals .....	9
<b>Table 3:</b>	Summary of biomarkers employed in this study and predicted response to pollutants.....	10
<b>Table 4:</b>	Summary of findings from previous studies on the effects of copper on marine mussels .....	16
<b>Table 5:</b>	Sources of common contaminants in the marine environment.....	33
<b>Table 6:</b>	Metal contaminant target sites in Mytilidae mussel tissues.....	35
<b>Table 7:</b>	Summary of the main effluent discharge points in close proximity to selected study sites.....	38
<b>Table 8:</b>	Measured and certified values for standard reference materials.....	40
<b>Table 9:</b>	Pearson's correlation coefficients between trace metal concentrations and biomarker responses.....	51
<b>Table 10:</b>	Mean physico-chemical water quality parameters of transplant sites over 2002 – 2007 during winter (June – July) and summer (December – January) months.....	67

## CHAPTER 1: General Introduction

South Africa has an estimated population of 40 million people, 30% of which reside along the coastline (Taljaard *et al.*, 2006). The extensive rate of urbanisation and industrialisation has introduced an array of toxic xenobiotics to the coastal waters of the country, mainly through the discharge of sewage, domestic waste water, industrial effluent, dumping of dredged materials and contaminated runoff from land (Moldan, 1995; Misheer *et al.*, 2006a,b,c; Taljaard *et al.*, 2006). Although there has been an increasing awareness for the urgency of marine conservation in the country, there is a substantial lack of marine pollution research in South Africa (Connell, 1988; Anandraj *et al.*, 2002; O'Donoghue and Marshall, 2003). The effects of pollution on the South African marine environment remain uncertain, and current monitoring procedures are inadequate (Anandraj *et al.*, 2002; O'Donoghue and Marshall, 2003). This prompted studies to identify the brown mussel *Perna perna* as a suitable biomonitor for use in South Africa's marine pollution biomonitoring programmes, using a suite of biomarker tests. The procedures for these biomarkers were first assessed in the laboratory to correlate a link between contaminant exposure and biomarker response, and thereafter the biomarkers were tested in field surveys.

Traditional marine pollution techniques include investigating species diversity and abundance, toxicity tests and analysing both sediment and water chemistry. Such techniques are inadequate as they are time consuming, expensive to perform and require specialised laboratory skills (Wedderburn *et al.*, 2000; Wells *et al.*, 2001). In addition, routine chemical analysis only provide specific information on contaminants present in both sediment and the water column, as well other details such as water temperature, salinity, oxygen and nutrient content of the water. However, this approach does not consider those contaminants present in undetectable levels in the environment, and neither does it provide information on the biological effects of these pollutants on biota (Soto *et al.*, 1995; Smolders *et al.*, 2003). In addition to these shortcomings, it is not feasible to conduct chemical analyses on a regular basis. Therefore the effects of a sporadic event such as adverse weather conditions, accidental leaks and spills will not be considered unless a chemical survey is undertaken directly after the event occurs (Shugart *et al.*, 1992).

In light of this, there has been a growing demand for marine pollution monitoring techniques that are quick, uncomplicated, inexpensive and sensitive enough to highlight the impacts on biota. In response to this need, monitoring techniques have evolved into low cost and easy to

use tools that provide rapid results (Galloway *et al.*, 2002). Possessing these attributes, biomarkers have successfully become a part of international marine pollution monitoring protocol (Shugart *et al.*, 1992; Abessa *et al.*, 2005). A biomarker can be defined as a xenobiotically-induced biological response from biota exposed to anthropogenic chemicals (Shugart *et al.*, 1992; Lagadic *et al.*, 1994; Handy *et al.*, 2003). Although they have been in use in marine pollution programmes for more than 35 years, biomarkers only gained global recognition in recent years (Handy *et al.*, 2003). These sensitive and useful tools measure a variety of responses from biota on different organism levels such as molecular, cellular, biochemical and physiological attributes (Shugart *et al.*, 1992; Moore *et al.*, 2006), thus providing an integrated approach on assessing the impacts of marine pollution that can be applicable over a wide range of both species and ecosystem types. Not only do biomarkers indicate the presence of toxic contaminants that may occur in too minute concentrations to be detected by chemical analysis, they also signify the presence of contaminants that are biologically available to biota and those that have already degraded in the environment (Handy *et al.*, 2003).

One other vital role of biomarkers is their service as an early warning distress signal that indicates contamination before irreversible damages can occur to ecosystems (Shugart *et al.*, 1992; Riveros *et al.*, 2002; Luk'yanova, 2006; Moore *et al.*, 2006; Nigro *et al.*, 2006). Once organisms are exposed to anthropogenic stress, the first variation in bodily response occurs on a molecular and cellular level, followed by changes on biochemical and physiological levels, after which changes to the entire body of the organism are observed (Shugart *et al.*, 1992). Biomonitoring is the evaluation of such biomarkers from biota in polluted environments (Boening, 1997) and serve to display the effects on individual organisms through to populations, communities and ecosystems.

Selecting a suitable organism from which biomarkers will be assessed is important as certain criteria must be met. Rainbow (1995) postulated that the selection of a biomonitor species should be dependent on the contaminant source that will be tested. Table 1 provides a brief description of biomonitors used in previous studies, and the main sources of anthropogenic contaminants these organisms are naturally exposed to.

According to Hennig (1984), Widdows and Donkin (1989), Ravera (2001) and Boening (1997) and Smolders *et al.* (2003), suitable biomonitor species should have the following traits:

- Wide geographic distribution and occur abundantly.

- Sedentary or have restricted movement.
- Long-lived to accommodate long-term studies.
- Survive laboratory conditions.
- Tolerant to a wide variety of contaminants that accumulate within body tissues.
- Easy to handle and able to cope with a fair amount of handling stress during collection and transplant experiments.
- Easily transplantable and survive prolonged captivity periods.
- Extensively studied in terms of their physiological responses and adaptations to intrinsic and extrinsic factors.

**Table 1:** Review of marine bioindicators and their sources of anthropogenic contaminants

<b>Bioindicator</b>	<b>Method / organ of contaminant acquisition</b>	<b>Source of contaminant</b>	<b>Reference</b>
Macroalgae	Easy absorption through large, free surfaces	Dissolved metals in seawater	Rainbow, 1995
Marine mussels, oysters and barnacles	Epibenthic suspension feeding	Dissolved metals and suspended particles in seawater	Rainbow, 1995 Wang and Fisher, 1999 Ravera, 2001
Deposit feeding bivalves	Newly deposited particles and seawater are taken up by inhalant siphons during feeding and respiration	Seawater, newly deposited particles on surface. Shell protects organism from contact with sediment	Rainbow, 1995 Wang and Fisher, 1999
Burrowing polychaetes	Soft tissues in contact with sediment, interstitial water and overlaying water currents. Suspension or deposit feeding.	Contaminated sediment, interstitial water and overlaying water. Suspended particles, newly deposited particles or contaminated food	Rainbow, 1995 Wang and Fisher, 1999
Amphipods	Detritivorous species that ingest sediment	Contaminated food, sediment and dissolved metals in seawater	Rainbow, 1995 Ravera, 2001
Lamellibranches	Filter feeding	Dissolved metals in seawater	Ravera, 2001
Gastropods (Prosobranchs)	Deposit feeding	Sediment particles	Ravera, 2001
Phytoplankton	Phytoplankton possesses free surfaces for absorption.	Dissolved metals in seawater	Ravera, 2001

Although various species have been employed as biomonitors, few meet all the above requirements of a model biomarker as suitably as marine bivalves (Goldberg, 1986; Widdows and Donkin 1989; Rainbow, 1995; Boening, 1997; Nasci *et al.*, 1999; Ravera, 2001; Smolders *et al.*, 2003; Domouhtsidou *et al.*, 2004). It is these characteristics that have lead to bivalves becoming prevalent in current marine biomonitoring programmes, as they are widespread in most marine ecosystems around the world (Smolders *et al.*, 2003; Domouhtsidou *et al.*, 2004). Mussels can be used in studies during all their life stages and their sedentary nature as well as their high bioaccumulation ability makes these specimens ideal study subjects. Being filter-feeders, they are exposed to suspended food and seawater, thereby providing an integrated measure of pollutant contaminant (Goldberg, 1986; Widdows and Donkin, 1989; Boening, 1997; Khessiba *et al.*, 2001; Domouhtsidou *et al.*, 2004; Nicholson and Lam, 2005).

Mussels were first established as biomarkers by Goldberg in 1975 in the Mussel Watch Programme, which aimed to analyse chemical contaminants within the marine environment and provide a better understanding of the quality of the marine water (Goldberg, 1986; Smolders *et al.*, 2003). The project was so successful in monitoring contaminants present in the coastal zones of USA that the programme was adopted internationally in the 1990s, thereby starting a new trend in biomonitoring techniques (Ravera, 2001).

Since then, mussels from the family Mytilidae have become well-established biomonitors (Nicholson, 1999a,b; Sze and Lee, 2000; Anandraj *et al.*, 2002). The brown mussel *P. perna*, native to the South African coast, also occurs in Venezuela, Brazil, Uruguay, Madagascar, Argentina and the West Indies (Anandraj *et al.*, 2002; Rajagopal *et al.*, 2003), and has already been the subject of numerous ecotoxicology studies undertaken around the world. Trace metal concentrations in soft tissues as well as byssus threads of *P. perna* have been successfully studied by Szefer *et al.*, 1997a,b; Costa *et al.*, 2000; Joiris *et al.*, 2000; Anandraj *et al.*, 2002; Baraj *et al.*, 2004; Rajagopal *et al.*, 2003; Sidoumou *et al.*, 2006. These mussels were also investigated for the accumulation of polycyclic aromatic hydrocarbons (PAHs), organochlorides (pesticides and PCBs) and furan by Alves *et al.* (2002), Otchere (2005) and Francioni *et al.* (2007) respectively.

Gregory *et al.* (1999; 2002) demonstrated the drastic effects of chronic exposure to metals on the gill morphology of *P. perna*, and Barracco *et al.* (1999) studied the morphology and chemistry of their haemocytes extensively and examined the role of phagocytosis in the species. Ferreira and Salomao (2000) studied the ionic sensitivity of the species' cardiac activity, and

biochemical responses from *P. perna* was researched by Bainy *et al.* (2000). The species' susceptibility of DNA damage of its digestive and mantle tissues was examined by de Almedia *et al.* (2003), and Stucchi-Zucchi and Salomao (1998) investigated how *P. perna* responded to chronic salinity stress and hyposmosis. The effects of temperature and hypoxia were well demonstrated by Hicks and McMahon (2002), and seasonal fluctuation in their biological functions by Dafre *et al.* (2004) and Almeida *et al.* (2003). This study focuses on highlighting the usefulness of *P. perna* as a biomonitor. The following biomarkers were used to establish *P. perna* as a suitable biomonitor:

- Cardiac activity.
- Lysosomal membrane stability.
- Body condition index.
- Malate dehydrogenase enzyme activity.
- Heavy metal bioaccumulation.

#### *Cardiac activity*

Previous studies have investigated the use of various physiological responses from invertebrates as biomarkers of contaminant exposure. Such responses include alterations in respiration, oxygen consumption, valve closure, pumping and filtration rates, growth, gonad maturity, cardiac activity and body condition indices (Wedderburn *et al.*, 2000). Cardiac activity is rapidly becoming a popular technique that is used in marine pollution monitoring programmes, especially since advances in technology has produced a non-invasive method of recording the heart rates of organisms (Wedderburn *et al.*, 2000; Bakhmet and Khalaman, 2006). The close link between heart rate and metabolic activity provides a useful technique to evaluate the physiological integrity of the organism (Abessa *et al.*, 2005). Marine mussels have an open circulatory system that consists of haemolymph vessels and sinuses that are regulated by a three chambered heart (Nicholson, 1999a,b). Mussels exhibit little fluctuation in cardiac activity, except when exposed to stress such as exposure to contaminants (Sabourin and Tullis, 1981; Nicholson, 1999a,b; Curtis *et al.*, 2000, 2001; Brown *et al.*, 2004; Marshall *et al.*, 2004; Abessa *et al.*, 2005). This is attributed to the lack of endothelial tissues in the bivalve auricular and ventricular cavities, which allows the heart to be in direct contact with contaminant loaded haemolymph (Nicholson 1999a,b). Hence, any significant alteration in cardiac activity can be attributed to an external stress such as contaminant exposure (Nicholson, 1999a,b; Curtis *et al.*, 2000; Brown *et al.*, 2004; Marshall *et al.*, 2004; Abessa *et al.*, 2005). This characteristic has made cardiac activity of marine mussels a widely used biomarker (Nicholson, 1999a,b; Curtis *et al.*, 2000, 2001; Brown *et al.*, 2004; Marshall *et al.*, 2004; Bakhmet and Khalaman, 2006).

Studies conducted by Nicholson (1999b) and Abessa *et al.* (2005) showed that some marine bivalves may display no significant variation in heart rate when exposed to increasing copper concentrations, due to the uptake of metals by humoral ligands, metallothionein binding and sequestration of the contaminant by lysosomes circulating in the haemolymph. It is postulated that the metal remaining in the haemolymph was not a high enough dosage to induce effects on the myocytes or cardio-regulatory neurons that would result in a change in the cardiac activity (Nicholson, 1999b; Abessa *et al.*, 2005). Another study by Nicholson (1999b) and Brown *et al.* (2004) showed mussels displaying elevated heart rates (tachycardiac), which was a result of elevated metabolic rates in order to rapidly detoxify and excrete the toxins from the organism's body (Nicholson, 1999b; Brown *et al.*, 2004; Marshall *et al.*, 2004). Another hypothesis that would explain such an elevation of heart rates is the increase in the organisms' body work rate in an attempt to atone for the impairment of other body organs by metal toxicity (Nicholson, 1999a). Such elevations in cardiac output has only been found in mussels exposed to low concentrations of contaminants (Nicholson, 1999a; Brown *et al.*, 2004) as the pollutant dosage was far too small to produce an immediate decline in heart rates. Nonetheless a rapid increase in heart rate will result in great energy loss to the organism and also inflict severe damage to the surrounding heart tissues and muscles (Nicholson, 1999a). Most studies on bivalve cardiac activity have reported a decline in heart rate (bradycardia) when mussels were exposed to metals (Nicholson, 1999b; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Galloway *et al.*, 2002; Marshall *et al.*, 2004), as a result of severe impairment of heart's nervous control system. The effects of contamination on the cardiac activity of mussels have now become widely studied (Nicholson, 1999a,b; Curtis *et al.*, 2000; Galloway *et al.*, 2002).

#### *Lysosomal membrane stability*

Lysosomes are membrane-bound organelles that are dominant in the digestive tissues of invertebrates (Lowe *et al.*, 1995; Wedderburn *et al.*, 2000; Matozza *et al.*, 2001; Petrovic *et al.*, 2004; Moore *et al.*, 2006). They have the primary function of breaking down biological material as lysosomes contain hydrolytic enzymes (Marigomez *et al.*, 1998; Kagley *et al.*, 2003; Nicholson, 2003; Moore *et al.*, 2006). Previous studies by Nicholson (2001; 2003) and Lowe *et al.* (1995) have shown that once organisms are exposed to anthropogenic chemicals, they accumulate an excessive amount of contaminants within their lysosomes, which becomes enlarged, rendering the lysosomal membrane non-functional. This occurrence of reduced lysosomal membrane stability is used as a biomarker of exposure to pollutants as the membrane instability is correlated with the concentration of the chemical contaminant (Lowe *et al.*, 1995; Marigomez *et al.*, 1998; Kagley *et al.*, 2003; Nicholson, 2003; Moore *et al.*, 2006). Since the

inside environment of lysosomes are maintained by a membrane  $\text{Mg}^{2+}$  ATPase-dependent  $\text{H}^{+}$  ion pump (Lowe *et al.*, 1995), excessive contaminant accumulation and lysosome enlargement will render the pump dysfunctional which consequents in an increase in pH within the lysosome. This in turn will create an equilibrium between the inner organelle contents and the cytosol exterior to the organelle, allowing the free passage of lysosomal contents to the organelle exterior (Lowe *et al.*, 1995; Nicholson, 2003). A method indicating a reduction of lysosomal membrane stability was developed by using the organelles' ability to take up and retain a neutral red dye, and as the stability of the lysosomal membrane decreases with increasing contaminant concentration, the time period for the dye to leak out of the damaged cell is monitored (Lowe *et al.*, 1995; Marigomez *et al.*, 1998; Viarengo *et al.*, 2000; Wedderburn *et al.*, 2000; Matozza *et al.*, 2001; Nicholson, 2001; Galloway *et al.*, 2002; Moore *et al.*, 2006).

### *Body condition index*

Factors such as temperature, salinity and food availability have different effects on the physiological responses of marine bivalves and have lead to investigations of various indices that evaluate the health of the organism (Nicholson, 1999b; Orban *et al.*, 2002); such indices include body condition index, metal burden index, and organ somatic indices. Studies by Dahlhoff and Menge (1996) and Nicholson (1999b) state that an unfavourable habitat condition, such as exposure to anthropogenic chemicals, induces a considerable degree of stress on the organism. This in turn will result in the organisms attempting to cope with such conditions, thus depleting energy that would otherwise be reserved for growth, reproduction and numerous other physiological body processes. There are numerous body condition indices that currently exist in international marine biomonitoring programmes (Lundebye *et al.*, 1997; Smolders *et al.*, 2003), which include the ratio between dry mass of soft tissue and internal shell cavity volume, wet mass of soft tissue and internal shell cavity volume, dry mass of soft tissue and shell length, and lastly dry mass of soft tissue and whole shell volume. Nevertheless, a detailed study by Lundebye *et al.* (1997) on the different body condition indices mentioned, revealed similar results between all of the condition ratios. Body condition index has proved to be a useful biomarker as mussels exposed to contaminants exhibit poor growth rates and body condition indices (Lawrence and Scott, 1982; Avery *et al.*, 1996; Dahlhoff and Menge, 1996; Lundebye *et al.*, 1997; Nicholson, 1999b; Orban *et al.*, 2002; Galloway *et al.*, 2002; Smolders *et al.*, 2003; Amiard *et al.*, 2004).



### *Malate dehydrogenase enzyme activity*

Biochemical responses from biota exposed to contaminants are becoming increasingly popular as biomarkers, specifically enzymatic biomarkers that are used in invertebrate marine biomonitors (Lushchak *et al.*, 1997; Fahraeus-Van Ree and Payne, 1999; Orbea *et al.*, 1999; Dahlhoff *et al.*, 2001; Dahlhoff *et al.*, 2002; Luk'yanova, 2006). They are indicators of metabolic activity of organisms (Dahlhoff, 2004), and are both directly and indirectly associated with biological functions that are vital for survival. Evaluation of the metabolic condition of organisms is a key component of understanding the extensive effects of environmental contamination on biota. Studies by Regoli *et al.* (1998), Dahloff (2003) and Kagley *et al.* (2003) document the use of metabolic enzymes as early warning indicators of chemical pollutants. Exposure to heavy metals have been shown to induce stress on enzyme processes such as protein degradation, lipid peroxidation of tissues and chemical carcinogenesis (Regoli *et al.*, 1998), as well as causing DNA damage and the inhibition of metabolic enzyme activities (Khessiba *et al.*, 2001). Malate dehydrogenase (MDH) is one such metabolic enzyme that has been the subject of numerous studies (Mizrahi and Achituv, 1989; Dahlhoff *et al.*, 2001; Dahlhoff *et al.*, 2002). The enzyme plays a vital role in the citric acid cycle and in the presence of MDH, malate is oxidized by nicotinamide adenine dinucleotide (NAD) to oxaloacetate (Mizrahi and Achituv, 1989; Cunningham *et al.*, 1997). The quantity of NADH that is formed in the reaction is thus proportional to the concentration of malate converted; therefore the reaction is monitored colorimetrically by measuring the rate of oxidation of NADH. Previous studies demonstrate a direct link between MDH activity and the overall metabolic activity of organisms (Cunningham *et al.*, 1997; Dahlhoff *et al.*, 2001; Dahlhoff *et al.*, 2002).

### *Heavy metal bioaccumulation*

Metals are widely used for various anthropocentric applications, some of which are outlined in Table 2, and those existing in the marine environment can be measured by a comprehensive chemical analysis of the water column and sediment (Soto *et al.*, 1995); however this methodology is inadequate as it can elude information on pollutants present in undetectable levels in the environment and little information on the biological effects of these contaminants on the organisms from polluted habitats. Most aquatic invertebrates have the ability to accumulate anthropogenic chemicals from their surrounding environment within their body tissues; however the extent of the accumulation between species may vary (Rainbow, 2002). Although heavy metals occur naturally in the marine environment, the contaminants discharged from anthropogenic activities such as waste disposal, industrial and sewage effluent, may be

sequestered from the water column and be deposited in sediment (Chong and Wang, 2000; Gregory *et al.*, 2002).

**Table 2:** Natural and anthropogenic sources / uses of metals (Atkins *et al.*, 1988).

<b>Metal</b>	<b>Natural occurrence</b>	<b>Common uses or sources of metal</b>
Cu	Weathering of rocks and mineral ores.	Electrical equipment, copper piping, treated sewage effluent, algacides, metal-plating, smelting, wood preservatives and anti-fouling paints on the hulls of ocean vessels.
Cd	Weathering of rocks, erosion of the earth's crust and deep sea volcanism.	Stabilisers for PVCs and pigments in plastics, alloy and glass manufacture, as well as in electroplating, treated sewage and smelting of non-ferrous metal ores.
Pb	Natural weathering of sulphide ores, forest fires and volcanism.	Emissions from motor vehicles, milling, mining, smelting of metals, industrial and domestic waste water, industries involved in the manufacture of car batteries, paints and pigments.
Zn	Weathering of rocks and mineral ores.	Galvanising, the manufacture of paints and dyes and paper milling.
Cr	Weathering of rocks and mineral ores.	Metal plating, manufacture of explosives and in the leather, paper, ceramic and paint industries.
Fe	Weathering of rocks and sulphide ores.	Manufacture of household detergents, petrochemicals and fungicides, burning of fossil fuel, mineral processing, sewage and corrosion of iron and steel.
Ni	Volcanic activity, forest fires and soil erosion.	Sewage sludge, incineration activities, electroplating, burning of fossil fuel, manufacture of asbestos, steel and cement, as well as emissions from Ni mining and refining.

A comprehensive study by Chong and Wang (2000) demonstrated the ability of marine mussels to assimilate heavy metals from sediment, and highlighted the significance of sediment ingestion by marine bivalves as major contributor of metal uptake. Although the measurement of metal concentrations from body tissue of mussels is a widely used biomarker, the biomarker has to be accompanied by other biomarkers as a fraction of the metals taken up by organisms is used by physiological and metabolic processes such as growth, excretion and reproduction. In addition, abiotic factors such as salinity, temperature and seasonality can affect metal

accumulation rates in mussels (Soto *et al.*, 1995; Boening, 1997; Francioni *et al.*, 2004). Nonetheless, the analysis of tissue concentrations as biomarkers remain a popular and informative approach that identifies toxic contaminants accumulated within soft body tissues (Shugart *et al.*, 1992; Boening, 1997; Ravera, 2001).

Table 3 provides a summary of all the biomarkers used in this study.

**Table 3:** Summary of biomarkers employed in this study and predicted response to pollutants.

<b>Biomarker</b>	<b>Category of biomarker</b>	<b>Documented response to anthropogenic contaminants</b>
Lysosomal membrane stability	Cytological	Significant reduction of lysosomal membrane stability
Cardiac activity	Physiological	Mild tachycardia to low levels of contaminants or significant bradycardia to high levels of contaminants
Body condition index	Physiological	Significant decline in growth and condition indices
Malate dehydrogenase activity	Biochemical	Significant MDH enzyme inhibition
Heavy metal bioaccumulation	Bioaccumulation	Significant metal accumulation within soft tissues and organs.

#### *Introduction to the study area*

Kwa-Zulu Natal has approximately 239 known freshwater outlets that exit into the ocean, which range from drains to rivers (Begg, 1978). However, only 73 of these outlets are considered to be significant rivers and estuaries. One such estuary is the Port of Durban, which was originally used for anchorage in the 1820s. However, the ever-increasing trade route recognised the estuary as a prime location for a permanent harbour. Thus the development of the harbour initiated the growth of a heavily industrialised and urbanised city on the periphery of the port. Despite its economic value to the city, the development and expansion of the harbour has had a negative impact on the coastal environment. The water quality of the rivers draining the city and emptying into the bay is in poor condition (Begg, 1978). Similar developments of urban and

industrial nodes along KZN's estuaries and coastline are also affecting their immediate coastal environments (Begg, 1978; Kalicharran and Diab, 1993; Grobler *et al.*, 1996). Durban South, in particular, is noted for being heavily industrialised with separate industrial outfalls along the coast.

Hence this study attempts to assess the pollution levels along the coast of Kwa-Zulu Natal, as well as Durban harbour, by employing *P. perna* as a bioindicator species.

#### *Aims and objectives of each chapter*

Chapter 2 is designed to test the hypothesis that exposure of mussels *P. perna* and *B. semistriatus*, sampled from two different sites on the Kwa-Zulu Natal coast, to increasing metal concentrations will induce alterations in physiological, cytological and biochemical responses. This chapter therefore aims to select a potential bioindicator from the two South African mussel species by investigating the following biomarker responses from specimens exposed to varying copper dosages:

1. Cardiac activity.
2. Lysosomal membrane stability.
3. Body condition index.
4. Malate dehydrogenase enzyme activity.

Chapter 3 tests the hypothesis that biomarker responses from *P. perna* can demonstrate a pollution gradient along the KZN coastline. Biomarkers were tested in a field survey of *P. perna* from Kwa-Zulu Natal coastal waters in order to provide an evaluation on the environmental state of the province's intertidal zones, and to firmly establish the species *P. perna* as a biomonitor for future pollution monitoring programmes in South Africa. The aims for this chapter are to:

1. Assess and compare the pollution status of KZN's coastline.
2. Investigate the usefulness of applying a suite of biomarker tests to evaluate the health of the marine environment.
3. Investigate whether *P. perna* is a suitable biomonitoring species in a field application by evaluating:
  - Heavy metal bioaccumulation in mussel soft tissues.
  - Cardiac activity.
  - Lysosomal membrane stability.
  - Body condition index.

Chapter 4 tests the hypothesis that biomarker responses from transplanted *P. perna* can demonstrate an underlying pollution gradient in Durban harbour. Cytological and physiological biomarker responses from *P. perna* transplanted to sites within the Port of Durban, were assessed to determine the environmental condition of the bay. Biomarker responses were also assessed for spatial differences. Thus this chapter aims to assess the health of the marine environment in Durban harbour by investigating:

1. Water quality in Durban harbour by identifying spatial trends in seawater metal concentrations.
2. The suitability of *P. perna* in transplant experiments by investigating:
  - Heavy metal bioaccumulation in *P. perna*.
  - Cardiac activity.
  - Lysosomal membrane stability.
  - Body condition index.
  - Spatial trends in biomarker responses.

Chapter 5 discusses the results and concluding remarks from the previous three chapters to assess if all research aims and objects have been successfully accomplished, provide recommendations and to conclude the study.

## **CHAPTER 2: Physiological, Cytological and Biochemical Responses to Copper Exposure of the Mussels *Perna perna* and *Brachidontes semistriatus***

### **Introduction**

Marine pollution has become of increasingly global concern. In recent years, rapid industrial growth and urbanisation has continuously introduced an array of toxic pollutants to the environment, which have the potential to cause dramatic impacts on ecosystems. Adverse biological effects such as reproductive failure, endocrine disruption and impairment of immune systems of aquatic biota have become well associated with man-made pollutants (Fossi *et al.*, 2002). The issue is exacerbated by the widespread presence of persistent toxic organic contaminants, which are bioaccumulative in body tissue and biomagnify through food chains existing in ecosystems (Rivera, 2001). Also, as a result of their slow degradation rates in the environment, these organic pollutants and their residues cause long-term effects, even long after the repository source has been removed (Handy *et al.*, 2003). Marine pollution monitoring has subsequently received global interest, and monitoring techniques are being extensively developed in an effort to assess, control and protect the stability of the environment.

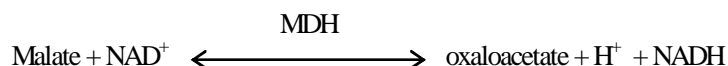
Much attention is currently drawn to marine organisms, their ability to accumulate contaminants within their body tissues, and their ability to display distinct physiological and cytological responses to varying levels of pollutant exposure. Mussels have been extensively studied and found to have significant potential as biological indicators compared to other aquatic organisms (Anandraj *et al.*, 2002; Smolders *et al.*, 2003). The brown mussel *P. perna* (Linnaeus, 1758), is native to the South African coast (Anandraj *et al.*, 2002; Rajagopal *et al.*, 2003), and has already been used in ecotoxicology studies undertaken around the world. The genus *Brachidontes* is also widely distributed in tropical and subtropical regions, (Rajagopal *et al.*, 2003), however the only documented occurrences of *B. semistriatus* (Krauss, 1848) occur on the east coast of South Africa and Mozambique (Branch *et al.*, 1994) as well as reported findings in the Suez Canal and eastern Mediterranean ocean (Kilburn and Rippey, 1982). Nevertheless, little work has been done on this species. Likewise, ecotoxicological studies in assessing and monitoring marine pollution in South Africa has been considerably neglected, and fallen behind the advances made by the rest of the global community (O'Donoghue and Marshall, 2003). This prompted investigations using these two mussel species as bioindicators and assessing biomarker responses from the mussels when exposed to varying concentrations of copper.

Infrared monitoring of cardiac activity is a non-invasive monitoring technique, developed by Depledge and Anderson (1990), that is extensively used in ecotoxicological experiments (Nicholson, 1999a; Curtis *et al.*, 2000; Galloway *et al.*, 2002; Brown *et al.*, 2004; Marshall *et al.*, 2004; Pereira *et al.*, 2007). This method requires that a sensor which encases a small infrared light emitter and phototransistor detector was attached onto the exterior shell surface of the animal in a position nearest to the organism's heart. The infrared light continuously illuminates the molluscan heart, whilst the phototransistor detects and transfers fluctuations in the heart rate to an oscilloscope. Variations in cardiac activity has become the subject of detailed studies in both laboratory and field tests assessing the biological effects of marine pollution (Nicholson, 1999a,b; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Brown *et al.*, 2004; Marshall *et al.*, 2004; Abessa *et al.*, 2005; Pereira *et al.*, 2007). Evidence from such studies shows a direct relationship between heart rate and exposure to varying levels of chemical pollutants, as otherwise mussels exhibit very little fluctuations in cardiac activity, except during valve closure (Nicholson, 1999a,b; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Marshall *et al.*, 2004). Significant fluctuations of heart rate can therefore be attributed to variations in the ambient environment such as the presence of chemical contamination, thus serving as a useful biomarker in monitoring pollution.

Lysosomes are concentrated in the digestive tissues of invertebrates (Lowe *et al.*, 1995; Nicholson, 2001; Moore *et al.*, 2006) and their main physiological function is the breakdown of food. These organelles are also capable of accumulating high amounts of contaminants, and are involved in contaminant detoxification (Lowe *et al.*, 1995; Matozzo *et al.*, 2001; Nicholson, 2001). However numerous studies have found that if the intracellular accumulation of chemical contaminants is excessive the health of the lysosomes deteriorates, and in doing so exhibit distinct stress signals, one of which is a reduction of lysosomal membrane stability (Lowe *et al.*, 1995; Nicholson, 2001; Galloway *et al.*, 2002). Lysosomal membrane stability is assessed by measuring the neutral red retention time of lysosome membranes. In such assays, lysosomes are incubated in neutral red dye solution, which is allowed to diffuse into, and thereafter be retained within the organelles. Membrane stability has been proven to decrease with exposure to contamination; therefore the stability capacity of lysosomes exposed to pollutants will be reduced (Nicholson, 1999a; Viarengo *et al.*, 2000; Galloway *et al.*, 2002). This process of dye retention and leakage is monitored over time under a microscope. Used as a biomarker, the assay has proven to be a very reliable and extremely accurate marker of contamination (Nicholson, 2001; Moore *et al.*, 2006).

Evidence shows that a negative impact of environmental conditions, such as chemical pollution, places the bioindicator specimen in a considerable amount of stress, resulting in depletion of energy that is normally allocated for ensuring optimum rates of growth, reproduction and numerous other body functions. Subsequently, organisms exposed to pollutants display reduced growth rates and poor body condition (Dahlhoff and Menge, 1996; Nicholson, 1999b). The condition index is expressed as a ratio of body mass and shell length and studies have been utilising a decrease in condition index as a sensitive biomarker of environmental stress (Nicholson, 1999a; Galloway *et al.*, 2002; Amiard *et al.*, 2004).

Measurement of metabolic enzyme activity is another newly developed indicator that assesses the effects of stress on the metabolic activity of biomonitors, and has been successfully used in numerous studies, particularly those that employ invertebrate marine organisms as bioindicators (Lushchak *et al.*, 1997; Fahraeus-Van Ree *et al.*, 1999; Orbea *et al.*, 1999; Dahlhoff *et al.*, 2001; Dahlhoff *et al.*, 2002; Luk'yanova, 2006). To assess the metabolic rate of *P. perna*, malate dehydrogenase (MDH), which has been the subject of many previous studies, was measured (Mizrahi and Achituv, 1989; Dahlhoff *et al.*, 2001; Dahlhoff *et al.*, 2002). The enzyme plays a vital role in the citric acid cycle, and research shows a direct link between MDH activity to the overall metabolic activity of organisms (Pellerin-Massicotte and Pelletier, 1987; Park *et al.*, 1995; Cunningham *et al.*, 1997; Dahlhoff *et al.*, 2001; Dahlhoff *et al.*, 2002). In the presence of MDH, malate is oxidised by nicotinamide adenine dinucleotide (NAD) to oxaloacetate,



(Mizrahi and Achituv, 1989; Cunningham *et al.*, 1997). The quantity of NADH that is formed in the reaction is thus proportional to the concentration of malate converted.

The experimental metal used for this study was Cu as it has been extensively used in previous biomarker studies and is a common contaminant in shallow coastal waters (Brown *et al.*, 2004; Curtis *et al.*, 2000; Marshall *et al.*, 2004). The metal occurs naturally in the environment in smaller concentrations; however, excessive quantities are introduced by anthropogenic wastes such as urban runoff, domestic and industrial wastes (Clark, 2001). Copper is an essential element for invertebrate metabolism (Nicholson, 1999a), however excessive concentrations of any metal in the natural environment has shown to inflict severe impairment of bodily functions. Table 4 provides a short summary on the effects of copper on marine mussels documented by previous experiments.



**Table 4:** Summary of findings from previous studies on the effects of copper on marine mussels

Category of effect	Effect	Reference
Biochemical	Inhibition of acetyl-cholinesterase, malate dehydrogenase, lactate dehydrogenase, cytochrome oxidase, $\alpha$ -amylase, catalase, malonedialdehyde, glutathione S-transferase activities.	Pellerin-Massicotte and Pelletier, 1987; Mizrahi and Achituv, 1989; Regoli <i>et al.</i> , 1998; Khessiba <i>et al.</i> , 2001; Narbonne <i>et al.</i> , 1999, 2005; Kagley <i>et al.</i> , 2003; Lionetto <i>et al.</i> , 2003; Brown <i>et al.</i> , 2004.
Cellular	Reduced lysosomal membrane stability. Impaired phagocytosis.	Nicholson, 1999a,b; Matozzo <i>et al.</i> , 2001; Nicholson, 2001; Riveros <i>et al.</i> , 2002; Nicholson, 2003; Brown <i>et al.</i> , 2004.
Physiological	Reduced protein catabolism in haemolymph. Reduced heart rate. Growth retardation. Changes in byssus and mucus production. Inhibited filtration. Suppressed O <sub>2</sub> uptake. Decline in faecal production. Limited shell production. Assimilation efficiency of organic matter from ingested food material.	Nicholson, 1999a,b; Curtis <i>et al.</i> , 2000, Sze and Lee, 2000; Curtis <i>et al.</i> , 2001; Matozzo <i>et al.</i> , 2001; Anandraj <i>et al.</i> , 2002; Nicholson, 2003; Brown <i>et al.</i> , 2004.
Bioaccumulation	Accumulation of Cu within soft tissues.	Phillips, 1976a,b; Amiard-Triquet <i>et al.</i> , 1986; Sze and Lee, 2000; Anandraj <i>et al.</i> , 2002; Riveros <i>et al.</i> , 2002.

Chapter 2 is designed to test the hypothesis that exposure of mussels *P. perna* and *B. semistriatus* to increasing metal concentrations will induce alterations in physiological, cytological and biochemical responses. The aim of this study was to determine the effects of copper on physiological, cytological and biochemical responses of *P. perna* and *B. semistriatus* from two different sites on the Kwa-Zulu Natal coast, in an attempt to investigate the suitability of these biomarkers for monitoring marine pollution. In these experiments, *P. perna* and *B. semistriatus* were subjected to increasing concentrations of copper, and thereafter, the following biomarker responses were investigated: cardiac activity, lysosomal membrane stability, body condition index and malate dehydrogenase enzyme activity.

The objectives of the study were to:

- Assess the selected biomarkers as useful tools for marine pollution monitoring.
- Compare biomarker responses from *P. perna* and *B. semistriatus* collected from a polluted and non-polluted site.
- Select a suitable biomonitoring species to be used in the following chapters.

## **Materials and methods**

### *Site overview*

Park Rynie Beach, (30° 19' S; 30° 43' E), located away from heavy industrial areas on the south coast of KZN, is regarded as a relatively unpolluted area, and was used as a reference site in this study as well as a collection site for test organisms for previous studies (Connell, 2001; Biseswar *et al.*, 2002; De Pirro and Marshall, 2005). In contrast, Isipingo Beach, (29 05'S; 30 56'E), also on the KZN south coast, is located alongside an estuary which drains two polluted rivers that flow through the heavily industrialised and urbanised areas of Prospecton and Isipingo Rail (Begg, 1978), (refer to Figure 1 in Chapter 3 for site locations). The Isipingo Estuary is documented by Begg (1978), Kalicharran and Diab (1993) and Grobler *et al.* (1996) to be greatly affected by chemical pollution due to industrial wastes, sewage effluent and agricultural runoff entering the water bodies from the surrounding areas. In addition to this, approximately 4 km on the opposite end of the beach, another polluted estuary drains the Umbogintwini River which also receives industrial effluent and stormwater runoff (Begg, 1978).

### *Collection and maintenance*

*Perna perna* of shell lengths between 55 – 60 mm were obtained from two localities within Park Rynie and Isipingo Beach ( $n = 150$  from each locality). *Brachidontes semistriatus* (shell lengths

45 – 50 mm,  $n = 150$  from each locality) was obtained from the same *P. perna* collection sites at Park Rynie; however no specimens were found at Isipingo. Mussels were removed from rocks at low tide and transported in plastic containers with seawater to the laboratory (University of KwaZulu-Natal). Prior to placing mussels in well aerated artificial seawater (35‰ Instant Marine) with a constant water temperature of 23 °C, shells were cleaned of any epizoans. Thirty specimens of *B. semistriatus* and *P. perna* from the two sites were randomly selected to assess biomarker responses immediately after collection from the field to compare any variances displayed by the mussels serving as a control group during the duration of the experiment. These specimens were allowed to acclimate to their tank environment for 24 hours before biomarker assays were undertaken. The remainder of the mussels collected from the field were divided into four different glass tanks (50 cm X 50 cm X 110 cm); each of which was divided into three mini aquaria by Perspex boards. Each mini aquaria was kept aerated during the entire experiment, and mussels were fed daily with unicellular alga, *Chlorella*.

Copper concentrations of 0; 10; 50 and 100  $\mu\text{g.L}^{-1}$ , ( $n = 30$  mussels from each species and site for each Cu treatment) were obtained by weighing out the relevant mass of analytical grade  $\text{CuSO}_4$  to prepare a stock solution, and thereafter diluting to obtain the required concentrations. Seawater was changed on a daily basis to eliminate accumulated waste, as well as to ensure a constant level of metal concentration of the water. Mussels were exposed to Cu treatments for 10 days before the majority of the biomarker responses were assessed. Mussels, randomly chosen for the condition indices, were exposed to copper for 21 days before the indices were measured, as a 10 day exposure period was considered too short to inflict significant alterations in mussel shell and body tissue growth. Ideally, each biomarker should be tested on separate specimens; however, due to the substantial numbers of specimens that were required, the sequence of biomarker tests were co-ordinated such that the least stressful biomarker (heart rate) was measured first, thereafter the invasive biomarker techniques were conducted.

#### *Mortality*

Specimens were declared dead if valves did not close when the organisms were disturbed.

#### *Cardiac activity*

Heart rate activity was measured by a non-invasive technique. An infra-red light-emitting diode was glued securely onto the shells of each individual mussel ( $n = 30$  individuals for each species and site). Once diodes were attached to the shells, the mussels were left undisturbed for at least 6 hours to ensure the organisms had a sufficient recovery time after the attachment of the

diodes. Cardiac activity was recorded for 20 minutes every hour, for 6 hours, after 10 days of exposure to the Cu treatments. The mean heart rate (beats.min<sup>-1</sup>) for each copper concentration was then obtained.

#### *Lysosomal membrane stability*

Methodology for neutral red assays was adapted from Lowe and Pipe (1994). Lysosomal membrane stability was determined for 30 mussels from each copper treatment ( $n = 30$  individuals for each species and site). Mussel valves were gently prised apart and approximately 0.5 ml of haemolymph was extracted from a sinus in the adductor muscle using a syringe, and thereafter placed in siliconised Eppendorf tubes, together with an equal volume of temperature adjusted physiological saline solution. The mixtures were then inverted at regular intervals to prevent cells from adhering to the tubes. Approximately 40 µl of the haemolymph solution was allowed to settle and then attach onto haemocytometers, and incubated at room temperature for 20 minutes. An equal volume of the dye neutral red (NR) was then introduced to the incubating slide, and allowed to infiltrate the cells for 15 minutes. Following the incubation period, cells were observed at 15 minute intervals microscopically, and the time taken for 50% of the cells to lose their dye was recorded as the membrane stability time.

#### *Body condition index*

The ratio of tissue dry mass (mg) of mussel body tissue to shell length (mm) was determined for 30 mussels from each copper treatment for each species and site (Lundebye *et al.*, 1997; Nicholson, 1999b; Orban *et al.*, 2002; Galloway *et al.*, 2002; Amiard *et al.*, 2004).

#### *Malate dehydrogenase (MDH) enzyme activity*

Twenty four mussels from each copper treatment for each species and site were removed from tanks and dissected. The adductor mussel tissue was isolated and placed on ice. The tissue samples were then weighed and immediately homogenised in a 5-fold dilution of chilled 50 mmol.L<sup>-1</sup> potassium phosphate buffer, pH 6.8 at 21°C. The homogenate was centrifuged for 5 minutes, and the supernatant was retrieved to be used for the MDH enzyme activities. A 50 ml stock solution of the assay medium used was freshly prepared for each Cu treatment, and comprised of 50 ml chilled 0.20 M imidazole-HCl buffer, pH 7.5, 5.4 mg NADH and 1.4 mg oxaloacetate. Two millilitres of this assay medium was pipetted into plastic cuvettes and thereafter 25 µl of the tissue homogenate was added into the cuvettes to initiate the enzyme reaction. Enzyme activities were assayed spectrophotometrically (Spectronic 20 Genesys) and

reported in International Units per gram wet mass (IU/g), (Young and Somero, 1993; Dahlhoff and Menge, 1996).

#### *Statistical analysis*

All statistical analyses were performed using SPSS version 15.0 (SPSS for Windows, Rel. 15.0. 2006. Chicago: SPSS Inc.). All data obtained were tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test). Not all the data obtained from the various biomarker tests conformed to normal distribution; hence the non-parametric Kruskal-Wallis H-test and Dunn's post-hoc comparisons were used to define differences between the treatments. Statistical comparisons between populations were unfortunately not undertaken because only two site localities each from Park Rynie and Isipingo Beach were available.

### **Results**

#### *Mortalities*

Only four mussels died over the 10 day exposure time period. One control *P. perna* collected from Park Rynie and three Isipingo Beach *B. semistriatus* individuals had died three and two days respectively, after haemolymph extraction. These deaths were possibly due to extensive damage that occurred to surrounding tissues while valves were pried apart. One *P. perna* mussel from Park Rynie and another two *B. semistriatus* individuals from highest concentration treatment died during the 21 day exposure time period. The mortality of these mussels could be due to the extended Cu exposure at the highest concentration.

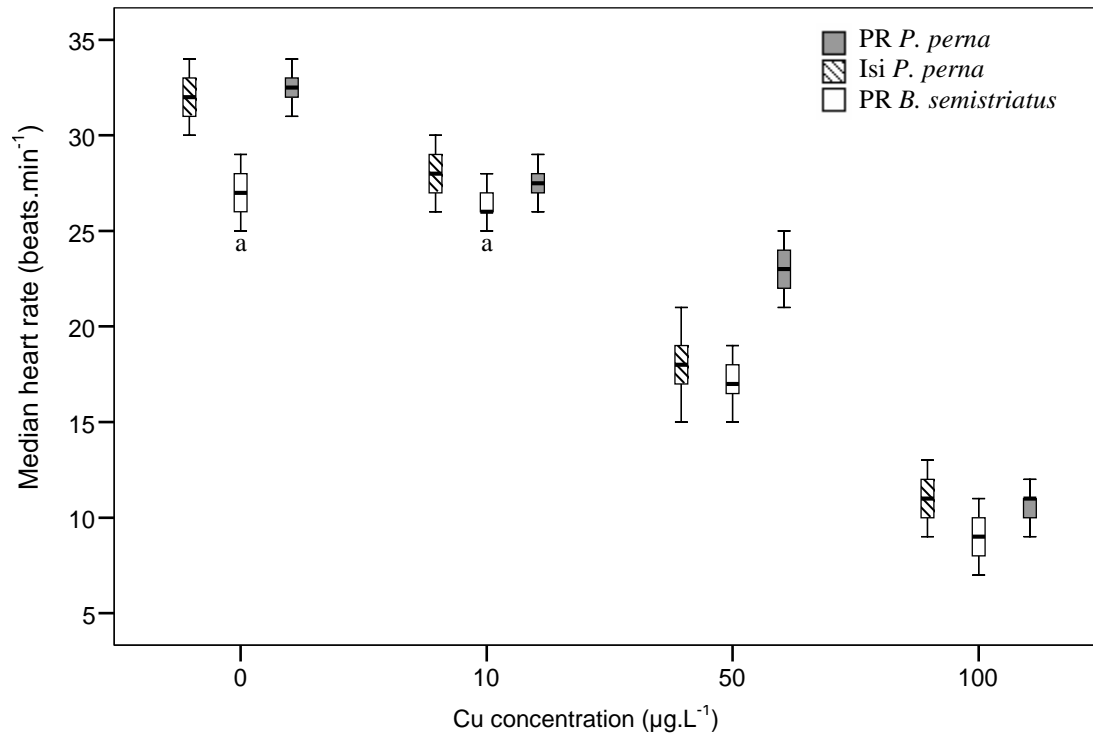
#### *Biomarker responses from mussels tested immediately after collection*

Inter-species biomarker responses showed no significant differences from the control mussels and those processed immediately after collection from the sites for both species and populations (Kruskal-Wallis,  $H = 53.16$ ,  $p > 0.05$ ).

#### *Cardiac activity*

Cardiac activity was successfully obtained from all mussels with relatively stable heart beats from all treatments. Median heart rates obtained from *P. perna* and *B. semistriatus* are presented in Figure 1. A steady decline in mean cardiac activity was observed as the concentrations of copper treatments were increased, thereby exhibiting a clear relationship between metal concentration and cardiac response. Dunn's post-hoc comparisons showed that *P. perna* from both Park Rynie and Isipingo Beach exhibited significant differences in heart rates between all

Cu treatments (Kruskal-Wallis,  $df = 3$ ,  $H = 17.89$  and  $15.46$  respectively,  $p < 0.001$ ). *Brachidontes semistriatus* displayed significant variations in heart rates when exposed to higher metal concentrations (Kruskal-Wallis,  $H = 11.26$ ,  $p < 0.001$ ); and similar cardiac activities from the control and the lowest Cu treatments (Dunn's test,  $p > 0.05$ ). *Perna perna* from Park Rynie exhibited highest heart rates at each Cu concentration whilst *B. semistriatus* displayed the lowest.



**Figure 1:** Median heart rates (beats.min<sup>-1</sup>) from *B. semistriatus* collected at Park Rynie (PR *B. semistriatus*) and *P. perna* collected from Park Rynie (PR *P. perna*) and Isipingo Beach (Isi *P. perna*). Box indicates the 25<sup>th</sup> and 75<sup>th</sup> percentile ranges and error bars represent a 95% confidence interval around the median. Similar medians between Cu treatments are indicated by common symbols (Kruskal-Wallis,  $p > 0.05$ ).

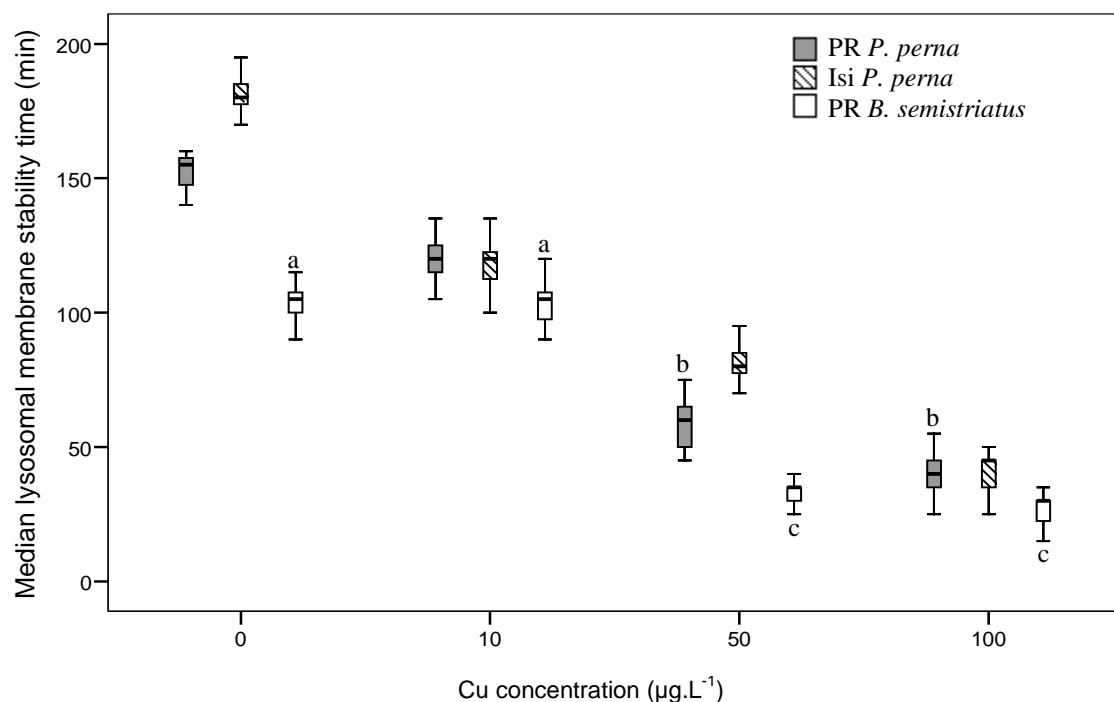
#### Lysosomal membrane stability

Figure 2 illustrates the decline of lysosomal membrane stability in haemocytes from Park Rynie and Isipingo mussels as the exposed Cu concentrations were increased. Isipingo Beach *P. perna* lysosomal membrane stability were longer than those from Park Rynie specimens, and exhibited significant differences in lysosomal membrane stability between all Cu treatments (Kruskal-Wallis,  $df = 3$ ,  $H = 56.06$ ,  $p < 0.001$ ). *Perna perna* from Park Rynie displayed similar lysosomal membrane stability between the two highest Cu concentrations (Kruskal-Wallis,  $df = 3$ ,  $H = 62.82$ ,  $p > 0.05$ ). Although lysosomes from *B. semistriatus* also displayed a reduction in lysosomal membrane stability, Dunn's *post-hoc* comparisons identified similar lysosomal

membrane stability between the control and the lowest Cu treatments, as well as between the two highest concentrations (Kruskal-Wallis,  $df = 3$ ,  $H = 46.99$ ,  $p < 0.001$ ).

#### Body condition index

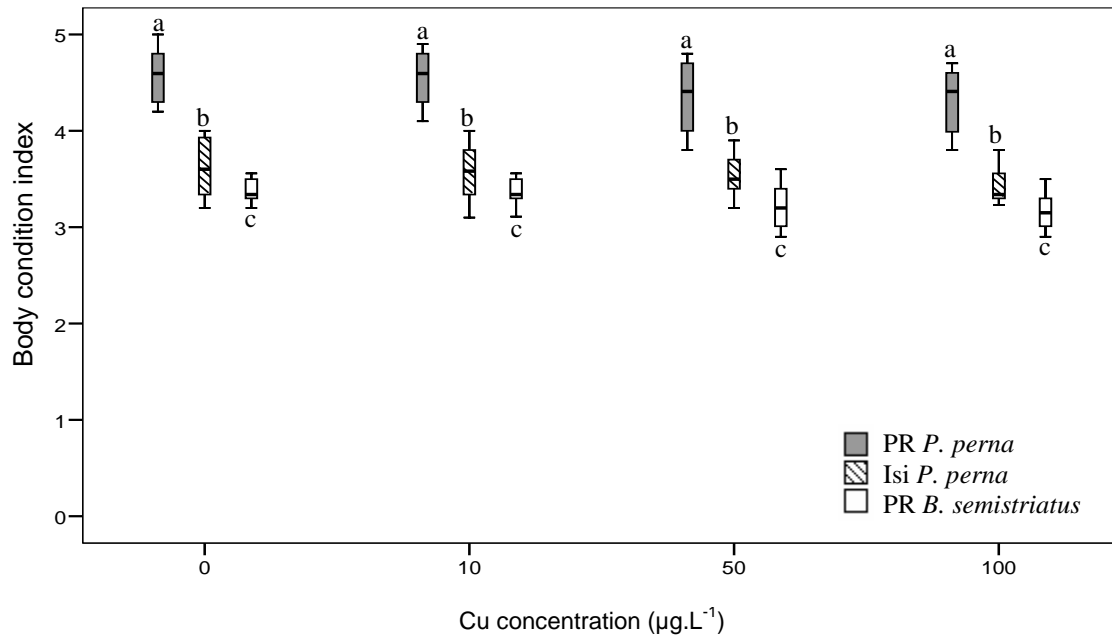
The condition indices measured did not reveal any significant difference between the Cu treatments for any of the mussels (Kruskal-Wallis,  $df = 3$ ,  $H = 18.35$ ; 10.41 and 14.63 for Park Rynie *P. perna*, Isipingo Beach *P. perna* and *B. semistriatus* respectively,  $p > 0.05$ ); however Figure 3 clearly shows *P. perna* collected from Park Rynie exhibiting healthier condition indices compared to those collected at Isipingo Beach and *B. semistriatus* mussels.



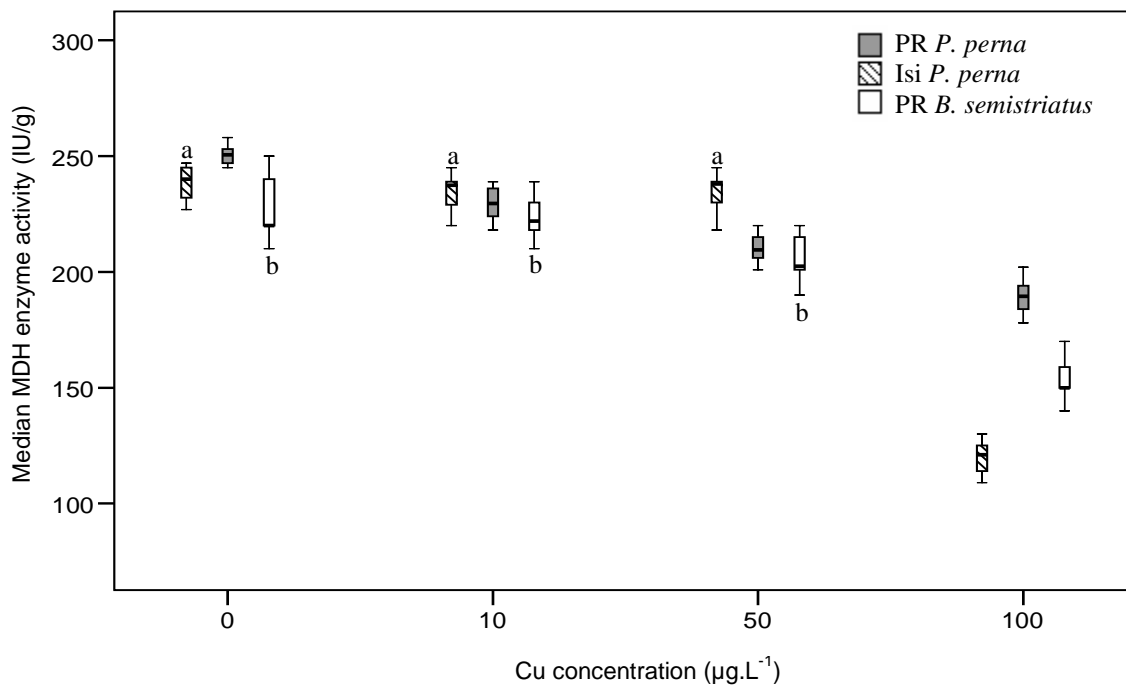
**Figure 2:** Median lysosomal membrane stability (min) from *B. semistriatus* collected at Park Rynie (PR *B. semistriatus*) and *P. perna* collected from Park Rynie (PR *P. perna*) and Isipingo Beach (Isi *P. perna*). Box indicates the 25<sup>th</sup> and 75<sup>th</sup> percentile ranges and error bars represent a 95% confidence interval around the median. Similar medians between Cu treatments are indicated by common symbols (Kruskal - Wallis,  $p > 0.05$ ).

#### Malate dehydrogenase activity

*Perna perna* from Park Rynie exhibited a significantly inverse relationship between metal concentration and MHD enzyme activity (Kruskal-Wallis,  $df = 3$ ,  $H = 36.14$ ,  $p < 0.001$ ), (Figure 4). Whilst *B. semistriatus* and *P. perna* sampled from Isipingo only exhibited significant responses at the highest Cu treatment (Kruskal-Wallis,  $df = 3$ ,  $H = 30.07$  and 23.19 respectively,  $p < 0.001$ ), mussels from Isipingo interestingly showed a slight elevation in MHD activity from the 50 µg.L<sup>-1</sup> Cu treatment.



**Figure 3:** Body condition indices from *B. semistriatus* collected at Park Rynie (PR *B. semistriatus*) and *P. perna* collected from Park Rynie (PR *P. perna*) and Isipingo Beach (Isi *P. perna*). Box indicates the 25<sup>th</sup> and 75<sup>th</sup> percentile ranges and error bars represent a 95% confidence interval around the median. Similar medians between Cu treatments are indicated by common symbols (Kruskal -Wallis,  $p > 0.05$ ).



**Figure 4:** Median MDH enzyme activities from *B. semistriatus* (collected at Park Rynie) and *P. perna* (collected from Park Rynie and Isipingo Beach). Box indicates the 25<sup>th</sup> and 75<sup>th</sup> percentile ranges and error bars represent a 95% confidence interval around the median. Similar medians between Cu treatments are indicated by common symbols (Kruskal -Wallis,  $p > 0.05$ ).



## Discussion

There has recently been a growing urgency to monitor and control marine pollution. In light of this, there has been a demand for marine pollution monitoring techniques that are simple, inexpensive and provide some understanding of the biological effects in marine biota. The biomarkers evaluated in this study were selected primarily for their low cost, ease of use and quick application. More importantly, they highlight the relationship between anthropogenic contaminants and long-term toxic effects in marine organisms. The present study tested a cellular biomarker (lysosomal membrane stability), physiological biomarkers (cardiac activity and condition index) and a biochemical biomarker (malate dehydrogenase enzyme activity) from mussels sampled at relatively pristine and polluted areas (refer to Chapter 3 for a survey of some heavy metals on the KZN coastline).

### *Cardiac activity*

Past studies assumed that metal-induced bradycardia was directly related to mussel valve closure in response to the elevated metal concentrations. Prolonged closure of the valves would result in hypoxia, which in turn results in a reduced heart rate (Davenport and Redpath, 1984). Since then extensive studies by Curtis *et al.* (2000; 2001) have contradicted this view by revealing the direct effects of metals on the nervous control system of the mussel heart. The effects of contamination on the cardiac activity of mussels have now become widely studied (Nicholson, 1999a,b; Curtis *et al.*, 2000; 2001).

Few studies have reported no difference in mussel cardiac activity when exposed to elevated metal concentrations (Nicholson, 1999a,b; Curtis *et al.*, 2001; Galloway *et al.*, 2002; Abessa *et al.*, 2005), which is due to metallothionein binding and the uptake of metal ions by the humoral ligands and haemocytal lysosomes. Therefore the remaining metal content available within the mussel body would not be a high enough dosage to inflict a response from the mussel myocytes or cardiac nervous system. In the present study, no such insensitivity of heart rate was observed; instead the data revealed a strong decline in cardiac activity from both populations of *P. perna* and *B. semistriatus* as the Cu dosages increased. This could be due to severe impairment of the heart from the metal exposure. Most studies on bivalve cardiac activity have reported a similar decrease in heart rate when mussels were exposed to heavy metals (Widdows, 1973; Sabourin and Tullis, 1981; Nicholson, 1999a,b; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Galloway, 2002; Marshall *et al.*, 2004; Abessa *et al.*, 2005), thereby highlighting cardiac activity as an effective and reliable biomonitoring tool.

### *Lysosomal membrane stability*

Previous studies have shown that mild alteration in lysosome membrane stability can be stimulated by external factors such as extreme thermal stress, hyposalinity, hypoxia and food deprivation (Domouhtsidou *et al.*, 2004). However, such effects are mild compared to the degree of membrane instability induced by pollutant exposure. Numerous studies in the field and laboratory have since verified lysosomal membrane stability to be a sensitive indicator of stress caused by contamination for a range of species including annelids, crustaceans, molluscs and fish (Lowe *et al.*, 1995; Marigomez *et al.*, 1998; Viarengo *et al.*, 2000; Wedderburn *et al.*, 2000; Matozza *et al.*, 2001; Nicholson, 2001; Galloway *et al.*, 2002; Moore *et al.*, 2006). The biomarker is based on the notion that neutral red dye is allowed to permeate into live lysosomes and then be retained inside the organelle by a membrane-bound proton pump (Nicholson, 1999a). Once the enzymes of the proton pump is denatured by the excessive swelling and increased autophagy (Wedderburn *et al.*, 2000) due to excessive contaminant accumulation, the pump is rendered useless resulting in an increase of pH within the lysosomes. This subsequently produces equilibrium between the inner organelle contents and the cytosol outside the organelle, and allowing the inner lysosomal contents to diffuse out of the lysosome. Thus lysosomes from healthy mussels will exhibit greater lysosomal membrane stability times of the dye. Conversely, mussels stressed by pollutant exposure will exhibit a reduction in lysosomal membrane stability (Nicholson, 1999a,b; Wedderburn *et al.*, 2000). Neutral red dye is used to indicate the dysfunction of the proton pump and its enzymes (Nicholson, 1999a,b) and the technique is low cost, quick and only a small sample of haemolymph is required from individuals.

This study shows that exposure to copper caused a considerable dose-dependent decrease in the lysosome membrane stability of mussels from Park Rynie, which is an unpolluted environment. These results are consistent with the findings from several other studies (Lowe *et al.*, 1995; Marigomez *et al.*, 1998; Viarengo *et al.*, 2000; Wedderburn *et al.*, 2000; Matozza *et al.*, 2001; Nicholson, 2001; Galloway *et al.*, 2002; Moore *et al.*, 2006). *Perna perna* collected from Isipingo Beach exhibited greater lysosome membrane integrity than those of Park Rynie. Wedderburn *et al.* (1998) postulated that the insensitivity of some lysosomes could be explained by the organisms' adaptation to their environment's elevated metal contamination. Furthermore several authors have demonstrated that mussels sampled from polluted urban and industrial areas have enlarged lysosomes in their digestive cells, suggesting that animals from areas exposed to elevated levels of pollution in their habitats will have developed a tolerance to the effects of toxic contaminants; thereby reducing the organisms' sensitivity to the copper treatments (Riveros *et al.*, 2001; Abessa *et al.*, 2005). This lower stability time of the Park

Rynie lysosomes can therefore be an indication of a greater stress experienced than the mussels from the polluted area of Isipingo, which has several industrial zones in the area. Lysosomes from *B. semistriatus* exhibited no response to the lowest copper treatment; and though there were a decline in lysosomal membrane stability, the membrane stability time for the highest two copper treatments were not significantly different from each other. This suggests that this organism is only tolerant to mild metal concentrations as their lysosomes cannot cope with excessive loads of contaminant accumulation, thereby excluding this species as a suitable bioindicator.

#### *Body condition index*

Numerous studies document a significant decrease in body condition indices with an increase in pollutant loads (Avery *et al.*, 1996; Nicholson; 1999a,b; Mauri and Baraldi, 2003; Bodin *et al.*, 2004). The findings in this study contrasts those reported previously as condition indices of *P. perna* and *B. semistriatus* however displayed no differences in their body indices between the copper treatments. Since Isipingo mussels may have the ability to tolerate exposure to mild levels of pollutants in their natural environment (Riveros *et al.*, 2001; Abessa *et al.*, 2005), it can be assumed that they will exhibit no effect on body condition index; however this seems an unlikely possibility as *P. perna* from Park Rynie also displayed similar condition indices for all Cu treatments. Another plausible explanation for such lack of response is that the duration of the experiments was too short to inflict drastic consequences on somatic growth rates. This hypothesis would also account for the insensitivity of *B. semistriatus* body indices to the copper exposure, as these mussels were also sampled from an unpolluted environment. However, from Figure 3, the difference in body condition indices between the two *P. perna* populations and *B. semistriatus* can be visually observed, and hence, suggests that *P. perna* obtained from Isipingo Beach have much lower body conditions than those sampled from Park Rynie. This could confirm the sensitivity of the condition index when testing for the history of contaminant exposure. Condition indices have been found to be affected by seasonal changes, reproductive activity and age of the organisms (Lundebye *et al.*, 1997); however in this study, such variability in results were reduced by collecting similar sized specimens from the different sites on the same day, and ensuring tank conditions were consistent for all treatment tanks. Although this biomarker has been proven in to be sensitive and effective in previous studies (Soto *et al.*, 1995; Avery *et al.*, 1996; Lundebye *et al.*, 1997; Galloway *et al.*, 2002; Smolders *et al.*, 2003), it is highly recommended that this biomarker be used for long-term monitoring, with consideration to the effects of external factors on the results.

### *Malate dehydrogenase activity*

Metabolic rate is fast becoming a popular biomarker (Dahlhoff, 2004) and biochemical indicators such as enzyme activity have already proven to be sensitive and useful markers for growth rate and metabolic stress. MDH activities have been used in several other studies to assess metabolism in marine invertebrates and fish (Dahlhoff, 2004), as the enzyme has a close link to metabolic rate. The production of oxaloacetate is essential to biological processes such as the tricarboxylic acid cycle, amino acid synthesis, oxidation/reduction balance and plays a role in the transference of metabolites between the cytoplasm and organelles (Goward and Nicholls, 1994; Park *et al.*, 1995). Thus the inhibition of MDH will result in a reduced production rate of oxaloacetate, which in turn will cause a negative cascading effect on important biochemical processes and metabolic pathways.

In this study, both *B. semistriatus* and *P. perna* from Isipingo Beach only exhibited a strong response in enzyme activities between the lower Cu treatments and the highest Cu treatment. The insensitivity of *P. perna* from Isipingo Beach to the low copper concentrations could also be a result of those organisms being constantly exposed to a mild level of pollutants in the environment, thus only exposure to a high concentration of Cu would inflict any effect on enzyme activity; however *B. semistriatus* from Park Rynie also did not display any sensitivity of enzyme activity to the lowest copper concentration even though the area from where the organisms were sampled was unpolluted. The data therefore suggest that the lower copper concentrations were far too low a dosage and the duration of the exposure period far too short to induce a hindrance on enzyme activity. This latter hypothesis is supported by the findings of a study by Pellerin-Massicotte and Pelletier (1987), which documented a decrease in MDH activity in mytilid mussels only after 22 days of exposure to both low and high concentrations of methyl mercury. In contrast, a study by Mizrahi and Achituv (1989) on the bivalve *Donax trunculus* exhibited a 4.8% drop in MDH activity after 24 hours of exposure at 50  $\mu\text{g.L}^{-1}$  Cu, and a further 28.5% decrease in the enzyme activity at 10  $\text{mg.L}^{-1}$  Cu after the same exposure time. Nevertheless the findings of this study conform to the results of these previous studies as metal exposure inhibits the MDH enzyme activity. Interestingly *P. perna* from Isipingo exhibited a slight increase in enzyme activity from the 50  $\mu\text{g.L}^{-1}$  Cu treatment. This can be accounted by the fact that mussels have been shown to elevate their metabolic rates in an attempt to rapidly detoxify and excrete contaminants from their tissues (Nicholson, 1999a,b). Evidence has revealed that numerous factors such as thermal stress, hyposalinity, hypoxia and starvation can influence enzyme activity (Dahlhoff, 2004), for that reason it is crucial that this

indicator is accompanied by multiple biomarkers to ensure a true reflection of the overall health of the organism.

#### *Experimental design considerations*

The basic elements of a scientific experiment are comprised of a hypothesis, an experimental design to test the hypothesis, implementation of the experimental procedure, data retrieval and manipulation by statistical analysis and lastly, a discussion on the results obtained which either accepts or rejects the hypothesis (Hurlbert, 1984). The consequences of improper experimental design have been the subject of numerous published articles (Hurlbert, 1984; Stewart-Oaten *et al.*, 1986; Eberhardt and Thomas, 1991; Wiens and Parker, 1995; Heffner *et al.*, 1996; Underwood, 1998; Chapman, 2000; McGregor, 2000; Oksanen, 2001; Hurlbert, 2004; Millar and Anderson, 2004; Kozlov, 2007; Velickovic, 2007). The most common mistakes in ecological experiments are inadequate sampling procedures, too small sample sizes, lack of controls and using inaccurate experimental units for statistical analysis, which results in statistical errors. Statistical treatment of different data sets require all replicate and control data sets to be independent of each other and that appropriate control groups for each experiment are designed so as to reduce any confounding factors. Hence, if any of the above requirements are not met, fundamental assumptions of statistical analysis are violated (Underwood, 1998). Hurlbert (1984) postulated that the most prevalent confounding factors that influence hypothesis testing in ecological experiments include temporal changes, handling effects of test organisms, bias on the part of the scientist and errors due to variability between results. Such factors can be minimised or eliminated by implementing proper control and replicate treatments, as well as practicing randomisation. Without appropriate replicate samples, there is difficulty in separating the effects of a treatment from confounding effects (Hurlbert, 1984; Eberhardt and Thomas, 1991; Cottenie and De Meester, 2003); thus the number of replicate and control groups of a treatment strengthens the test of the hypothesis.

In simple terms, pseudoreplication is the misidentification between the number of observations or measurements made during the experiment and the number of independent replicates required for statistical analysis. A true replicate of an experimental unit is independent, and in the event sub-samples from one experimental treatment or inter-dependent measurements are treated as true independent replicates, pseudoreplication is committed (Hurlbert, 1984; Mundry and Sommer, 2007). The occurrence of pseudoreplication in ecological experiments is a common mistake among biologists with several published reviews citing some documented studies showing this error in experimental design (Hurlbert, 1984; Heffner *et al.*, 1996; Millar and

Anderson, 2004; Kozlov, 2007). According to Hurlbert (1984), there are different types of pseudoreplication:

1. Simple pseudoreplication - occurs when there are no 'true' replicates in the experiment, or replicates are not independent from each other.
2. Temporal pseudoreplication - occurs when multiple non-independent replicates from one experimental treatment are taken over a period of time.
3. Sacrificial pseudoreplication - occurs when the data values of each experimental unit is pooled and averaged before any statistical analysis is undertaken, thereby providing an error in the variability of the variance.

In this study, despite having the appropriate number of replicates ( $n = 3$  for each concentration), the replicate tanks for each treatment were not independent from each other, thus representing a single experimental unit and pseudoreplication was committed. As stated by Musset (2006), when test organisms are contained in an inter-dependent holding facility, confounding effects may be brought on by either an alteration in the holding facility, such as any malfunction of the system shared by the 'replicate units' or accidental contamination which will spread to the rest of the inter-dependent systems, or by test organisms within the holding facility affecting each other's responses, for example death. In Hurlbert's article (1984) various types of basic experimental designs that are commonly used in current biological experiments are described, and with regards to the potential occurrence of pseudoreplication, these designs are categorised by spatial interspersal and segregation of individual experimental units. The experimental design of this study can be classed as having 'physically inter-dependent replicates'. Pseudoreplication is most common in studies involving large systems such as rivers, lakes and watersheds, where replicate sampling is either too costly or impossible (Oksanen, 2001); however in this study, the error of inter-dependent replicates were a consequence of a lack of forethought in the experimental design stage of the study and poor knowledge on proper experimental design and statistical protocols. As a result, the statistical results of this chapter have to be reviewed with caution as it is difficult to exclude the effect of confounding factors on the biomarker responses.

Considering the weaknesses and pitfalls in the experimental design of this chapter, some fundamental concepts in experimental design were highlighted. Seeing how an inappropriate design set-up can result in statistical results being inconclusive, the importance of using clear and logical thought at the onset of an experimental design phase is emphasised. Even more important, the experiment must be designed in a fashion that tests the hypothesis of the study. In

the case of this chapter, to test the effects of a treatment on a response of an organism, it is necessary for the experiment to include a sufficient number of treatment levels, i.e. different Cu concentrations. In addition, the sample size of a treatment ( $n$ ) must also be suitable as small sample sizes will reduce the power of the statistical test being applied. Each treatment level (in this case concentration) must have an appropriate number of completely independent replicates, as the number of replicates also affects the power of the statistical tests used. Furthermore, control treatments with independent sets of replicates are customary for any experiment as they are required to test whether the different treatments had a significant effect on the test organism. Lastly, thorough randomisation at each step of the experimental process must be executed to further reduce confounding effects, and randomisation also ensures the independence of experimental treatments and replicates.

However, despite the problem of pseudoreplication, the present results do not differ greatly from as the findings from numerous published articles that support the biomarker results obtained in this study.

## **Conclusion**

Biomarkers have numerous advantages to the traditional techniques of analysing trace metals from the environment and biota. Besides being low cost and low technology, they indicate the effects of biologically-available contaminants instead of an inert form of the chemical pollutant. Various biomarkers also provide an indication of damage to physiological, cellular and biochemical processes, thus illustrating the effects of pollution on a whole-organism level. They also serve as an early warning signal of environmental stress before detrimental damages can occur to ecosystems. Despite the experimental design and statistical considerations, the findings in this study show that the selected biomarkers tested proved to be useful tools that can be employed in marine pollution monitoring programs as the exposure to a widespread contaminant inflicted an inverse effect on most biomarker responses from mussels. Nevertheless MDH activity did not provide as a distinct relation to low Cu treatments in most of the mussels as the rest of the biomarkers, hence this biomarker is not recommended for short-term monitoring in areas of low pollution. Condition index must be used for long-term monitoring experiments as the short exposure time in this study failed to inflict any considerable differences between indices from the Cu treatments. Furthermore, previous studies have documented that the index is influenced by biotic and abiotic variables, and it is therefore important that these factors are considered during monitoring programs. Cardiac activity and lysosomal membrane

stability proved to be highly suitable biomarkers by exhibiting distinct responses to metal concentrations.

Bivalve molluscs have been extensively used in biomonitoring programmes as they have proved to be excellent bioindicator. In this study, *B. semistriatus* failed to exhibit any sensitivity to low contaminant exposure for three out of the four biomarker tests and are not as widely distributed as *P. perna*, thus highlighting their inappropriateness as a bioindicator. In contrast *P. perna* proved to be the more sensitive bioindicator and has a wide global distribution which suggests that the species has a high tolerance to substantial variation in environmental factors such as temperature and salinity. Nevertheless the importance of using a suite of biomarkers rather than a single biological evaluation is emphasised as each of the biomarkers tested in this study indicate general stress of the organism, and can be influenced by other external factors such as hypoxia and food deprivation.



## **CHAPTER 3: Biomonitoring of Heavy Metals and Biomarker Responses from *Perna perna* in KwaZulu-Natal Coastal Waters**

### **Introduction**

Approximately half of the world's population lives within 60 km of the coastline (Turner *et al.*, 1996; Creel, 2003), resulting in coastal waters functioning as a free and convenient depot for waste materials (Lapointe and Clark, 1992; Sumich, 1992; Turner *et al.*, 1996). As coastal cities are continuously expanding, the need for waste disposal is proportionately increasing; however the capacity of the ocean to receive such pollutant loads will shortly be exceeded, resulting in irreversible marine degradation (Sumich, 1992; Moldan, 1995).

South Africa has the longest coastline on the African continent, stretching from Ponta do Ouro on the east coast to the Orange River on the west. Covering a distance of approximately 2954 km (Oelofse *et al.*, 2004), the coastline is an ideal repository for wastes (McClurg, 2006; Oelofse *et al.*, 2004; Misheer *et al.*, 2006a,b). Sixty three licensed pipelines discharge nearly 800 000 m<sup>3</sup> of effluent into South African waters on a daily basis (Misheer *et al.*, 2006a,b,c). The majority of these outfalls dispose of their wastes in deep waters, however an estimated 27 older outfall pipes discharge effluent above the high water mark (Misheer *et al.*, 2006a,b,c). Half of the offshore pipelines dispose industrial effluent, one third dispose domestic effluent and the rest of the pipelines discharge a mixture of both domestic and industrial wastes (Moldan, 1995). Seeing as the KwaZulu-Natal province has experienced rapid expansion of urbanisation and industrialisation, it is not surprising that the majority of the toxic outfall pipes are located on the eastern seaboard of the country (Moldan, 1995; Misheer *et al.*, 2006a,b,c). Although there has been an increasing awareness of marine pollution and the need for marine conservation, there is a large gap between marine pollution research in the country and the latest research methodologies being developed internationally. In addition, current marine pollution monitoring procedures in South Africa are inadequate (Anandraj *et al.*, 2002; O'Donoghue and Marshall, 2003).

Heavy metals can be naturally introduced to marine environments by events such as volcanic activity, forest fires, wind blown dust and erosion of ore-bearing rock (Clark, 2002; Gregory *et al.*, 2002). Nonetheless, the vast majority of heavy metals introduced to coastal ecosystems are due to anthropogenic activities (Chong and Wang, 2000, Gregory *et al.*, 2002; Irato *et al.*, 2003; Petrovic *et al.*, 2004). Another increasingly recognised route of metal exposure to marine environments is atmospheric precipitation. Significant sources of coastal water pollution are summarised in Table 5.

**Table 5:** Sources of common contaminants in the marine environment (adapted from Schachter and Serwer, 1971; Gross, 1978; Moldan, 1995; Taljaard *et al.*, 2006).

<b>Atmospheric sources</b>	<p>Deposition of fine contaminants.</p> <p>Air-sea gas exchange of fluorocarbons, fluorochlorocarbons and halogenated hydrocarbons from combustion and petroleum consumption.</p>
<b>Marine sources</b>	<p>Natural minerals derived from the ocean floor.</p> <p>Dredging of shallow marine environments.</p> <p>Deposition of dredged spoil.</p> <p>Wastes and accidental leaks from operational ships.</p> <p>Oil spills.</p>
<b>Land-based sources</b>	<p>Polluted riverine input.</p> <p>Surface runoff.</p> <p>Stormwater drainage.</p> <p>Disposal of municipal and domestic wastes.</p> <p>Discharge of sewage and industrial effluent.</p> <p>Seepage of contaminated groundwater.</p>

The fate of heavy metals in the marine environment has been the subject of numerous studies (Bryan, 1971; Chong and Wang, 2000; Blackmore and Wang, 2003). Other than the dilution of contaminants, metals from seawater are rapidly removed by both precipitation and deposition in sediments (Bryan, 1971; Chong and Wang, 2000). As most metals enter the marine environment in the form of inorganic compounds or hydrated ions, they are easily adsorbed to sediment particles via chemical bonds (Pempkowiak *et al.*, 1999). Sediments therefore act as a sink for deposited metals and contaminant concentrations of this medium are often higher than those found in seawater (Chong and Wang, 2000; Lowe and Day, 2002; Yap *et al.*, 2002; Beiras *et al.*, 2003). As a result, contaminants present in sediment become bioavailable once it is ingested by marine organisms, resulting in sediments being a major source of metal contaminants (van Hattum *et al.*, 1996; Pempkowiak *et al.*, 1999; Chong and Wang, 2000; Lowe *et al.*, 2002; Phillips, 2002; Blackmore and Wang, 2003). However, before metal ions are removed from the water column, a fraction of the contaminants are directly absorbed by marine organisms (Bryan, 1971; Ravera, 2001). Metals can be absorbed by organisms via the ingestion of contaminated food particles (Wang and Fisher, 1996). This pathway of metal uptake has been shown by Bryan (1971), Chong and Wang (2000) and Ke and Wang (2002) to be a vital source of bioavailable

metals in marine bivalves. Heavy metals, both essential metals such as Fe, Cu and Zn, as well as non-essential metals, which include Cd, Pb, Cr and Ni, in excessive concentrations, are toxic to marine organisms (Andersen, 1996; Irato *et al.*, 2003).

Bivalves are highly resistant to several pollutants which have shown to be lethal to other common biomonitoring species such as fish and crustaceans (Ravera, 2001). Marine mussels are also very effective accumulators of metals as these organisms are exposed to contaminants existing in the water column, sediment and ingestible food particles (Chong and Wang, 2000). Metal assimilation efficiency and absorption by marine bivalves have been studied widely (Reinfelder *et al.*, 1997; Roditi and Fisher, 1999; Wang and Dei, 1999). These studies show that there are several mechanisms of uptake and transport of metals within molluscan tissues (Wang and Dei, 1999), which include:

- passive diffusion of dissolved metals;
- facilitated diffusion of metals across the cell plasma membrane;
- endocytosis (which is an active transport process whereby a substance gains entry into a cell by the inward folding of plasma membrane);
- pinocytosis of dissolved metal ions (which is the introduction of substances into a cell by invagination of the cell membrane).

Studies have found that a fraction of essential metals is absorbed by body tissues and are partially regulated for biological functions. Non-essential metals absorbed by the organisms are usually excreted from the body or accumulated (Fisher *et al.*, 1996; Ravera, 2001; Anandraj *et al.*, 2002). Mussels have been found to concentrate heavy metals by factors of  $10^2 - 10^5$  compared to levels present in ambient seawater (Farrington *et al.*, 1983; Galloway, 2002). Hence, there have been several investigations on the cell and tissue localisation of metals in marine mussels. Specific tissues such as the digestive gland, gills and kidney, as well as specific cells such as lysosomes, digestive cells and brown cells which underlie the mantle are target sites for metal accumulation (Marigomez *et al.*, 2002). These target sites for different metal elements are summarised in Table 6.

Mussels are sedentary and therefore reflect pollutant levels from the area they are situated in. More importantly however, mussels display extremely low metabolism or biotransformation of xenobiotics, providing an accurate account of ambient contaminant levels (Smolders *et al.*, 2003). On account of these attributes, these marine organisms were chosen as a sentinel species to assess the state of pollution in the coastal waters of USA in 1975 in the Mussel Watch

**Table 6:** Metal contaminant target sites in Mytilidae mussel tissues

<b>Metal</b>	<b>Target cells / tissues in Mytilidae mussels</b>	<b>Reference</b>
Cu	Brown cells of the mantle Cytosolic ligands Digestive cells Epithelial cells Gills Haemocytes Labial palp Lysosomes Nephrocytes Stomach cells	Smith, 1985 Soto <i>et al.</i> , 1996 Domouhtsidou and Dimitriadis, 2000 Marigomez <i>et al.</i> , 2002
Cd	Brown cells of the mantle Cytosolic ligands Digestive cells Haemocytes Lysosomes Nephrocytes Stomach cells	Janssen and Scholz, 1979 Scholz, 1980 Soto <i>et al.</i> , 1996 Marigomez <i>et al.</i> , 2002
Pb	Cytosolic ligands Digestive cells Extracellular granules Gill filaments Lysosomes	Domouhtsidou and Dimitriadis, 2000 Marigomez <i>et al.</i> , 2002 Dimitriadis <i>et al.</i> , 2003
Zn	Brown cells of the mantle Cytosolic ligands Digestive gland Endothelial cells Haemocytes Kidney Lysosomes Mantle Nephrocytes Stomach cells	Lobel, 1986 Soto <i>et al.</i> , 1996 Tsangaris <i>et al.</i> , 1997 Marigomez <i>et al.</i> , 2002
Cr	Amoebocytes Cytosolic ligands Cytosolic ligands Digestive cells Digestive gland Gills Haemocytes Kidney Lysosomes Muscle fibres Nephrocytes	Boening, 1997 Tsangaris <i>et al.</i> , 1997 Walsh and O'Halloran, 1998 Parlak <i>et al.</i> , 2004 Marigomez <i>et al.</i> , 2002

**Table 6 continued:** Metal contaminant target sites in Mytilidae mussel tissues

Fe	Amoebocytes Cytosolic ligands Digestive gland Foot Gills Gonads Hepatopancreas Kidney Labial palp Lysosomes Mantle Valve muscles	Bootsma <i>et al.</i> , 1990 Tsangaris <i>et al.</i> , 1997 Marigomez <i>et al.</i> , 2002
Ni	Connective tissue Cytosolic ligands Digestive gland Kidney Lysosomes Mantle cells	Zaroogian and Johnson, 1984 Tsangaris <i>et al.</i> , 1997 Marigomez <i>et al.</i> , 2002

monitoring programme (Smolders *et al.*, 2003). Since then, the concept has been adopted internationally, and numerous countries currently use marine mussels in marine pollution monitoring programmes (Rainbow, 1995; Ravera, 2001; Rittschof *et al.*, 2005). Different species of mussel have been employed, the most common belonging to the family Mytilidae such as *Mytilus edulis* (USA), *Septifer virgatus* and *Perna viridis* (Hong Kong), *Trichomy hirsute* (Australia), *Perna canaliculus* (New Zealand), *Dreissena polymorpha* (Europe) and *Perumytilus purpuratus* (Chile), (Rainbow, 1995; Riveros *et al.*, 2001; Smolders *et al.*, 2003). The brown mussel *P. perna* occurs in abundance on rocky shores along Southern Eastern Africa and compared to other species from the Mytilidae family, not much work has been done on this species. Although *P. perna* has been used as accumulators of trace metals in the coastal waters of Brazil, Ghana, Morocco and Yemen (Anandraj *et al.*, 2002), the use of this species as a fully fledged biomonitor has not been established.

The hypothesis of this study is that biomarker responses from *P. perna* can be used to demonstrate a pollution gradient along the KZN coastline. The aims for this chapter are to therefore:

- Assess and compare metal contamination along KZN coastline.
- Investigate the usefulness of applying a suite of biomarker tests to evaluate the health of the marine environment.
- Investigate whether *P. perna* is a suitable biomonitoring species in a field application by evaluating the biomarkers cardiac activity,

lysosomal membrane stability, body condition index and heavy metal bioaccumulation in mussel soft tissues.

## **Materials and methods**

### *Site overview*

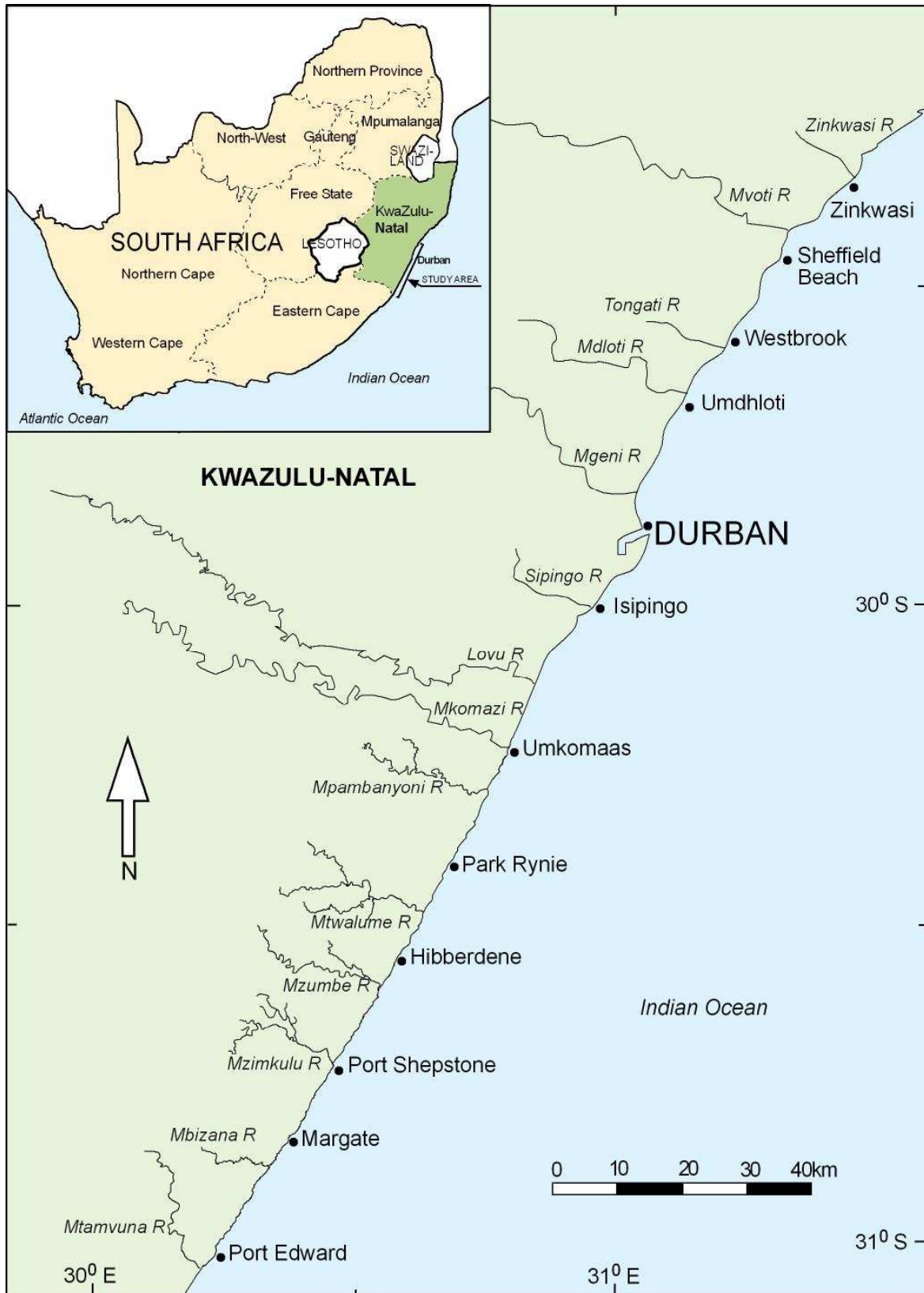
KwaZulu-Natal has an eastern seaboard that spans across approximately 570 km along the Western Indian Ocean, with 73 significant rivers and estuaries exiting into the sea (Begg, 1978). Most of these water bodies have been severely degraded over time. Sites along the coastline were selected to represent polluted and non-polluted coastal areas. Several points along the coast are discharge points for either domestic sewage, industrial effluent or both. As outlined by Connell (1988), there are at least 47 effluent discharge points along the coast, and although this coastal assessment was undertaken in 1988, present conditions of the coast are still very much the same. A summary of the main points of effluent discharge located in near proximity to the sites sampled in this study are summarised in Table 7.

### *Collection and maintenance*

Specimens of *P. perna* with shell lengths between 55 – 60 mm, ( $n = 60$  from each site) were collected by hand from the same intertidal depth at low tides from rocky shores between Zinkwazi and Port Edward (Figure 5). Each site was subdivided into three localities, hence  $n = 20$  from each replicate locality. Mussels were transported in plastic containers with seawater to the laboratory (University of KwaZulu Natal). The specimens were then cleaned of any epizoans and thereafter maintained in well aerated artificial seawater (35‰ Instant Marine) with a constant water temperature of 23 °C. *Perna perna* were allowed to depurate for 24 hours before biomarker tests were conducted. Although this holding period may have partially allowed biomarker responses to recover to some degree, this time frame was assumed to be adequate time to allow for the recovery of organisms from any stress incurred by handling and transportation, which would have a greater influence on biomarker responses. Thus any external influences on biomarker responses would have been minimal. In addition, this holding period was necessary due to the time taken to collect specimens from the field, return to the laboratory, and allow for the removal of epizoans and attach the diodes. At each site approximately 3 cm of surface, intertidal sediment was collected using a wooden scoop, and sieved through a 0.5 mm nylon mesh sieve on site. Sieved particles were collected, sealed in plastic bags and transported in a cooler bag to the laboratory. Three localities from each site were sampled for sediment metal analysis.

**Table 7:** Summary of the main effluent discharge points in close proximity to selected study sites (adapted from Connell, 1988); (\* denotes significant quantities of effluent discharged ( $> 20\,000\text{ m}^3\cdot\text{day}^{-1}$ )).

Site	Effluent type	Discharge point	Reference
Zinkwazi	Domestic sewage	- Surf zone	Begg, 1978 Connell, 1988
Sheffield	Domestic sewage	- Surf zone	Begg, 1978 Connell, 1988
Westbrook	Domestic sewage and industrial effluent	- Tongati River	Begg, 1978 Connell, 1988 Pillay, 2006a
Umdhloti	Domestic sewage	- Surf zone - Mdloti River	Begg, 1978 Connell, 1988 Thomas <i>et al.</i> , 2005 Pillay, 2006b
Durban	Domestic sewage and industrial effluent *	- Umgeni River - Submarine outfalls	Begg, 1978; Connell, 1988; Macleod, 1982; McClurg <i>et al.</i> , 2006.
Isipingo	Domestic sewage and industrial effluent *	- Surf zone - Submarine pipeline - Umlaas River - Isipingo River - Umbogintwini River	Begg, 1978 Connell, 1988 Kalicharran and Diab, 1993 Gregory <i>et al.</i> , 2005 Newell <i>et al.</i> , 1991
Umkomaas	Domestic sewage and industrial effluent *	- Surf zone - Submarine pipeline	Begg, 1978 Connell, 1988 Schleyer <i>et al.</i> , 2006 Blair <i>et al.</i> , 2006
Port Shepstone	Domestic sewage and industrial effluent	- Surf zone	Begg, 1978 Connell, 1988
Margate	Domestic sewage	- Surf zone	Begg, 1978 Connell, 1988
Port Edward	Domestic sewage	- Surf zone	Begg, 1978 Connell, 1988



**Figure 5:** KZN coastline showing sampling sites and major rivers that drain the province.



### *Metal analysis*

Mussels were thawed and dissected by stainless steel instruments. After separating the soft tissues from the shells, tissues were pooled in groups of 5 specimens per inter-site replicate for each site ( $n = 3$  pooled samples from each site). Tissues were weighed and then homogenised in a stainless steel blender. The samples were dried at 60 °C for 2 days in an oven and then prepared for metal analysis by digesting approximately 2 g of sample in 5 ml 60% concentrated nitric acid for at least 10 hours. Samples were then further digested by microwave digestion. Microwave digestion was conducted in three stages (Baldwin et al., 1994) at the following power settings: two stages at 600 W for 2 min, with 2-min intervals, and a third stage at 200 W for 15 min. Once the samples were filtered through Whatman N°42 filter paper, metal concentrations were determined by atomic absorption spectrophotometry. Samples were analysed for copper (Cu), cadmium (Cd), lead (Pb), zinc (Zn), chromium (Cr), iron (Fe) and nickel (Ni), and are presented on a dry mass basis. Sediment was thoroughly stirred in a pestle for homogeneity and approximately 1g of sample was analysed for the same metals as the mussel tissue metal analysis using the same procedure described above. Internal standards were used to verify metal concentrations obtained, and recoveries of the internal standards always fell within 90% of this value. Mussel tissue metal concentrations were validated using certified reference material (National Institute of Standards and Technology), (SRM 2976 mussel tissue). Analysed and certified values for standard reference materials are presented in Table 8.

**Table 8:** Measured and certified values for standard reference materials

<b>Metal</b>	<b>NIST 2976 mussel tissue (µg/g)</b>	<b>Analysed value (µg/g)</b>
Cu	4.02 ± 0.33	4.21 ± 0.12
Cd	0.82 ± 0.05	0.79 ± 0.03
Pb	1.19 ± 0.18	1.17 ± 0.22
Zn	137 ± 12	141 ± 3
Cr	0.5 ± 0.16	0.46 ± 0.02
Fe	171 ± 4.9	165 ± 3.8
Ni	0.93 ± 0.12	1.02 ± 0.06

### *Cardiac activity*

A non-invasive technique was used to measure cardiac activity. An infra-red light emitting diode was glued onto the shell of each individual mussel at the position nearest to the heart ( $n = 30$  from each site, 10 specimens per replicate locality). Once diodes were attached to the shells, the mussels were left undisturbed for 6 hours. Cardiac activity were recorded, thereafter the mean heart rate (beats.min<sup>-1</sup>) for each site was calculated.

#### *Lysosomal membrane stability*

Lysosomal membrane stability was determined for each mussel ( $n = 30$  from each site, 10 specimens per replicate locality). Approximately 0.5 ml of haemolymph was extracted from a sinus along the adductor muscle using a 3.0 ml, 23G syringe, and thereafter placed in Eppendorf tubes. This was mixed with an equal volume of temperature adjusted physiological saline solution. Approximately 40  $\mu$ l of the haemolymph solution was allowed to attach onto haemocytometers, and were incubated at room temperature for 20 minutes. An equal volume of the neutral red dye (NR) was then allowed to infiltrate the cells for 15 minutes. The number of haemocytes that absorbed and retained the dye was observed at 15 minute intervals microscopically, and the time taken for 50% of the cells to lose their dye was recorded as the lysosomal membrane stability time (Nicholson, 1999a,b).

#### *Body condition index*

The ratio of tissue dry mass (mg) of mussel body tissue to shell length (mm) was determined for 30 mussels from each site ( $n = 10$  specimens per replicate locality), (Lundebye *et al.*, 1997; Galloway *et al.*, 2002).

#### *Statistical analysis*

Statistical analyses were performed using SPSS version 15.0 (SPSS for Windows, Rel. 15.0. 2006. Chicago: SPSS Inc.). All data obtained were tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test). All the data obtained from the various biomarker tests conformed to normal distribution and the variances were homogenous. Differences in metal concentrations in mussel tissue and sediment between sites were determined by ANOVA. Comparisons of biomarker responses among and within sites were investigated by a nested ANOVA, where localities were nested within sites. Tukey's HSD *post-hoc* comparisons were conducted to define significant differences between sites. Multidimensional scaling (MDS) was employed as a graphical presentation to differentiate sites by mussel tissue metal concentration by using PRIMER version 6 software. The Pearson's correlation coefficient was applied to test for significant correlations between pooled biomarker responses and tissue metal concentrations from all sites, in addition to correlations between pooled sediment and tissue metal concentrations.

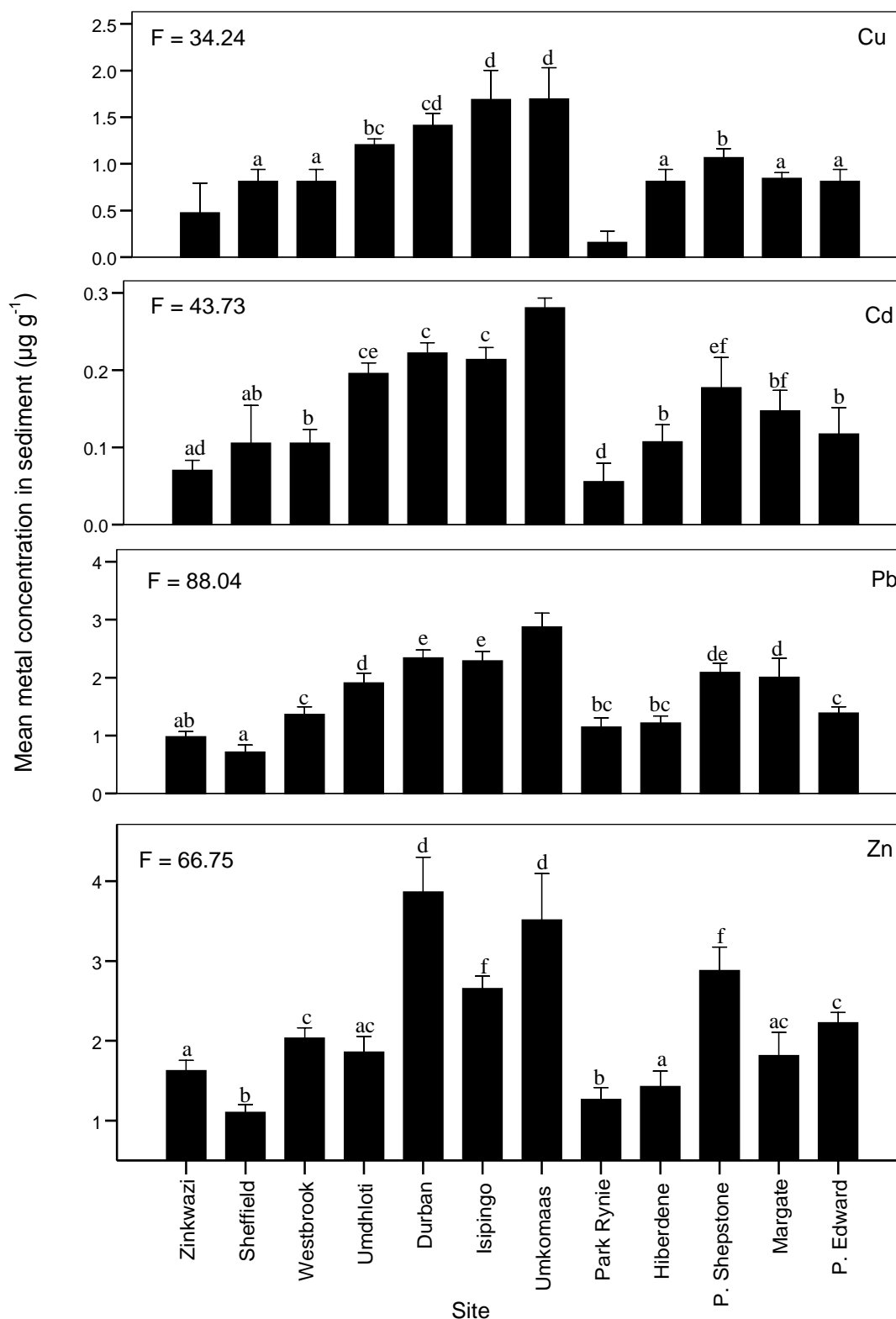
## Results

### *Sediment concentrations*

Figure 6 presents the mean metal concentrations in sediment collected along the KZN coastline and the ANOVA results for each metal analysed. The sediment metal results exhibited an increase in metal concentrations from the northern sites (Zinkwazi and Sheffield) to Umkomaas before significantly decreasing in Park Rynie sediments. Umkomaas had significantly higher sediment metal concentrations for Cd, Pb and Ni compared to the rest of the sediment results (ANOVA,  $df=11$ ,  $F = 43.73$ ; 88.04 and 28.65 respectively,  $p < 0.05$ ), whereas Zn and Fe however reached their maximum sediment concentrations at Durban. In contrast, Park Rynie displayed the lowest sediment metal concentrations for Cu and Cr compared to most of the other sites along the coast (ANOVA,  $df=11$ ,  $F = 34.24$  and 16.72 respectively,  $p < 0.05$ ).

### *Perna perna tissue metal concentrations*

MDS was used to graphically differentiate sites by *P. perna* tissue metal concentrations (Figure 7). MDS grouped sites by a stress factor of 0.02, resulting in Park Rynie and Zinkwazi being separated from the rest of the sites, as well as Durban, Isipingo and Umkomaas. Hence a clear distinction of these sites from the rest of the sampling sites along the KZN coast was established. Figure 8 illustrates a distinct latitudinal gradient in *P. perna* tissue concentrations and presents the ANOVA results for each metal analysed. A similar latitudinal trend as the sediment metal concentrations sampled at the same sites was found, as most metals such as Cu, Cd, Pb, Zn and Cr exhibited a gradual increase in soft tissue metal concentrations from the northern sites (Zinkwazi and Sheffield) which peaked at Durban, Isipingo and Umkomaas. These latter three sites displayed the highest tissue metal concentrations for most metals, particularly Isipingo and Umkomaas, and were mostly different from the northern most sites and Park Rynie and Hibberdene; except for tissue Ni concentrations which were did not show such large differences across sites (ANOVA,  $df=11$ ,  $F = 6.95$ ,  $p < 0.05$ ). Mussels collected at Umkomaas displayed significantly higher Cr tissue concentrations than the rest of the sites (ANOVA,  $df=11$ ,  $F = 19.95$ ,  $p < 0.05$ ). In contrast to Isipingo and Umkomaas, Park Rynie and Zinkwazi exhibited the lowest Cr mussel tissue concentrations and displayed significant differences in tissue metal concentrations for Pb and Cd from the rest of the sites (ANOVA,  $F = 39.98$  and 19.31 respectively,  $p < 0.05$ ). Zinkwazi also exhibited significantly low Cr concentrations in mussel soft tissues compared to the other sites (ANOVA,  $df=11$ ,  $F = 19.95$ ,  $p < 0.05$ ). The southern most sites sampled (Port Shepstone, Margate and Port Edward) did not show large variations in mussel tissue metal concentrations for metals, except for Cu, Zn and Fe



**Figure 6:** Spatial variations in metal concentration ( $\mu\text{g g}^{-1}$  dry mass) in sediment sampled from sites along the KZN coastline. Bars and error bars represent the mean sediment concentrations  $\pm$  standard error of the mean ( $n = 3$  localities from each site). Bars with common symbols denote similar means (ANOVA,  $p > 0.05$ ) between sites.

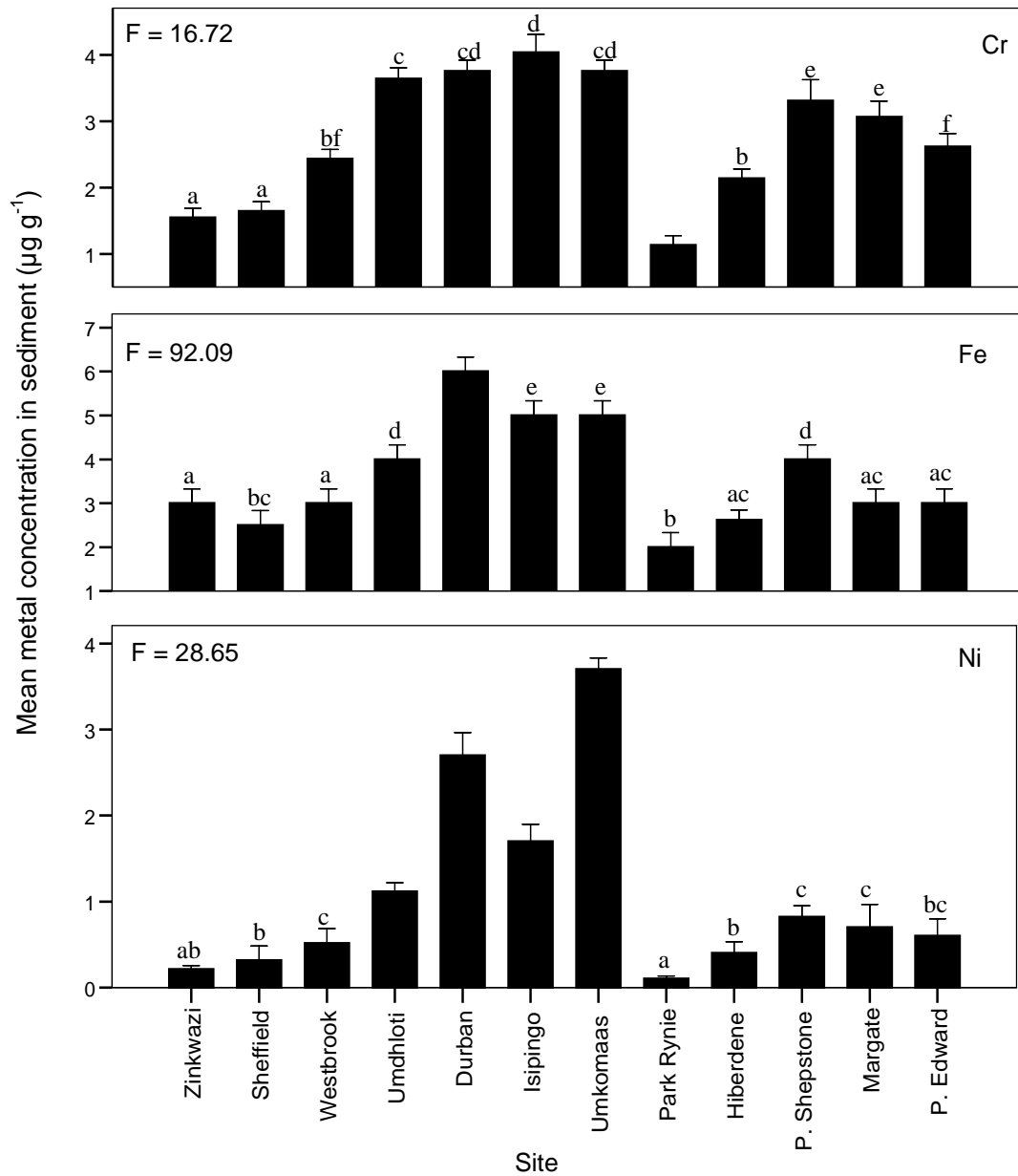


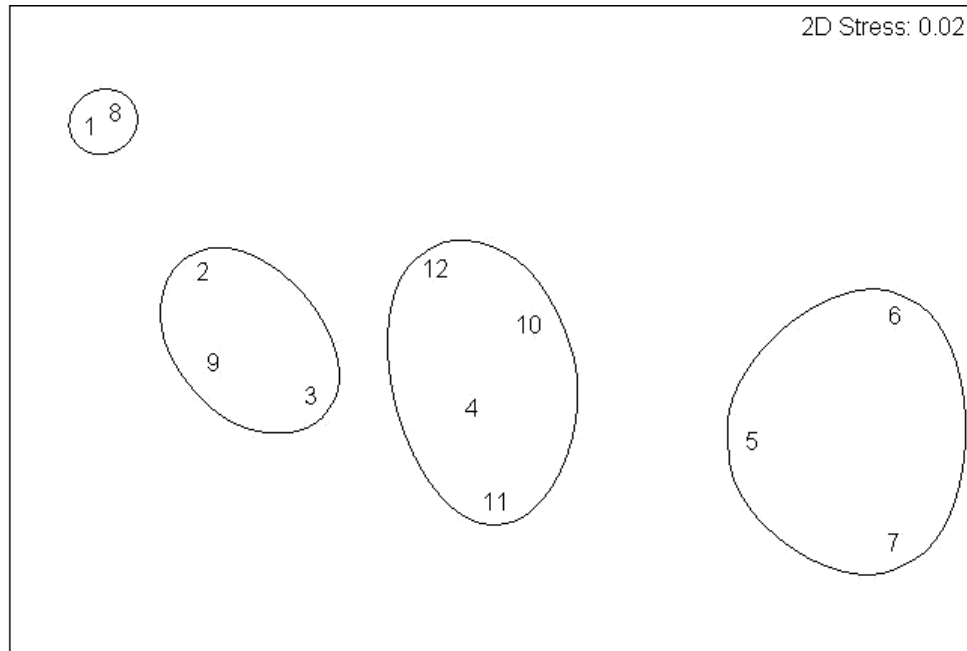
Figure 6 continued. Spatial variations in metal concentration ( $\mu\text{g g}^{-1}$  dry mass) in sediment sampled from sites along the KZN coastline. Bars and error bars represent the mean sediment concentrations  $\pm$  standard error of the mean ( $n = 3$  localities from each site). Bars with common symbols denote similar means (ANOVA,  $p > 0.05$ ) between sites.

tissue concentrations. Each of these metals displayed either significantly higher or lower mussel tissue concentration than the other two southern most sites.

#### *Mussel tissue and sediment concentration correlation analysis*

Figure 9 illustrates the correlations between tissue metal concentrations and sediment metal concentrations. Metals Cd, Pb, Zn, Cr and Fe exhibited strong correlations between tissue and

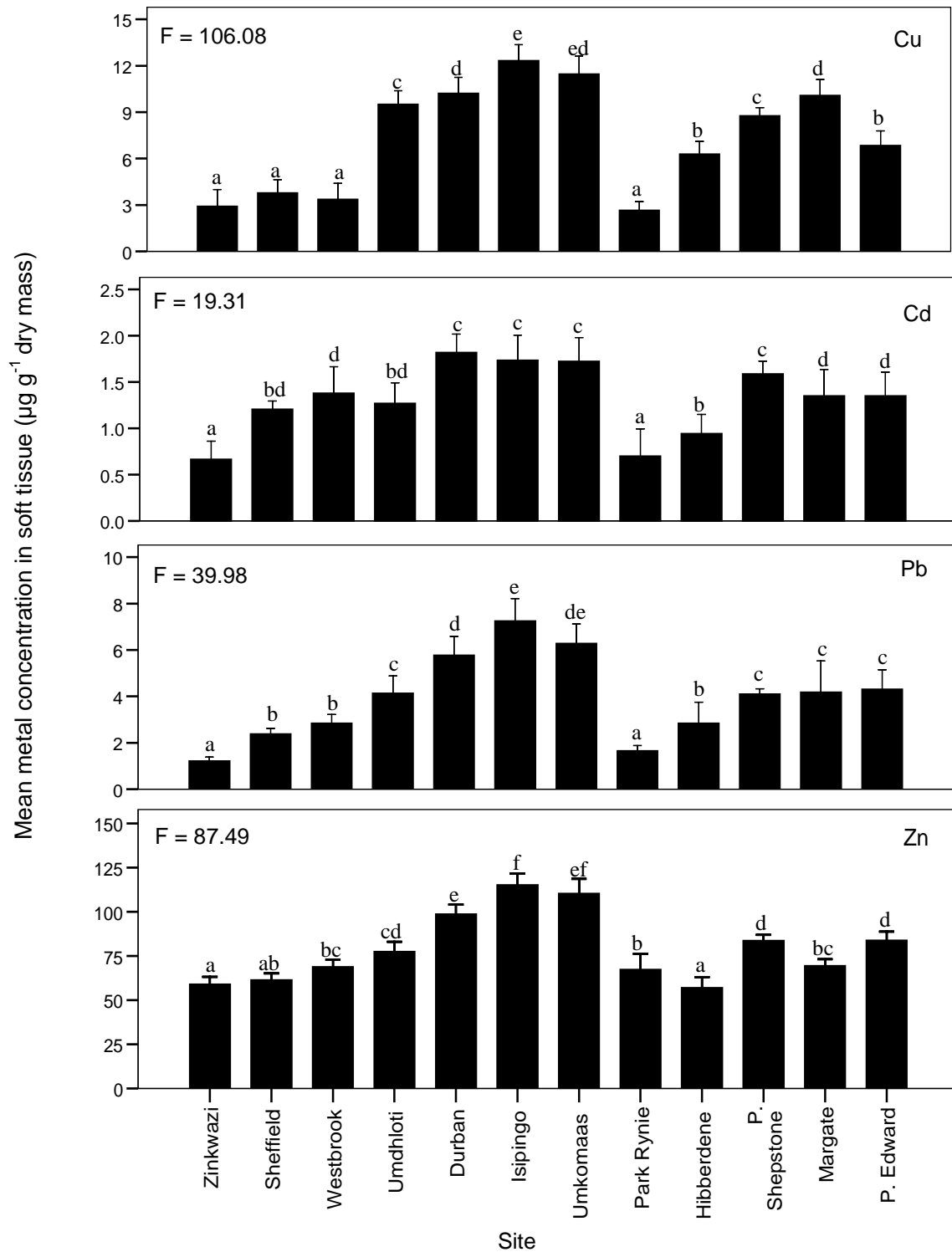
sediment concentrations, ( $r = 0.66, 0.71, 0.76, 0.69$  and  $0.79$  respectively,  $p < 0.05$ ). In contrast, there was no correlation between Ni tissue and sediment concentrations ( $r = -0.21, p > 0.05$ ), and Cu tissue and sediment concentration displayed a weak correlation ( $r = 0.36, p < 0.05$ ).



**Figure 7:** MDS representation of differentiation of sites by tissue metal concentrations by a stress factor of 0.02. Site 1 = Zinkwazi, 2 = Sheffield, 3 = Westbrook, 4 = Umdhloti, 5 = Durban, 6 = Isipingo, 7 = Umkomaas, 8 = Park Rynie, 9 = Hibberdene, 10 = Port Shepstone, 11 = Margate, 12 = Port Edward.

#### *Cardiac activity*

Heart rates were successfully measured from mussels from all sampling sites, and are presented in Figure 10. Mean heart beats from *P. perna* indicated a decreasing trend in cardiac activity from Zinkwazi to Umkomaas, before displaying a significant increase at Park Rynie; thereafter the sites south of Park Rynie exhibited an insignificant decline in heart rates. *Perna perna* heart rates differed significantly between sites, however there was little variation within sites (nested ANOVA,  $df = 24$ ,  $F = 121.43$ ,  $p > 0.05$ ). Cardiac activities of specimens from Durban, Isipingo and Umkomaas exhibited significantly reduced responses compared to mussels sampled from other sites (nested ANOVA,  $df = 11$ ,  $F = 50.98$ ,  $p < 0.05$ ), with mussels from Umkomaas showing the lowest heart rates ( $17.0 \pm 0.7$  beats.min<sup>-1</sup>), closely followed by those from Isipingo ( $17.6 \pm 0.9$  beats.min<sup>-1</sup>). The highest heart rates however were recorded from mussels sampled at Park Rynie ( $38.5 \pm 0.7$  beats.min<sup>-1</sup>).



**Figure 8:** Spatial variations in soft tissue metal concentration ( $\mu\text{g g}^{-1}$  dry mass) in *P. perna* retrieved from sites along the KZN coastline. Bars and error bars represent the mean tissue concentrations  $\pm$  standard error of the mean ( $n = 3$  pooled samples from each site). Bars with common symbols denote similar means (ANOVA,  $p > 0.05$ ) between sites.

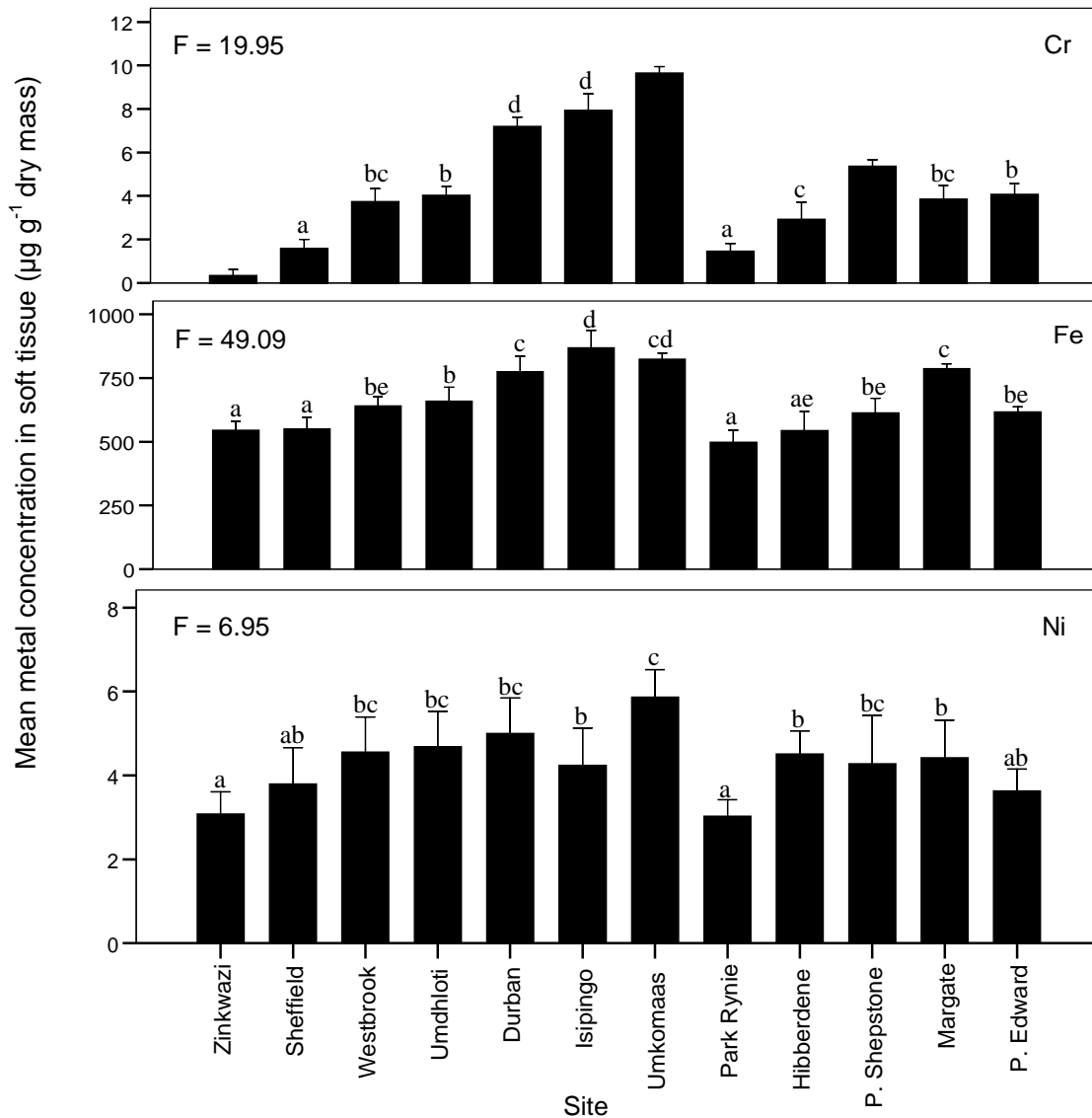
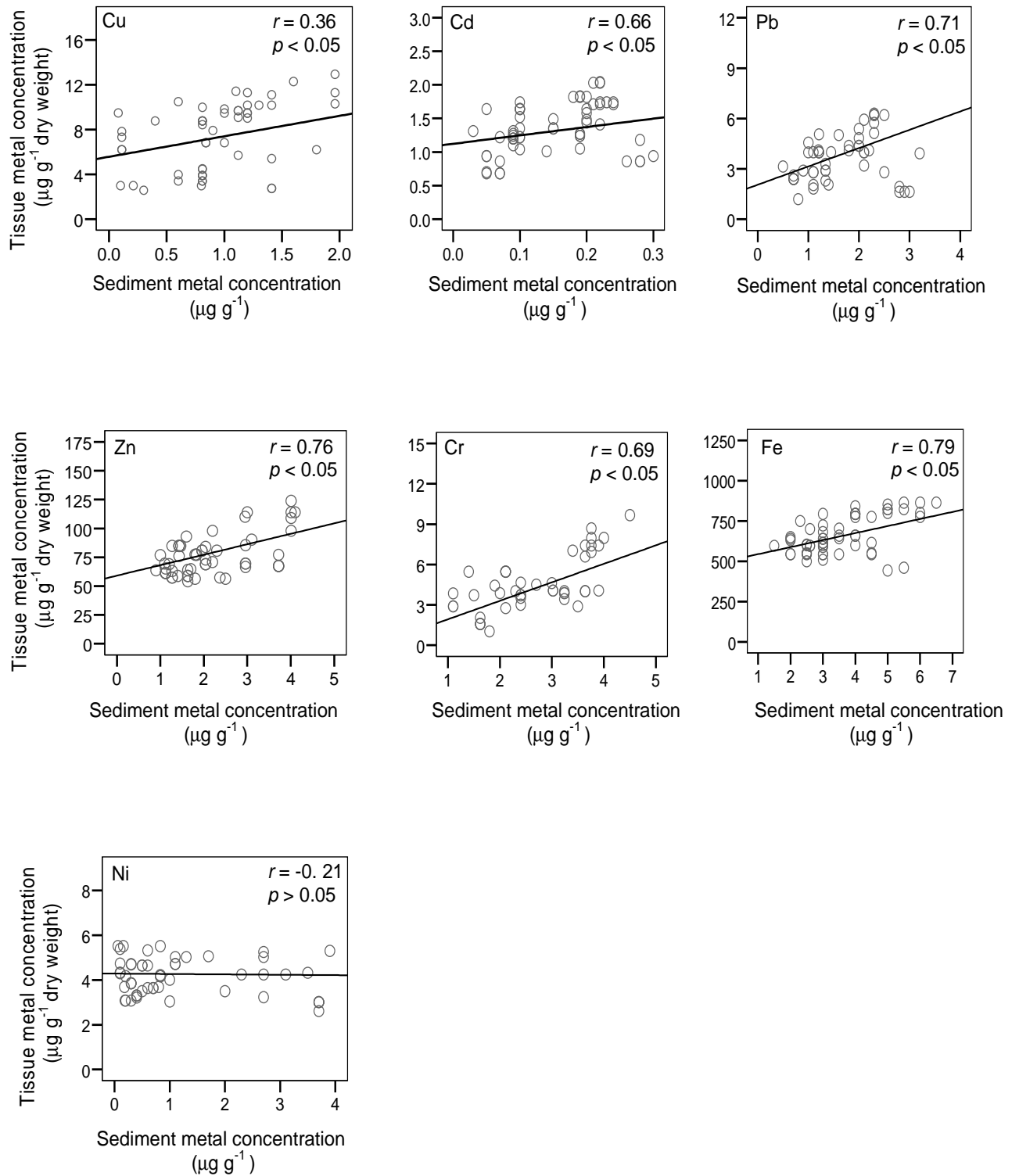


Figure 8 continued. Spatial variations in soft tissue metal concentration ( $\mu\text{g g}^{-1}$  dry mass) in *P. perna* retrieved from sites along the KZN coastline. Bars and error bars represent the mean tissue concentrations  $\pm$  standard error of the mean ( $n = 3$  pooled samples from each site). Bars with common symbols denote similar means (ANOVA,  $p > 0.05$ ) between sites.

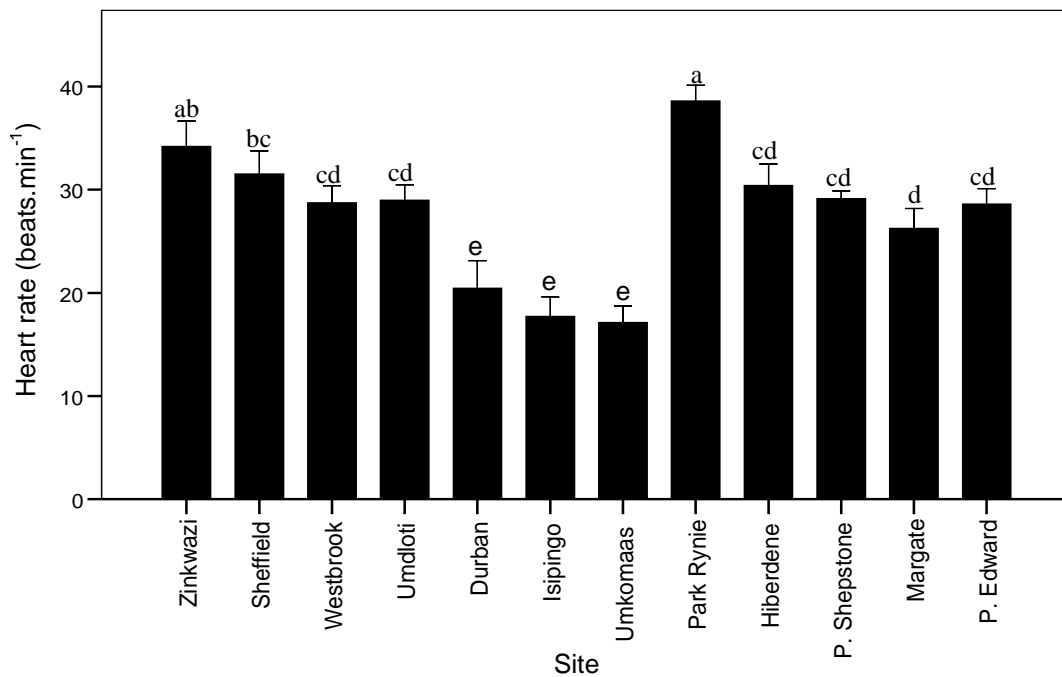
#### Lysosomal membrane stability

The lysosomal membrane stability of *P. perna* along the KZN coast adopted a similar pattern as cardiac activity (Figure 11); however reduced lysosomal membrane stability from mussels were observed at Margate. Lysosomal membrane stability differed significantly between sites, however there were no differences amongst localities per site (nested ANOVA,  $df = 24$ ,  $F = 104.91$ ,  $p > 0.05$ ). Durban, Umkomaas and Isipingo specimens displayed the lowest lysosomal membrane stability, and were significantly different from membrane stability times of mussels sampled from rest of the sites (nested ANOVA,  $df = 11$ ,  $F = 26.16$ ,  $p < 0.001$ ).





**Figure 9:** Correlations between *P. perna* tissue and sediment metal concentrations. ( $r$  = Pearson's correlation coefficient)

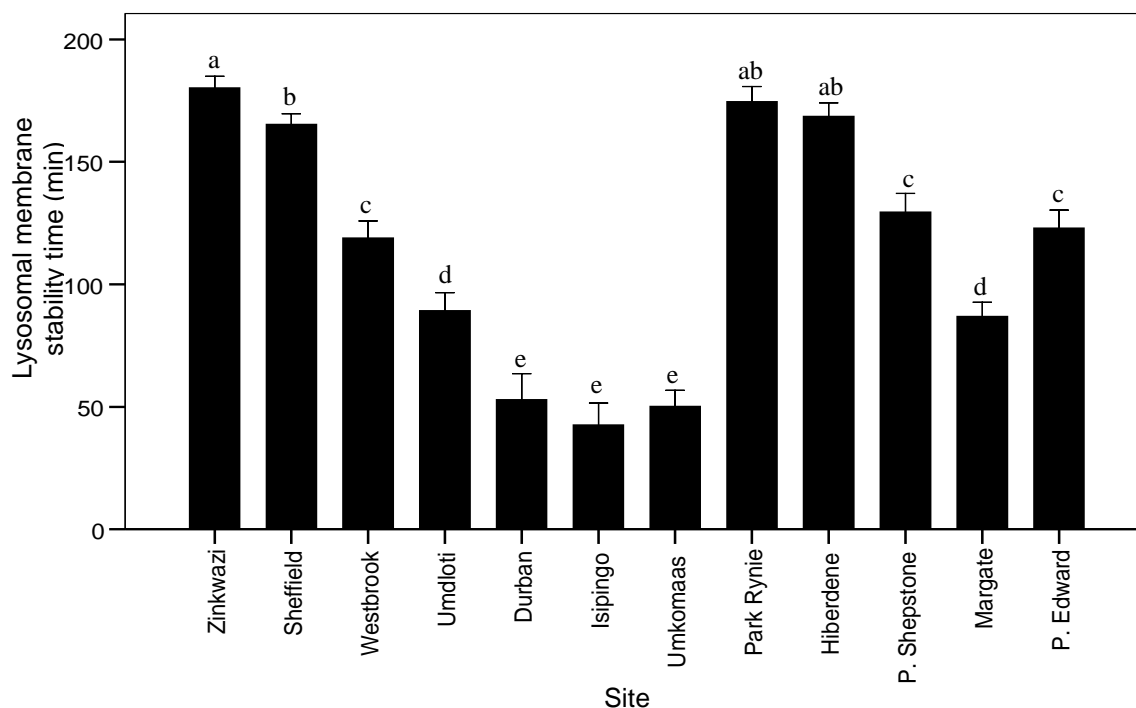


**Figure 10:** Spatial variations in mean heart rate (beats.min<sup>-1</sup>) from *P. perna* along the KZN coastline. Bars and error bars represent the mean tissue concentrations  $\pm$  standard error of the mean ( $n = 30$ ). Bars with common symbols denote similar means (nested ANOVA,  $p > 0.05$ ) between sites.

The lowest lysosomal membrane stability was observed from mussels sampled at Isipingo ( $42.3 \pm 4.3$  min), whereas the highest lysosomal membrane stability was observed from specimens obtained at Zinkwazi Beach ( $180.0 \pm 2.3$  min), followed closely by lysosomal stability recorded from Park Rynie, Sheffield and Hibberdene, all of which were significantly different from other sites sampled.

#### *Body condition index*

Condition indices from *P. perna* sampled along the coast are presented in Figure 12. The condition indices obtained from mussels at each site were consistent with the two other biomarker results. No significant differences were found within sites (nested ANOVA,  $df = 24$ ,  $F = 126.16$ ,  $p > 0.05$ ). The lowest indices from *P. perna* were obtained from Umkomaas ( $1.9 \pm 0.5$ ), which was also significantly different from the rest of the indices obtained along the coast (nested ANOVA,  $df = 11$ ,  $F = 41.90$ ,  $p < 0.001$ ). The healthiest conditions were obtained from Park Rynie specimens ( $4.9 \pm 0.2$ ), however the condition index from this site were not different to the high indices from Zinkwazi and Sheffield.



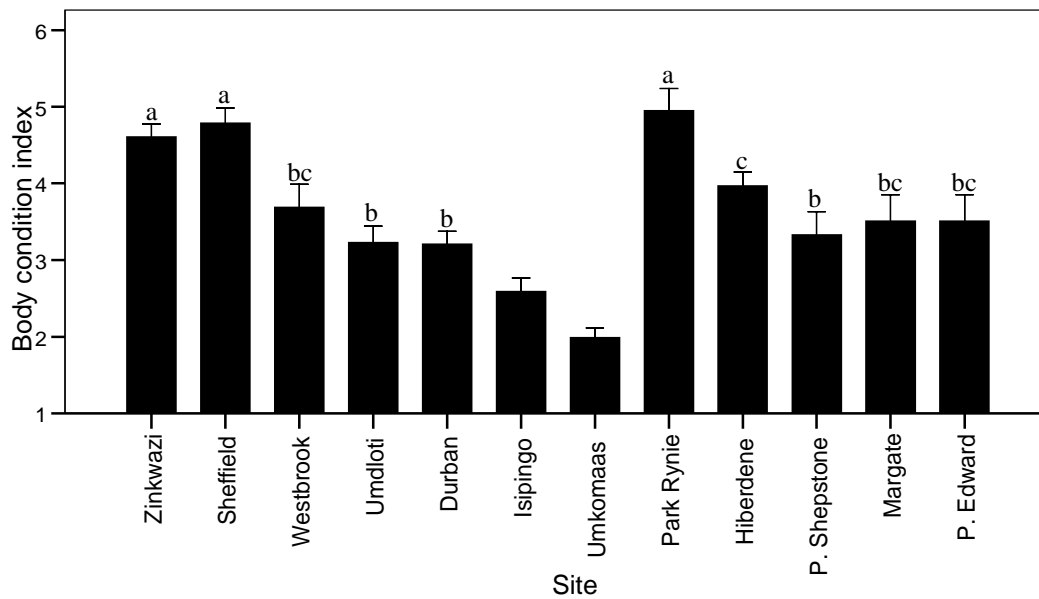
**Figure 11:** Spatial variations in mean lysosomal membrane stability from *P. perna* along the KZN coastline. Bars and error bars represent the mean tissue concentrations  $\pm$  standard error of the mean ( $n = 30$ ). Bars with common symbols denote similar means (nested ANOVA,  $p > 0.05$ ) between sites.

#### *Biomarker and tissue concentration correlation analysis*

Table 9 presents the correlations between biomarker responses from *P. perna* and tissue metal concentrations. Body condition indices were significantly correlated to tissue concentrations; however the other two biomarkers used in the study only showed strong associations with selected metal tissue concentrations. Lysosomal membrane stability had significant correlations only for Cu, Cd, Zn and Cr tissue concentrations ( $r = -0.66, -0.72, -0.59$  and  $-0.65$  respectively,  $p < 0.05$ ); whereas cardiac activity only display significant correlations with Cu, Pb, Zn and Fe tissue concentrations ( $r = -0.75, -0.68, -0.49$  and  $-0.69, p < 0.05$ ).

#### **Discussion**

Heavy metals are natural elements that occur in marine environments; however, coastal waters, particularly areas adjacent to industrialised and urbanised zones, are vulnerable to the excessive contaminant loads entering the environment (Anderson *et al.*, 1996; Wells, 1999; Chase *et al.*, 2001). The coastal waters along KZN are particularly at risk as the rapid rate of development along the coast has highlighted the urgency for marine pollution monitoring (Moldan, 1995; O'Donoghue and Marshall, 2003; Oelofse *et al.*, 2004; Misheer *et al.*, 2006a,b,c). This study demonstrates how a suite of biomarkers from *P. perna*, accompanied by chemical analysis, can distinguish a pollution gradient in coastal waters off KZN.



**Figure 12:** Spatial variations in mean body condition indices from *P. perna* along the KZN coastline. Bars and error bars represent the mean tissue concentrations  $\pm$  standard error of the mean ( $n = 30$ ). Bars with common symbols denote similar means (nested ANOVA,  $p > 0.05$ ) between sites.

**Table 9:** Pearson's correlation coefficients between trace metal concentrations and biomarker responses.

Metal	Body condition index	Lysosomal membrane stability	Cardiac activity
Cu	-0.84 *	-0.66 *	-0.75 *
Cd	-0.75 *	-0.72 *	0.35
Pb	-0.86 *	-0.13	-0.68 *
Zn	-0.82 *	-0.59 *	-0.49 *
Cr	-0.91 *	-0.65 *	-0.12
Fe	-0.73 *	-0.21	-0.69 *
Ni	-0.61 *	-0.07	-0.06

(\* denotes significant  $p$ -values at  $p < 0.05$ )

#### Metal analysis

Heavily industrialised areas are well associated with pollution of coastal waters, and prominent metal concentrations in sediment and mussel soft tissues have been documented in numerous studies (Phillips, 1976a,b; Davies and Pirie, 1980; Shugart *et al.*, 1992; Boening, 1997; Rainbow *et al.*, 2000; Ravera, 2001; Blackmore and Wang, 2002; Lui and Kueh, 2005; Szefer *et al.*, 2006). Similar results as these studies have been found in this study as sediment and *P. perna* soft tissue metal concentrations were generally greater at industrial and urban zones along

the KZN coast. Sediments concentrations of Cu, Cd, Pb, Cr, Fe and Ni conformed to the general latitudinal pattern displayed by tissue metal concentrations. Sediment concentrations of Zn however, failed to display a distinct latitudinal gradient but showed maximum levels at Durban, Umkomaas and Port Shepstone, and low levels at Sheffield and Park Rynie. The results generated from the MDS analysis (Figure 7) reflected the magnitude of industrialisation and heavy metal contamination at each site, and grouped Durban, Isipingo and Umkomaas together, as well as differentiated the unpolluted and undeveloped areas of Park Rynie and Zinkwazi from the rest of the sites. Metals Cu, Cd, Pb, Zn and Cr in *P. perna* soft tissues exhibited significant concentration gradients along the coastline, with pronounced levels at Durban, Isipingo and Umkomaas and significantly low levels at Park Rynie and Zinkwazi. Fe and Ni tissue concentrations however did not display as large variations across sites as did the other metals.

Considering the correlation results between tissue and sediment metal concentrations, it can be deduced that *P. perna* is an effective bioaccumulator species, especially for metals Cd, Pb, Zn, Cr and Fe. Zn and Fe concentration levels, in particular were found to be much higher in *P. perna* tissues compared to sediment. Similarly, Yap *et al.* (2002) found a significant correlation between metal concentrations in mytilid mussel tissue and sediment for Cd, Cu and Pb; whereas Beiras *et al.* (2003) found strong correlations only between Cu and Pb tissue and sediment concentrations. In addition, both these studies document weak correlations between Zn tissue and sediment concentrations. This is in contrast with the results of this study which found a significant correlation between tissue and sediment Zn concentrations, and no such significance for the correlation between tissue and sediment Cu concentrations. A study by Hummel *et al.* (1997) however, does support the findings of a weak Cu tissue and sediment correlation. The metals Zn, Fe and Cu are essential metals to organisms, hence the varying correlation strengths of the tissue and sediment concentrations of these metals can be attributed to regulation within the body or rapid detoxification and elimination (Hummel *et al.*, 1997; Yap *et al.*, 2002). A significant correlation between Pb mytilid tissue and sediment concentration was also found by Beiras *et al.* (2003), as was the correlation of Cr in Zebra mussel (*Dreissena polymorpha*) tissue and sediment by Lowe and Day (2002); thus further supporting the findings of this study. The above study also supports the lack of correlation between Ni tissue and sediment concentrations and attributes this result to rapid detoxification and excretion of the metal. However, in this study, higher Ni concentrations were found in mussel tissue than in sediment. According to McGeer *et al.* (2003) and Muysen *et al.* (2004), bioconcentration factors are independent of contaminant exposure, as metal uptake and elimination are controlled by complex physiological mechanisms. Marine organisms commonly eliminate excess metals via their kidneys, liver or

gills; and mussels in particular have been found to detoxify and store metal contaminants such as Ni. Mussels contain calcium-phosphate based, insoluble granules that store excessive amounts of Ni, thereby making the metal non-bioavailable to the organism (McGeer et al., 2003; Muyssen et al., 2004). Hence, this reasoning can be used to explain the higher concentration of Ni found in mussel body tissues than in sediment.

Durban is the largest city in KZN with its most productive industrial and commercial nodes located in central Durban and the port (Parsons, 2002; Pillay, 2003). Two large submarine outfalls have been responsible for discharging the city's industrial and domestic effluent since 1970 (Connell, 1988; McClurg *et al.*, 2006). As industry and informal settlements in the city are continuously expanding, the coastal waters of the city have been receiving increasing amounts of toxic wastes and untreated sewage (Begg, 1978; Mardon and Stretch, 2002). The biomarker responses from *P. perna* and the metal concentrations from soft tissues and sediment indicate that Durban is the third most contaminated site along the KZN coast. Although the Tongati, Umhlanga and Umdhloti Rivers and estuaries on the north of Durban are under pressure from receiving industrial and sewage discharges, as well as agriculture runoff, the contamination levels in *P. perna* were not as severe as those from central Durban and the region south of Durban.

Called the South Durban Basin, the region south of Durban supports numerous major industries. Some of the larger industries situated in this industrial belt are Engen (the largest South African petroleum group), Sapref (the largest crude oil refinery in Southern Africa), AECI (which produces various chemicals), Huntsman Tioxide (manufacturer of chemicals and pigments) and Mondi Paper Mill (which is one of the largest individual paper mills in the world), (Newell *et al.*, 1991; Nurick and Johnson, 1998; Matooane and Diab, 2001; Diab *et al.*, 2002, Lund-Thomson, 2002; Batterman *et al.*, 2007; Van Alstine, 2007). Other industries include the Illovo and Hullels sugar mills, manufactures of plastics, textiles, asbestos products, galvanising plants, landfill sites, large chemical tank farms and paint industries (Batterman *et al.*, 2007). It is therefore not surprising that the Isipingo and Umbogintwini Rivers and the Umlaas and Reunion canals that discharge into the sea are severely polluted, as these water bodies receive large quantities of toxic wastes from the entire industrial region, together with sewage, stormwater runoff and leached agrochemicals (Kalicharran and Diab, 1993; Grobler *et al.*, 1996). Still in the south coast, the coastal environment of Umkomaas is also severely polluted by industrial effluent from extensive paper and pulp industry (SAPPI-SAICCOR) and municipal domestic wastes, as well as agricultural runoff from numerous farms alongside the river (Begg, 1978;

Connell, 1988; Schleyer *et al.*, 2006; Blair *et al.*, 2006). Considering the substantial number of industries along the coast of KZN is localised in central Durban, Isipingo and Umkomaas, it stands to reason that these environments would be the most contaminated. The results from this study shows that although the highest contamination levels of heavy metals in *P. perna* and sediment were generally limited to these industrial localities, metal contamination along the KZN coastline is prevalent. While stormwater drainage, leached agrochemicals and river inputs contribute to the metal contamination levels, the main contributors of marine pollution are the point sources of effluent discharges off the KZN coastline (Connell, 1988).

Chemical analysis of water and sediment provide a valuable insight into which chemicals are present in the ambient environment, however the approach fails to consider the biological effects of such contaminant levels. Metal tissue determinations on the other hand provides an indication of metals that are bioavailable to organism in the environment, however it must be noted that essential metals such as Cu and Zn are regulated for bodily functions (Anandraj *et al.*, 2002; Nicholson, 2003), whilst the more toxic metals are either stored in lysosomes or rapidly detoxified and eliminated from the mussel body. Additionally, external factors such as salinity and temperature fluctuations can influence metal uptake in mussels (Phillips, 1976a; Nicholson 1999a,b; Anandraj *et al.*, 2002). Hence, in order to attain an accurate assessment of the effects of pollution levels in the environment, a suit of biomarkers must be employed (Wedderburn *et al.*, 2000).

#### *Cardiac activity*

Numerous authors have shown a distinct change in various physiological responses from marine bivalves such as gill filtration rate, respiration rate, valve closure and heart rate when exposed to anthropogenic pollutants (Nicholson, 1999a,b; Wedderburn *et al.*, 2000; Galloway *et al.*, 2002; Brown *et al.*, 2004; Marshall *et al.*, 2004; Petrovic *et al.*, 2004; Abessa *et al.*, 2005). Bradycardia has been well documented in bivalves exposed to metal contaminants (Nicholson, 1999a,b; Curtis *et al.*, 2001, Galloway *et al.*, 2002). The findings in this study are consistent with these previous studies as mussels sampled along the coastline displayed a strong geographic trend in declining cardiac activity from relatively undeveloped sites to highly industrial zones supporting various industries. *Perna perna* sampled at Durban, Umkomaas and Isipingo in particular were under significant physiological stress as indicated by significantly depressed cardiac activity. In contrast, mussel heart rates from Park Rynie and Zinkwazi did not exhibit reduced cardiac activities. Nicholson (1999a) explained that very mild metal levels will not induce bradycardia as a result of effective metal assimilation by humoral ligands and

metallothionein binding of pollutants, which restricts the quantity of the contaminant present in the haemolymph before any organelle damage can occur. The difference in mussel cardiac activity between sites are thus consistent with the findings from several other studies which compared cardiac activities from mussels obtained from polluted and unpolluted sites (Nicholson, 1999a; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Galloway, 2002).

#### *Lysosomal membrane stability*

Lysosomes are hydrolytic enzyme containing organelles that are the target sites for accumulated metal contaminants as these organelles are involved in contaminant storage and detoxification (Wedderburn *et al.*, 2000; Petrovic *et al.*, 2004). Lysosome membrane destabilisation has been well documented for mussels experiencing elevated contaminant conditions from polluted sites (Lowe *et al.*, 1995; Nasci *et al.*, 1999; Wedderburn *et al.*, 2000; Nicholson, 2001; Riveros *et al.*, 2002; Domouhtsidou *et al.*, 2004; Petrovic *et al.*, 2004; Abessa *et al.*, 2005; Regoli *et al.*, in press), as the excessive contaminant load on the lysosome ceases the membrane proton pump and denatures important lysosomal membrane enzymes which aid in the functioning of the organelle (Nicholson, 1999b). The results of this study are consistent with the findings of these previous studies, as the lysosomal membrane stability assays carried out on *P. perna* from sites that supported industrialised and urbanised activities were significantly shorter than those recorded from relatively unpolluted areas along the KZN coastline. Hence the clear geographic trend exhibited by mussel cardiac activity was also displayed by lysosomal membrane stability in *P. perna*.

#### *Body condition index*

The condition indices measured in *P. perna* from the KZN coast exhibited significant differences between sites. In particular, mussels sampled from Umkomaas and Isipingo displayed the lowest condition indices when compared to the rest of the sites. The findings in this study is similar to that of previous work done by Avery *et al.* (1996), Nicholson (1999a,b), Mauri and Baraldi (2003) and Bodin *et al.* (2004) which also documented low condition indices from contaminated sites. As pointed out by Dahlhoff and Menge (1996) and Nicholson (1999b), the exposure of marine mussels to elevated anthropogenic contaminants will induce a considerable degree of stress on the organisms. This will result in the organisms attempting to cope with such conditions by rapidly assimilating, detoxifying and excreting much of metal contaminant from its body, thereby depleting energy reserves that would ordinarily be reserved for growth, reproduction and numerous other physiological body processes. In contrast to the indices obtained from mussels sampled at industrial and urban zones, *P. perna* sampled at Park



Rynie and Zinkwazi displayed the healthiest conditions indices. The results of the condition indices from mussels of KZN support the adverse responses indicated by significant bradycardia and reduced lysosomal membrane stability of *P. perna* from highly industrialised areas. Although condition indices have also been found to be affected by seasonal changes, reproductive activity and nutritional health of the organisms (Lundebye *et al.*, 1997), this biomarker has been proven in to be sensitive and effective in numerous studies (Soto *et al.*, 1995; Avery *et al.*, 1996; Lundebye *et al.*, 1997; Nicholson, 1999a; Galloway *et al.*, 2002 and Smolders *et al.*, 2003).

Although cardiac activity and lysosomal membrane biomarker responses of *P. perna* exhibited strong correlations with only selected metals, body condition index displayed strong correlations for all metals analysed in body tissues, thereby emphasising the biomarker's accurateness in indicating contaminated environments.

In this study, the results of metal analysis and the adverse biomarker responses indicated by significant bradycardia, low lysosomal membrane stability and poor condition indices of mussels from these regions support the notion that Durban, Isipingo and Umkomaas are hotspots for marine pollution. Furthermore, biomarker responses were directly related to elevated metal concentrations, thus highlighting these marine pollution monitoring techniques as valuable tools for assessing the overall condition of the KZN coastal environment. These biological responses are well established biomarkers which have been extensively studied under laboratory conditions for their effectiveness as biomonitoring tools (Wedderburn *et al.*, 2000). Nevertheless, environmental factors from different locations can influence biomarker responses from mussels. To overcome this, Phillips (1976b) designed a sampling protocol for *in situ* sampling and comparisons of bioindicator responses from different locations. The most important considerations taken into account here were:

1. All samples were collected over the shortest period as possible (over 2 days), to reduce the occurrence of sudden events that could affect biomarker responses, e.g. sudden storms.
2. Since *P. perna* displays distinct annual growth and reproductive cycles, tissue mass and metal uptake rates are also affected. Hence mussels were collected in later winter in order to minimise the effects of gonad maturity on metal uptake. Also, mussels generally show a higher metal tissue concentration in winter.

3. Studies have shown a distinct relationship between mussel size (shell length and wet mass) and metal accumulation rates; hence adult mussels only from a set size class were sampled (shell lengths between 55 – 60 mm).
4. Mussels were all collected from similar environments e.g. highly exposed rocky shores from the same intertidal depths, to minimise variation in mussel exposure to air, food, salinity and temperature.

Mussels from the Mytilidae family are a well established family of bioindicator species. The mussels used in this study occur abundantly on KZN shores and proved to be a suitable bioindicator species for this *in situ* study. Being an indigenous species, *P. perna* is well adapted to local environmental conditions. Thus the distinct biological responses obtained from the mussels for all biomarker assays highlights the species sensitivity to contamination on a localised scale, and to its ability to display biomarker responses that are proportional to the degree of pollution.

### **Conclusion**

In conclusion, this study illustrates how the use of a suite of biomarkers that incorporate physiological and cytological responses from *P. perna* can be a diagnostic tool of stress in mussels that serve as early warning signals of marine environment degradation. The usefulness of assessing these biomarkers from field studies provides a reliable and cost-effective approach to rapidly assess marine pollution in coastal waters of KZN and clearly differentiate contaminated areas from unpolluted sites.

## **CHAPTER 4: Spatial Variations in Physiological and Cytological Biomarkers of *Perna perna* Transplanted in Durban Harbour (KZN).**

### **Introduction**

Estuaries have been defined as semi-enclosed systems that are still freely connected to the open sea (Begg, 1978; Moore, 1999; Kennish, 2002). These systems exhibit complex hydrology dynamics as they are subject to the continuous mixing of freshwater and seawater (Kennish, 2001), and hence produce unique and highly productive ecosystems that support complex food webs consisting of both terrestrial and aquatic biota. Estuaries also function as vital spawning and nursery grounds for a diverse range of species (Cyrus and Forbes, 1996; Forbes *et al.*, 1996; Vandermeulen, 1996; Whitefield, 1997; Abuodha and Kairo, 2001; Alongi, 2002; der Boer, 2002). Despite their ecological value, these ecosystems are being degraded beyond repair on a global scale (Chase *et al.*, 2001; Kennish, 2002), particularly those in southern Africa where development encroachment has a severe impact on shallow coastal and estuarine systems (Begg, 1978; Cyrus and Forbes, 1996; Forbes *et al.*, 1996; Whitefield, 1997; Abuodha and Kairo, 2001).

Strategically located along one of the world's most important shipping routes, South Africa has had an exponential increase in international trade over the last decade (Siko, 1996; Marshall and Rajkumar, 2003). As a consequence, there has been extensive port development along the coastline (Vermeulen and Wepener, 1999; Fatoki and Mathabatha, 2001; Marshall and Rajkumar, 2003; Wepener and Vermeulen, 2005), particularly on the eastern seaboard. To accommodate such rapid development, two of the largest estuaries in Kwa-Zulu Natal have been converted into productive ports, namely Durban and Richards Bay Harbours (Forbes *et al.*, 1996; Vermeulen and Wepener, 1999; Marshall and Rajkumar, 2003).

Port environments are continuously being threatened by a range of contaminants, which originate from shipping and dry dock activities, accidental leakage of toxic substances, illegal dumping and spillage from tankers (Chase *et al.*, 2001; Taljaard, 2006). Harbours also receive vast amounts of ballast water discharges and it is estimated that approximately 20 million tons of ballast water is discharged in South African harbours annually (Taljaard, 2006). Furthermore, dredging activities that take place in such environments reintroduce contaminants that have accumulated within the sediment, particularly heavy metals and hydrocarbons, into the environment (Taljaard, 2006). Since harbours are sheltered from strong tidal action, there is poor water circulation within these environments to flush pollutants out of harbour. As a result most contaminants that empty into bays accumulate within harbour environments (Taljaard,

2006), and pollutants that are commonly associated with such systems include heavy metals, petroleum contaminants, suspended solids, litter and biodegradable organic matter (Vermeulen and Wepener, 1999; Fatoki and Mathabatha, 2001; Wepener and Vermeulen, 2005; Taljaard, 2006).

Originally a shallow and permanently open estuary, the Port of Durban was developed in 1824 and is regarded as the most productive port in Africa. The harbour is still strongly influenced by the continuous mixing of sea water and freshwater (Harris and Cyrus, 2000); hence a distinct salinity gradient exists from the harbour entrance to the upper reaches of the bay which receive freshwater inputs from three rivers. Although the bay is used as a fully operational port, Durban Harbour still sustains estuarine and marine environments that support high species diversity, which have documented by numerous studies (Cyrus and Forbes, 1996; Forbes *et al.*, 1996; Vermeulen and Wepener, 1999; Harris and Cyrus, 2000; Weerts and Cyrus, 2002). This prompted a study to assess the environmental health of the bay by using biomarkers from the brown mussel *P. perna* transplanted in the bay, as well as investigating the seasonal and spatial variations of these responses and heavy metal contamination in the harbour.

Biomarker responses from indicator organisms have become an appreciated tool used in assessing environmental condition and include cytological and physiological responses from organisms as indicators of environmental stress (Riveros *et al.*, 2002; Luk'yanova, 2006; Nigro *et al.*, 2006). Active biomonitoring is defined by Nasci *et al.* (1999) as the relocation of biomonitoring organisms from an unstressed and unpolluted site to another monitoring area in order to investigate the change in their biological responses for monitoring marine contamination. The brown mussel *P. perna* occurs abundantly on the east coast and has exhibited distinct cytological and physiological responses to varying levels of chemical contaminants (see Chapters 2 and 3). Hence this species was selected as a suitable biomonitor for this study.

Chapter 4 tests the hypothesis that biomarker responses from transplanted *P. perna* show a response with regards to a gradient in pollution in Durban harbour. This chapter aims to assess the health of the marine environment in Durban harbour by investigating:

1. Water quality in Durban harbour by identifying spatial trends in seawater metal concentrations.
2. The suitability of *P. perna* in transplant experiments by investigating:
  - Cardiac activity from transplanted organisms.

- Lysosomal membrane stability from mussels.
- Body condition index from *P. perna*.
- Spatial trends in biomarker responses from mussels.
- Heavy metal bioaccumulation in biomonitor mussels.

## **Materials and methods**

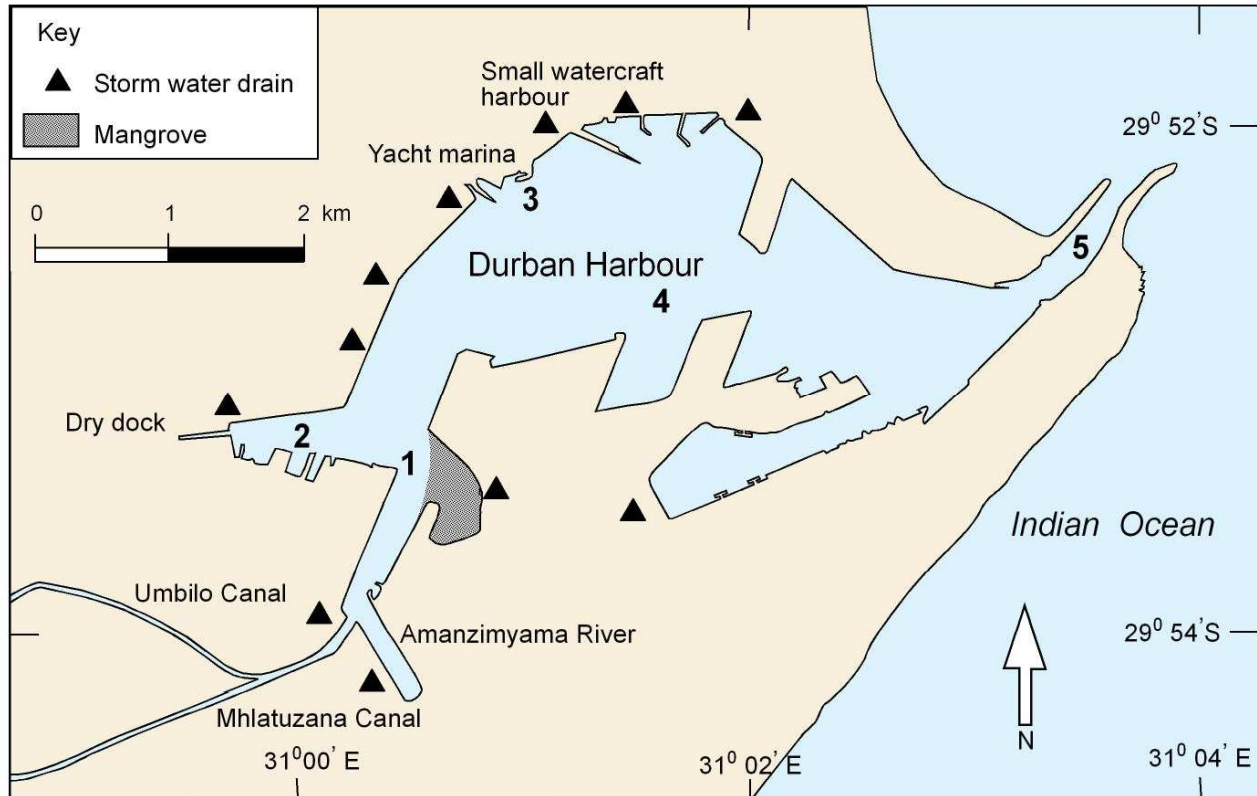
### *Site overview and station selection*

Durban harbour (29° 52° S; 31° 02° E) has a total land and surface water area of approximately 1854 ha and operates numerous terminals, the largest being Durban Container, Multi-purpose and Car Terminals, as well as the Maydon Wharf Terminal which supports a sugar, bulk, wood chip terminals and other privately owned facilities. The harbour also supports a coal terminal, oil and petroleum complex, a passenger terminal and two fresh produce terminals. In addition to these, there are three marinas within the harbour that are allocated for yachts and small watercrafts. The harbour is surrounded by the recent Point Waterfront development and central business district at the north eastern region, Maydon Wharf on the west, the Bluff Peninsula and Salisbury Island on southern region and the Bayhead ship repair area (dry dock) at the inner reaches of the port. The area that surrounds the harbour is therefore heavily urbanised and industrialised, with many storm water drains emptying into the bay (Figure 13), together with three rivers (Umbilo and Mhatuzana Canals and Amanzimyama River). Stations in Durban harbour were selected by their proximity to stormwater drains and freshwater inflows, as well as the harbour entrance to obtain a fair representation of the harbour. Station 1 was located at the nearest possibly area to the freshwater inputs, however salinity and depth were also taken into account. Station 2 was chosen due to its location near the dry dock area. Station 3 was positioned in the vicinity of the yacht and small watercraft harbour, as well as directly opposite the main stormwater outfalls that drain the immediate city centre. Stations 4 and 5 were positioned near the entrance of the harbour. Park Rynie was chosen as a reference site (see Chapter 2, page 25).

### *Construction of mesh bags*

Polyethylene mesh bags were constructed using plastic cable ties, each bag consisted of five separate compartments. Each of these compartments held only five *P. perna* individuals to ensure free circulation of water, to allow each organism equal exposure to the ambient environment, to allow valve movements and to reduce stress from over-crowding. Four replicate mussel bags were secured at each station, onto existing navigational bouys positioned around the harbour using nylon rope at depths of 1.5 meters to ensure the test specimens were still fully

submerged during low tide. At Park Rynie, mussel bags were secured to floats and existing rocks below the infra-tidal zone.



**Figure 13:** Map of Durban Harbour showing sampling stations.

#### *Mussel collection and retrieval*

Transplant experiments began at the beginning of June 2005 and the entire procedure was repeated at the beginning of December. Healthy *P. perna* specimens, between 55 – 60 mm in length, were collected by hand from Park Rynie at low tide and transported in plastic containers with seawater to the laboratory (University of Kwa-Zulu Natal). Mussels were carefully cleaned of any epizoans, and 30 specimens were randomly selected to be used as a reference group that represented baseline information on shell measurements, tissue mass, body metal concentrations and biomarker responses. The remaining mussels were slotted into the bag compartments and thereafter maintained in well-aerated artificial seawater (35‰ Instant Marine) with a constant water temperature of 23 °C. The transplant bags were deployed into the field 24 hours after collection, and deployment commenced the following morning. Mussel bags were transplanted at five stations within Durban Harbour, and control bags were transplanted back at Park Rynie. Each station had four replicate mussel bags spread out approximately 2.5 m apart. One

compartment per replicate bag was retrieved from each station every 14 days for biomarker and tissue metal analysis, over a total transplant period of 70 days.

#### *Metal analysis*

*Perna perna* individuals from each station were dissected by stainless steel instruments ( $n = 20$  from each station, i.e. five mussels per replicate bag). After separating the soft tissues from the shells, the individual tissues were dried at 60 °C for 2 days in an oven. Due to budget constraints, mussel tissues from each station were pooled. Tissues were then prepared for metal analysis by digesting approximately 2 g of sample in 5 ml 60% HNO<sub>3</sub> for at least 10 hours. Samples were further digested by microwave digestion, which was conducted in three stages (Baldwin et al., 1994) at the following power settings: two stages at 600 W for 2 min, with 2-min intervals, and a third stage at 200 W for 15 min. Once the samples were filtered through Whatman N°42 filter paper, metal concentrations were determined by atomic absorption spectrophotometry. All samples were analysed for Cu, Cd, Pb, Zn, Cr, Fe and Ni. Seawater samples were obtained after the 70 day period from each station for metal analysis. Water samples ( $n = 2$  from each station) were with obtained with 1 litre polyethylene sampling bottles, which were first rinsed twice with seawater and then filled with water samples from each station. Samples were filtered, digested using 1% nitric acid, and then analysed for the same metals as the mussel tissue metal analysis using the same procedure described above. Internal standards were used to verify metal concentrations obtained, and recoveries of the internal standards always fell within 90% of this value. Mussel tissue metal concentrations were validated using certified reference material (National Institute of Standards and Technology), (SRM 2976 mussel tissue). Analysed and certified values for standard reference materials are presented in Table 8 (Refer to Chapter 3).

#### *Physico-chemical water quality parameters*

Salinity at each transplant station was measured using a hand-held refractometer at the onset of mussel transplants. The rest of the physico-chemical water quality parameters were obtained from previous Durban Harbour monitoring reports (Pillay, S., 2003; 2007; pers comm.).

#### *Cardiac activity*

Mussels from each station were assessed for cardiac activity ( $n = 20$  from each station). Once the diodes were attached, the mussels were left undisturbed for 6 hours. Cardiac activity was recorded for 6 hours and the mean heart rate (beats.min<sup>-1</sup>) for each station was calculated.

#### *Lysosomal membrane stability*

Lysosomal membrane stability were determined for mussels from each station ( $n = 20$ ). Approximately 0.5 ml of haemolymph was extracted from the adductor muscle using a 3.0 ml, 23G syringe, and thereafter placed in Eppendorf tubes. This was mixed with an equal volume of temperature adjusted physiological saline solution. Approximately 40  $\mu$ l of the haemolymph solution was allowed to attach onto haemocytometers, and were incubated at room temperature for 20 minutes. An equal volume of the neutral red dye (NR) was then allowed to infiltrate the cells for 15 minutes. The number of haemocytes that absorbed and retained the dye was observed at 15 minute intervals microscopically, and the time taken for 50% of the cells to lose their dye was recorded as the lysosomal membrane stability time (Nicholson, 1999a,b).

#### *Condition index*

The ratio of tissue dry mass (mg) of mussel body tissue to shell length (mm) was determined for 20 individual mussels from each station (Lundebye *et al.*, 1997; Galloway *et al.*, 2002).

#### *Statistical analysis*

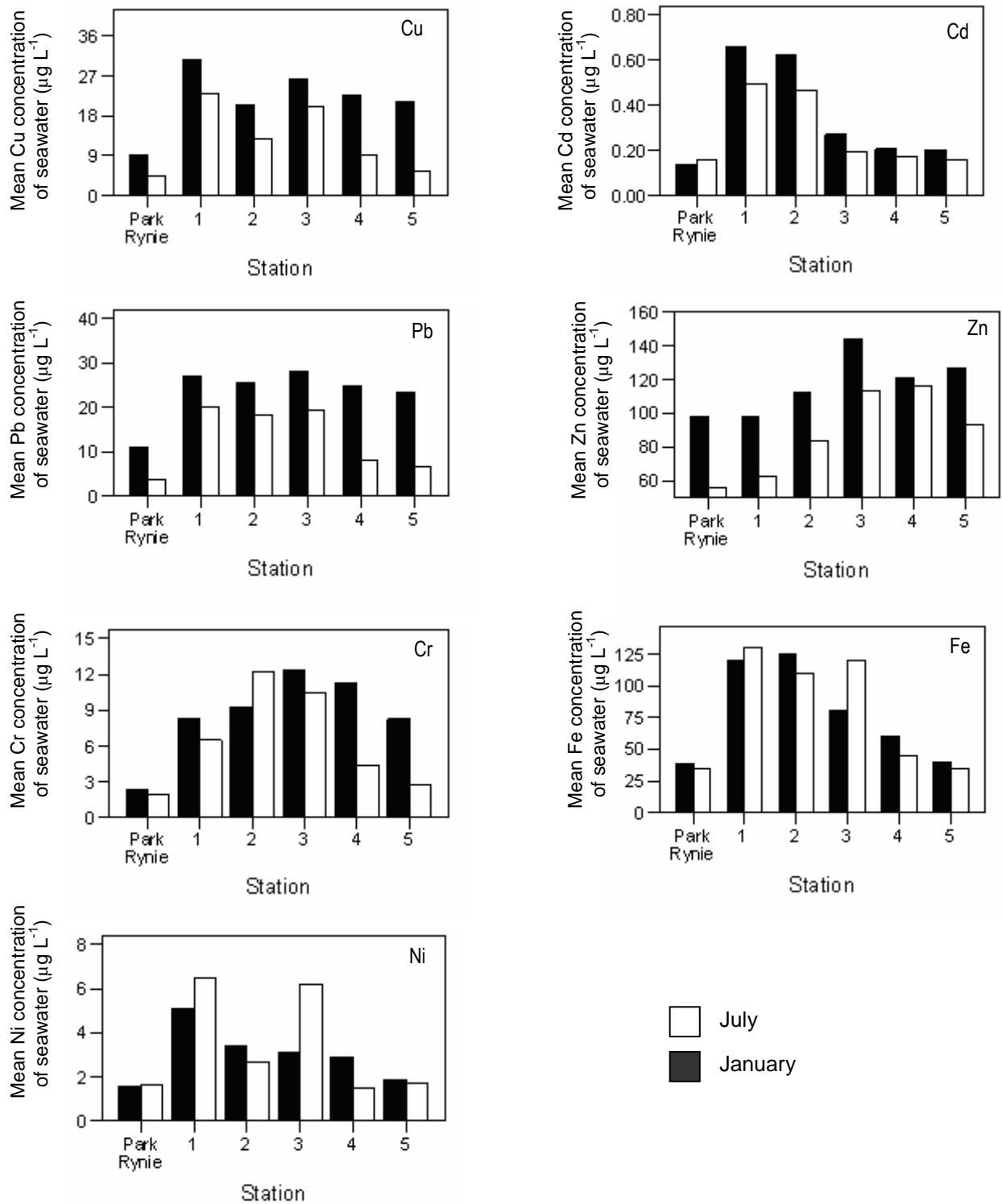
All statistical analyses were performed using SPSS version 15.0. (SPSS for Windows, Rel. 15.0. 2006. Chicago: SPSS Inc.). All data obtained from the experimental study were tested for normality (Shapiro-Wilk) and homogeneity of variance (Levene's test). All the data obtained from the various biomarker tests and metal concentrations conformed to normal distribution and the variances were homogenous. Between station differences in biomarker responses over time were investigated by regression analysis using log-transformed data for heart rate and condition index. Analysis of covariance (ANCOVA) was used to determine whether the slopes of the regression lines were significantly different among stations, with the precondition that all slopes were parallel. Due to the pooling of tissue samples ( $n = 1$ ), and number of water samples collected ( $n = 2$  from each station), statistical analysis of tissue and seawater metal concentrations between stations were not possible, however the mean metal concentrations of the pooled mussel tissues and seawater from each station were calculated and presented graphically. Spatial differences in seawater metal concentrations were analysed by one-way analysis of variance (ANOVA) and Tukey's *post-hoc* tests.

### **Results**

#### *Seawater metal analysis*

Figure 14 represents the metal concentrations in seawater collected at the selected stations in Durban harbour and Park Rynie during the months of July and January. Seawater seemed to





**Figure 14:** Spatial variations of Cu, Cd, Pb, Zn, Cr, Fe and Ni in seawater samples collected at transplant and reference stations at the end of the transplant period.

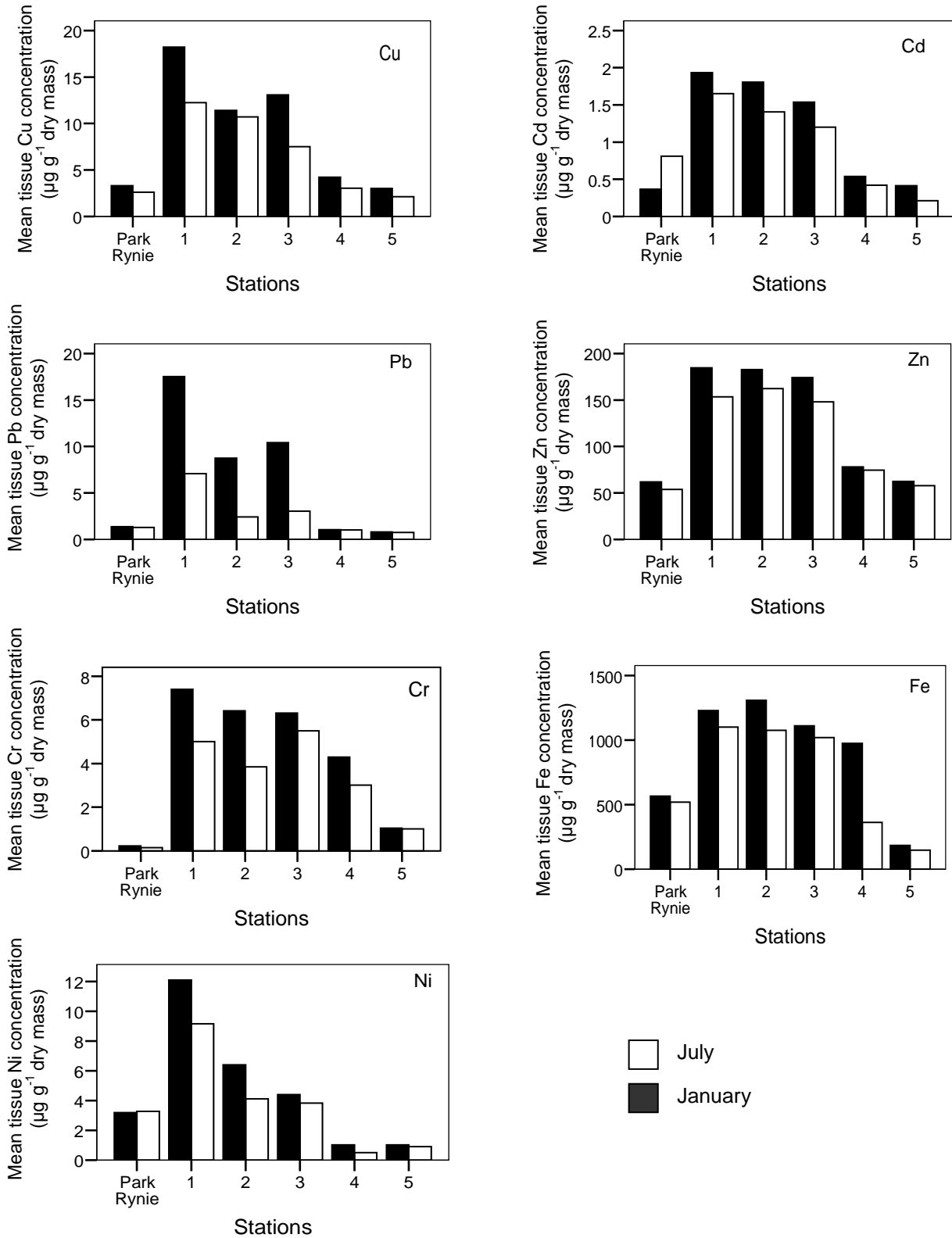
display higher metal concentrations in January than July for most metals, except Fe and Ni which were higher in July at Stations 1 and 3 in Durban harbour. For most metals, an observational trend between stations was evident with Stations 1 – 3 exhibiting the highest seawater metal concentrations, particularly Station 1. This station seemed to display the highest Cu concentration than the rest of the stations during July and January, and for Fe during July only. However, for the metal Cd, both Stations 1 and 2 seemed to display higher seawater metal concentrations during both sampling months, and for Fe in January only. During July, Stations 1 and 3 showed higher concentrations for Ni. Station 3 also displayed a high concentration for Zn during January. In contrast, Park Rynie displayed seemingly lower seawater metal concentrations for Cu, Cd, Pb and Cr during January, and Pb during July only.

#### *Perna perna soft tissue metal analysis*

Variations in soft tissue concentrations in *P. perna* are presented in Figure 15. Tissue metal concentrations exhibited accumulation patterns, with tissue samples obtained in January having seemingly higher metal concentrations than those obtained in July. However tissue metal concentrations obtained from the entrance of the harbour (Stations 4 and 5) and Park Rynie did not exhibit strong fluctuations between the different sampling periods. Trends between stations were seemingly evident with Station 1 displaying the highest soft tissue metal concentration for metals Cu, Cd, Pb and Ni during sampling months, as well as Cr and Zn in January and Fe in July. These high tissue metal concentrations were closely followed by Station 2, whereas Stations 5 and Park Rynie seemed to exhibit the lowest tissue metal concentrations, except for Ni during July, which occurred at Station 4.

#### *Physico-chemical water quality parameters*

Table 10 displays the mean values for salinity, temperature, pH and dissolved oxygen at the transplant stations during summer and winter periods, over a seven year period, as well as salinity readings taken at the beginning of each transplant period. Although temperature, pH and dissolved oxygen measurements were not taken on the exact days during the transplant study, the mean values provided does provide some indication of the physico-chemical conditions experienced at each of the transplant sites during summer and winter seasons. Salinity varied little between sites, with Station 1 having the lowest salinity during summer. Temperature did not vary much between stations; however, there was a great difference between winter and summer months. The rest of the physico-chemical parameters (pH and dissolved oxygen) did not seem to vary greatly between sites.



**Figure 15:** Between station variations in pooled soft tissue metal concentrations ( $\mu\text{g g}^{-1}$  dry mass) in *P. perna* retrieved from Durban Harbour and Park Rynie (Reference site).

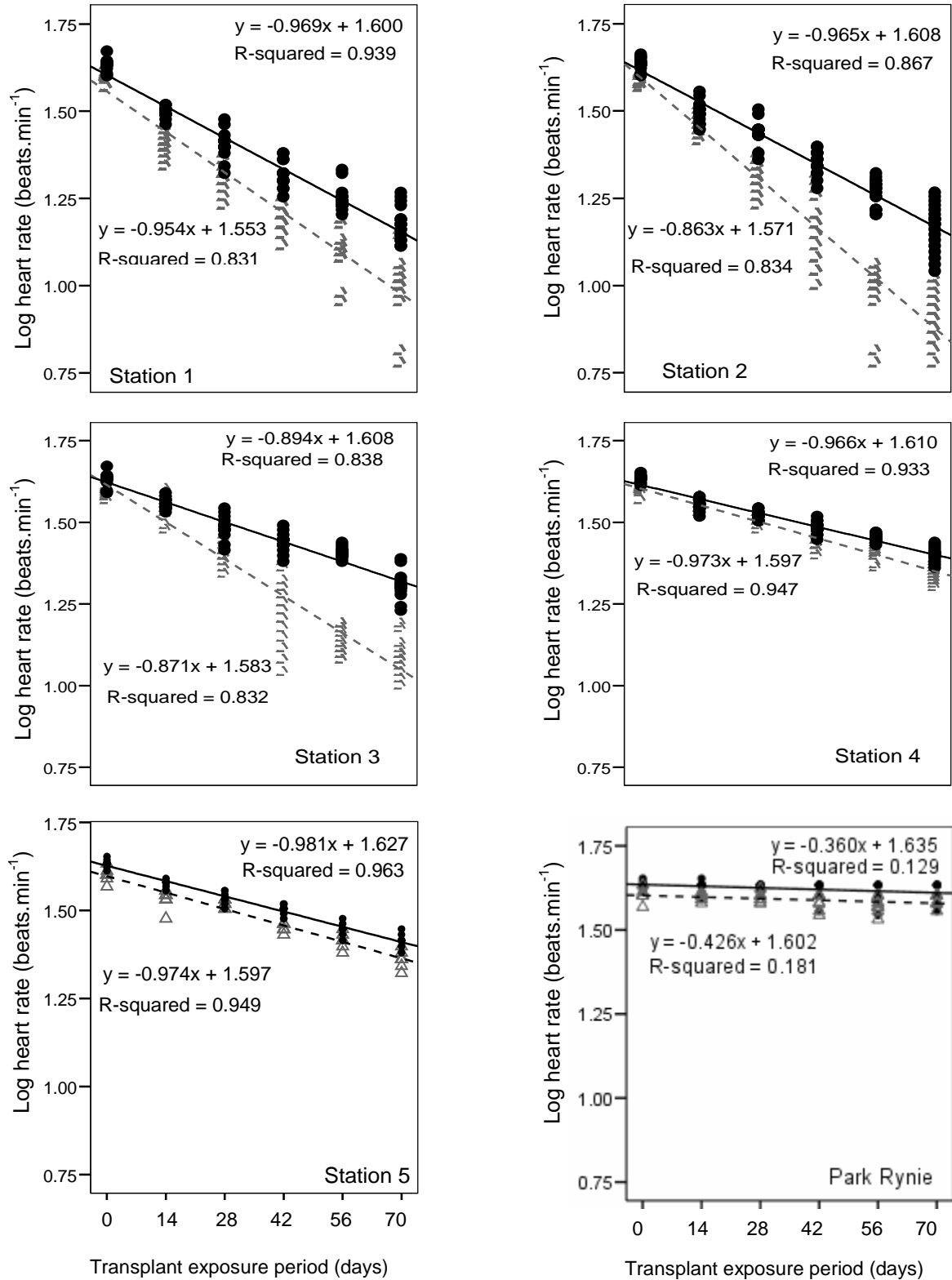
**Table 10:** Mean  $\pm$  standard deviation of physico-chemical water quality parameters of transplant sites over 2002 – 2007 during winter (June – July) and summer (December – January) months, (Pillay, S., 2003; 2007; pers. comm.).

Winter					
Sites	1	2	3	4	5
Salinity (‰) *	32	33	34	35	35
Salinity (‰)	31.8 $\pm$ 2.5	32.2 $\pm$ 2.3	32.1 $\pm$ 2.0	33.7 $\pm$ 0.6	34.7 $\pm$ 0.6
Temperature (°C)	20.1 $\pm$ 1.7	19.8 $\pm$ 1.4	19.7 $\pm$ 0.7	20.6 $\pm$ 1.6	20.6 $\pm$ 1.6
pH	7.9 $\pm$ 0.7	8.2 $\pm$ 0.3	8.1 $\pm$ 0.1	8.0 $\pm$ 0.5	8.0 $\pm$ 0.5
Dissolved Oxygen (mg.L <sup>-1</sup> )	5.8 $\pm$ 0.7	5.5 $\pm$ 0.6	5.8 $\pm$ 1.2	6.4 $\pm$ 0.8	6.4 $\pm$ 0.8
Summer					
Sites	1	2	3	4	5
Salinity (‰) *	29	33	33	35	35
Salinity (‰)	31.0 $\pm$ 1.6	32.0 $\pm$ 0.2	33.2 $\pm$ 0.8	34.9 $\pm$ 0.9	34.9 $\pm$ 0.9
Temperature (°C)	24.5 $\pm$ 1.8	24.4 $\pm$ 1.9	24.7 $\pm$ 2.0	24.7 $\pm$ 1.6	24.7 $\pm$ 1.7
pH	8.0 $\pm$ 0.3	8.0 $\pm$ 0.8	8.0 $\pm$ 0.8	8.3 $\pm$ 0.2	8.4 $\pm$ 0.6
Dissolved Oxygen (mg.L <sup>-1</sup> )	6.1 $\pm$ 0.7	5.6 $\pm$ 0.8	6.1 $\pm$ 0.9	6.9 $\pm$ 0.4	6.9 $\pm$ 0.4

(\* represents salinity measurements taken by a hand-held refractometer at the onset of each mussel transplant).

#### Cardiac activity

The linear relationship between heart rate over time from each station is depicted in Figure 16; it provides a clear indication of the biomarker responses of transplanted *P. perna*. All stations displayed significant negative slopes (biomarker response over time;  $p < 0.05$ ), with slopes for January heart rates from mussels ( $R^2 = 0.831, 0.838, 0.832, 0.947$  and  $0.949$  for Stations 1 – 5 respectively,  $p < 0.05$ ) being steeper than those recorded in July ( $R^2 = 0.939, 0.867, 0.838, 0.933$  and  $0.963$  for Stations 1 – 5 respectively,  $p < 0.05$ ). At the end of the 70 day transplants, the lowest mean heart rates were found at Station 1 ( $11 \pm 1$  beats.min<sup>-1</sup> in January and  $13 \pm 2$  beats.min<sup>-1</sup> in July). On the contrary, mussels at Park Rynie exhibited the highest mean heart rates ( $37 \pm 1$  beats.min<sup>-1</sup> and  $39 \pm 1$  beats.min<sup>-1</sup> in January and July respectively), and therefore



**Figure 16:** Linear regression relationships between log-transformed cardiac activities over time for transplanted *P. perna*. All slopes are significant at the 0.05 probability level ( $n = 20$  mussels from each station).  $R^2$  = regression coefficient. Black circles with solid lines represent heart rates and best-fit lines for July. Grey triangles with broken lines represent heart rates and best-fit lines for January.

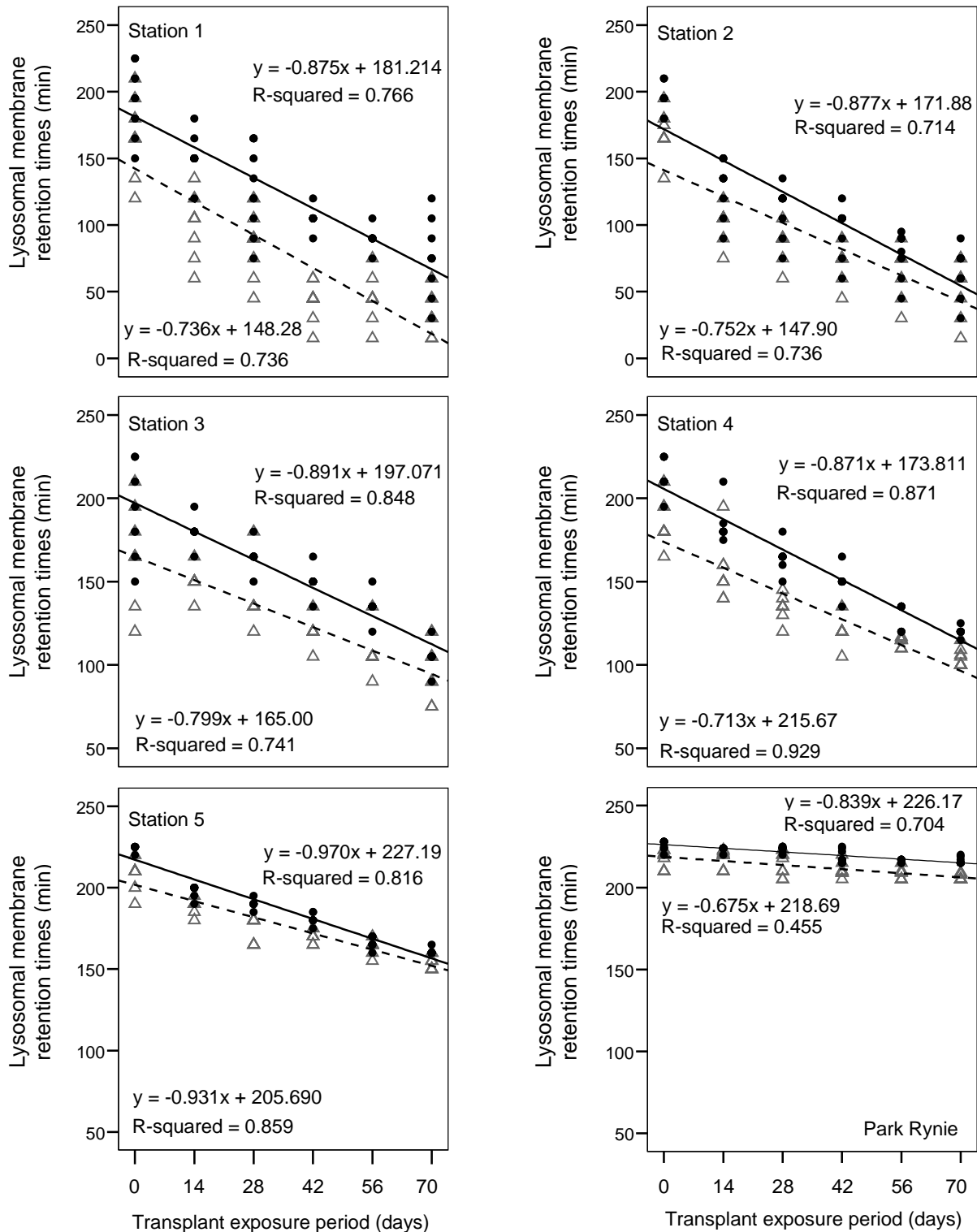
produced the weakest regression relationship over time ( $R^2 = 0.181$  and  $0.129$  respectively,  $p < 0.05$ ). Regression slopes obtained from Park Rynie mussels were significantly different from the rest of the stations (ANCOVA,  $df = 113$ ,  $F = 101.6$  and  $71.8$  in January and July respectively, ( $p < 0.05$ ), however in Durban Harbour, Stations 2 and 3 were similar for both transplant periods. In July, Stations 4 and 5 adopted the same trend as Stations 2 and 3; however Station 1 displayed a significantly different slope from all other stations.

#### *Lysosomal membrane stability*

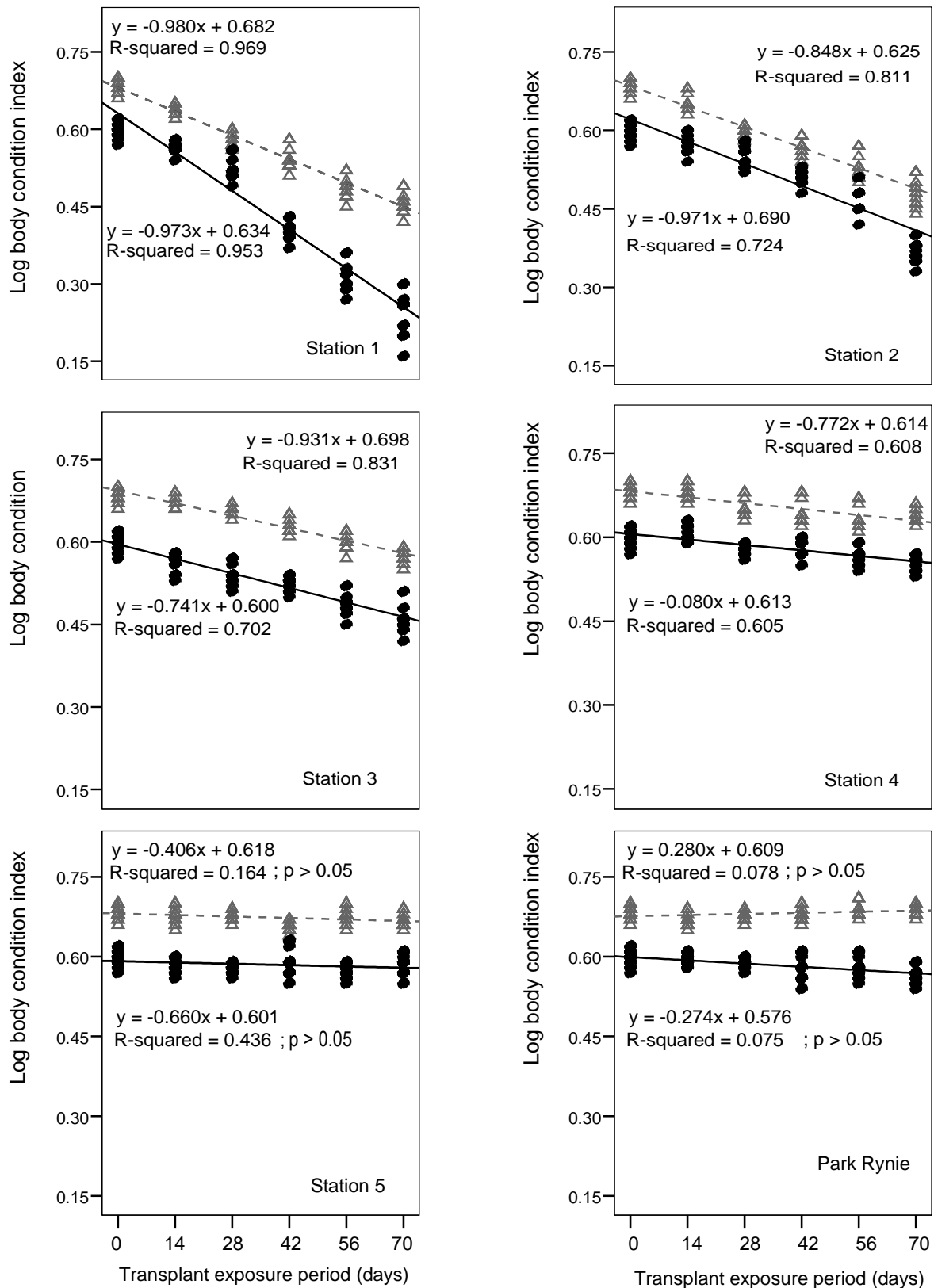
Most stations displayed a distinct variation in mussel lysosomal membrane stability between the two sampling periods, with membrane stability in January being considerably lower than those recorded in July, particularly at Station 1 ( $R^2 = 0.766$  and  $0.736$  in July and January respectively,  $p < 0.05$ ), (Figure 17). In contrast, mussels from Station 5 and Park Rynie did not exhibit such strong variations in lysosomal membrane stability over the different sampling periods. As with the recorded cardiac activities, mussels from Park Rynie displayed the highest mean lysosomal membrane stability for both transplant periods ( $170 \pm 3$  min in January and  $180 \pm 5$  min in July), and was closely followed by Station 5. The lowest means were obtained from Station 1 ( $39.5 \pm 3$  min) in January and Station 2 in July ( $71.04 \pm 7$  min). Linear regression analysis showed a considerable reduction in lysosomal membrane stability over time for all stations, (Figure 5). Slopes from mussels at Park Rynie were significantly different from all other stations (ANCOVA,  $df = 113$ ,  $F = 88.2$  and  $55.2$  in January and July respectively,  $p < 0.05$ ), however Stations 1, 2 and 3 exhibited similar slopes during July, as did Stations 2 and 3 in January.

#### *Body condition index*

Mussels of all stations exhibited a considerable difference in body condition indices between the two transplant periods (Figure 18). In contrast to the results of the other biomarkers tested, condition indices of *P. perna* obtained in July were distinctly lower than those obtained in January. Stations 1 – 3 exhibited pronounced declines in condition indices ( $R^2 = 0.953$ ,  $0.724$  and  $0.702$  in July, and  $R^2 = 0.969$ ,  $0.811$  and  $0.831$  in January for Stations 1 – 3 respectively,  $p < 0.05$ ). On contrary, mussels from Park Rynie and Station 5 did not display strong declines in condition, particularly in January ( $R^2 = 0.078$  and  $0.164$  respectively,  $p > 0.05$ ) and subsequently displayed the highest condition indices at the end of the transplant period (Park Rynie,  $4.93 \pm 1$  in January and  $3.82 \pm 1$  in July). Mussels at Station 1 had the lowest index for both transplant periods ( $2.89 \pm 1$  in January and  $1.78 \pm 1$  in July). The Park Rynie January body



**Figure 17:** Linear regression relationships between lysosomal membrane stability over time from transplanted *P. perna*. All slopes are significant 0.05 probability level ( $n = 20$  mussels from each station).  $R^2$  = regression coefficient. Black circles with solid lines represent lysosomal membrane stability and best-fit lines for July. Grey triangles with broken lines represent lysosomal stability and best-fit lines for January.



**Figure 18:** Linear regression relationships between log-transformed body condition indices over time. All slopes are significant 0.05 probability level, unless otherwise stated ( $n = 20$  mussels from each station).  $R^2$  = regression coefficient. Black circles with solid lines represent body condition indices and best-fit lines for July. Grey triangles with broken lines represent condition indices and best-fit lines for January.



condition index of *P. perna* also displayed a positive slope; hence this data set was not included in ANCOVA. ANCOVA showed most stations were significantly different from each other, except Stations 2 and 3 during July and January (ANCOVA,  $df = 113$ ,  $F = 162.4$  in July and  $F = 124.7$  ( $df = 94$ ) in January,  $p > 0.05$ ).

## Discussion

This study shows how physiological and cytological biomarkers from *P. perna* can identify spatial and seasonal variations of contamination in Durban Harbour.

### *Metal analysis*

Seawater and mussel tissue metal concentrations displayed varied trends, with high concentrations of different metals being spread across stations; however those stations nearest to the freshwater inputs of the bay (Stations 1 – 3) seemed to display higher tissue metal concentrations than the rest of the stations. The Amanzimnyama, Mbilo and Mhlatuzana Rivers drain the most industrialised and urbanised areas of the city, and thus carry a high amount of pollutants. Stations 1 and 2 were subjected to this inflow of contaminants, as well as contaminants originating from dry dock activities. These stations also experience a regular build-up of land-derived sediment from the rivers and therefore also subjected to periodic dredging, which releases sediment-trapped contaminants into the water column. Therefore the prime locations of these two stations would account for them displaying the highest seawater and soft tissue concentrations. Station 3 which also displayed high metal concentration in seawater and soft tissues, particularly for metals Zn, Cr and Cu, was exposed to contaminants originating from stormwater runoff and the small watercrafts and yacht harbours. Stations near the harbour entrance (Stations 4 and 5) were more influenced by fresh tidal seawater entering the harbour; hence most metals occurred in low concentrations there. However Zn, Pb and Cr had surprisingly high concentration at these stations during the transplant period in January. Durban experiences its peak rainfall season over during this month; hence a possible explanation for the high Zn, Pb and Cr concentrations found at these stations is that the heavy rainfall during this transplant period would have drained excessive amounts of these metals into the harbour. Park Rynie also displayed an unusually high Ni concentration in mussel tissue during January. Again, this can only be attributed to the high summer rainfall which would cause high riverine outputs from neighbouring rivers to disperse numerous contaminants around the coastal area.

Numerous studies have found a distinct trend in accumulated metal concentrations in mussel soft tissue during winter and summer periods (Phillips, 1976a; Latouche and Mix, 1981; Amiard *et al.*, 1986; Borchardt *et al.*, 1988; Regoli and Orlando, 1994; Soto *et al.*, 1995; Bodin *et al.*, 2004; Sokolowski *et al.*, 2004), with winter months exhibiting much higher metal accumulation rates in marine mussels than summer months. Many authors have attributed this pattern to an inverse relationship with tissue mass, i.e. mussels have low tissue mass in winter months with high metal content and high tissue mass with low metal content in summer months (Phillips, 1976a; Amiard *et al.*, 1986; Borchardt *et al.*, 1988; Soto *et al.*, 1995). These studies suggest that this inverse relationship between tissue metal concentration and tissue dry mass can be due to a 'dilution effect' on the metals that accumulate in high flesh mass in summer. A study by Cossa *et al.* (1980) also supports this hypothesis as the findings of the study concluded that smaller mussels with lower tissue masses are richer in metal content than their larger counterparts. A study by Latouche and Mix (1981) attempted to overcome the seasonal fluctuation in gonad mass by excluding the reproductive organs during metal analysis; however this method was unsuccessful as the rest of the soft tissue in the test organisms also showed seasonal changes in growth and mass. Another possible hypothesis is that mussels have more energy available for rapid contaminant excretion in summer months than in winter months when energy is depleted by maturing of the gonads and spawning (Phillips, 1976a,b; Amiard *et al.*, 1986; Soto *et al.*, 1995). Although the experimental design of this study does not permit any valid deductions on seasonal variations in tissue metal accumulation, the findings in this study were in contrast to the previous studies mentioned above in that soft tissues exhibited higher tissue metal concentrations from the January transplant period (summer months) rather than the July transplant period (winter months), hence emphasising that biological responses from *P. perna* transplanted in Durban harbour and Park Rynie were more influenced by the excessive seawater metal concentrations present in the summer transplant period. Similar results were documented by Farrington *et al.* (1983), Boalch *et al.* (1981) and Amiard *et al.* (1986), who show that seasonal metal accumulation in tissue can be suppressed by short-term contaminant exposure such as pollutant overloading during rainy seasons. Furthermore, a study by Soto *et al.* (1995) postulated that mussels gain mass during sexual maturation which occurs before spawning, and gonad tissue and other organs usually associated with contaminant storage do not accumulate metals. This explanation, together with the hypothesis of 'metal dilution' by high flesh masses would also contribute to the lower metal content in *P. perna* during the July transplant period.

#### *Cardiac activity*

Numerous studies have examined the effects of anthropogenic contaminants on the heart rates of invertebrates (Widdows, 1973; Sabourin and Tullis, 1981; Nicholson, 1999a,b; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Galloway, 2002; Marshall *et al.*, 2004; Abessa *et al.*, 2005). Most of these studies report no significant differences in heart rates when the test organisms were exposed to low concentrations of contaminants, however as the metal concentration were increased, the measured cardiac output decreased proportionately (Nicholson, 1999a,b; Curtis *et al.*, 2001; Galloway *et al.*, 2002) even after exposure times of less than four hours (Curtis *et al.*, 2000). Studies by Nicholson (1999b), Wedderburn *et al.* (2000) and Brown *et al.* (2004) documented significant increases in heart beats from Mytilid mussels exposed to low contaminants over a short period of time. Brown *et al.* (2004) found tachycardia from mussels exposed to a Cu concentration of  $38.2 \mu\text{g.L}^{-1}$  for 7 continuous days; however Wedderburn *et al.* (2000) and Nicholson (1999b) found similar results from mussels transplanted in unpolluted areas. The authors of these studies attributed the increase in cardiac activity to an increased metabolic rate by the test specimens to rapidly detoxify and purge the pollutants; however, these sites were reported to be relatively unpolluted. It is unlikely that an environmental conditions would have effected the cardiac activity of the transplanted mussels in these studies, as the same effects would have been observed at all transplant areas of the study (as distances between the transplant sites were not as great to experience different environmental conditions). In addition, the study by Nicholson (1999b) only used transplant sites that had similar physio-chemical properties. Rather, the increase in mussel heart rate could have been due to a sudden flush of contaminants in that particular region. Seeing as Nicholson (1999b) documented the frequency of dredging near that transplant site, it can be assumed that newly released contaminants or fine silt from such activities could have influenced the biomarker response, even though the area is relatively unpolluted. According to Nicholson (1999b), tachycardia in mussels occurs when the organisms are exposed to too low contaminant concentrations for bradycardia to be induced, however the sudden increase in metabolic activity and physiological response can cause injury to fragile tissues and organs such as gills, muscles, the heart and its surrounding tissues. Hence the elevation of mussel heart rates from mildly polluted sites is not uncommon, however tachycardia was not observed during either of the transplant periods. This suggests that although the reference site (Park Rynie) was exposed to a flush of anthropogenic contaminants into the marine environment, particularly during the heavy rainfall period in December and January, the level of contaminants present at this station were too low to induce such a response. In contrast, stations in Durban harbour contained metal contaminants high enough for distinct bradycardia to occur. Stations 1 – 3 in particular exhibited a drastic decline in heart rates. These findings are

consistent with the findings from several other studies which compared heart rates from mussels obtained from polluted and unpolluted stations (Nicholson, 1999a; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Galloway, 2002).

Nevertheless, biological biomarkers are variable to external factors such as salinity, temperature, sex, age and reproductive status (Lundebye *et al.*, 1997), and during the sampling months of December and January, the bay could have been exposed to fluctuating salinities and high temperatures, especially at Station 1. Most marine mussels are osmoconformers (Neufeld and Wright, 1996; Berger and Kharazova, 1997; Nicholson, 2002); hence, a reduction in salinity may consequent in a 'dilution effect' of extravisceral fluids and alterations of the ionic balance of cells. However, studies by de Vooy (1991), Neufeld and Wright (1996), Berger and Kharazova (1997), Nicholson (2001) and Braby and Somero (2006) have documented various mechanisms of salinity adaptations in mussels. One such mechanism is the use of free amino acids to stabilise cell volume induced by low salinity stress, (de Vooy, 1991; Neufeld and Wright, 1996; Berger and Kharazova, 1997; Nicholson, 2001), as well as transient changes in protein and RNA synthesis in isolated tissues and cells, particularly during acclimation to low salinities. However, the most frequently documented adaptation to salinity stress is valve closure, which isolates the organisms' internal body fluids from the ambient environment, thereby reducing salt loss and preventing contact of internal tissues with the ambient water (Phillips, 1977; Berger and Kharazova, 1997; Nicholson, 2002; Braby and Somero, 2006). A reaction to the above mechanisms is a reduction in physiological and metabolic processes, including bradycardia (Phillips, 1977; Nicholson, 2002; Braby and Somero, 2006).

Experimental work on the heart rate of *M. edulis* by Bakhmet *et al.* (2005) concluded that large variations in salinity can induce significant bradycardia when mussels were exposed to a drop in salinity from 25 – 15‰. The study also found that mussels can become acclimated to lowered salinities within five days, and display a heart rate that is similar to those recorded at the original salinity. When the test mussels were returned to control conditions, the recovery time taken to reach control heart rates fell just over 24 hrs; thereby indicating the organisms' ability to physiologically compensate and adjust to natural fluctuations in salinity. According to Berger and Kharazova (1997), this observation of an increase in physiological activity such as heart and respiration rates, after the return to normal salinity from a higher or lower salinity is another typical adaptive mechanism of marine molluscs to salinity variations.

Nicholson (2002) determined the effects of a wide range of salinities on the heart rate of mussels from the same genus as those used in this study, and found that at a salinity change rate of  $3\text{‰}\cdot\text{day}^{-1}$ , *P. viridis* were able to physiologically adjust to salinities ranging from 15 – 35‰ without exhibiting significant bradycardia. Stickle and Sabourin (1979) found similar results in their studies on the heart rate of *M. edulis*, as no significant variations in mussel heart rate were observed during gradual exposure to salinities as low as 10‰. The salinities tested in these previous represented extremely low levels which are unlikely to occur in field situations. Referring to Table 10, the average salinity levels found in study area do not fall below 29‰, even during wet months; and according to previous literature, the effects of a dip just below 30‰ in salinity will be transient (Berger and Kharazova, 1997; Bakhmet *et al.*, 2005). Hence it can be assumed that any effects on cardiac biomarker responses due to salinity variations may have been minimal.

According to the Nicholson (2002), although mussels from the genus *Perna* are subtropical and intertidal, they have a limited capacity to regulate their cardiac activities to extremes in ambient temperatures. Mechanisms such as valve closure, which attempts to isolate the organisms' internal tissues from exposure to ambient conditions and avoid desiccation, are commonly observed particularly during extremely high temperatures ( $\geq 30^{\circ}\text{C}$  during emersion). Hence, bradycardia may be induced as valve closure inhibits physiological processes to some degree (Nicholson, 2002; Braby and Somero, 2006). Tachycardia during elevated temperatures may also be observed as mussel attempt to rapidly regulate heart rate as a response to the thermal shock (Nicholson, 2002).

Experimental investigations on *P. viridis* (Nicholson, 2002) showed that water temperatures at  $31^{\circ}\text{C}$  and above represented the species upper tolerance limit for stable heart rates. The study determined the effects of gradual thermal alterations on mussel heart rate, and found that at a rate of  $2^{\circ}\text{C}\cdot\text{day}^{-1}$ , there were no differences in mussel heart rates exposed to a low temperature of  $15^{\circ}\text{C}$ , as well as to an elevated temperature of  $25^{\circ}\text{C}$ . However, when exposed to  $30^{\circ}\text{C}$  and above, mussel heart rates were significantly different from those of the control treatment. The investigation also found that when extreme temperatures were allowed to return to ambient conditions, the mussel heart rates also returned to a rate similar to the control treatment. Hence extremes in temperature can induce bradycardia or tachycardia, which will cease when normal temperature levels are returned. Pickens (1965) studied the effects of thermal stress on marine mussel heart rates, and found the upper thermal tolerance limit for stable heart rates to be between  $25 - 30^{\circ}\text{C}$  for *M. edulis*, *M. galloprovincialis* and *M. californianus*. Widdows (1973)

also found significant reduction in heart rates from *M. edulis* when exposed to 30°C. However, for *P. perna*, which are found in warmer waters, the thermal tolerance limit for stable heart rate was 32°C (Pickens, 1965). Chang and Acuna (1981) found the upper thermal lethal limits of *P. perna* to range from 37.44 – 42.61°C, and concluded that the species' ability to rapidly adapt to the thermal shock was dependent on the rate of temperature elevation. A study on other physiological processes in *P. perna* by Resagalla *et al.* (2007) found that only extremes in temperatures ( $\leq 15^{\circ}\text{C}$  and  $\geq 30^{\circ}\text{C}$ ) have induced significant effects on respiration and excretion rates. The average temperature conditions in Durban Harbour do not reach such extremes in temperature (Table 10); it can therefore be reasoned that temperature fluctuations during the course of the transplant study may have not had a large influence in heart rate from *P. perna*, as any effects would have been transient before returning to normal.

With regards to the possible effects of low dissolved oxygen on cardiac activity, a study by Nicholson (2002) investigated the effects of low dissolved O<sub>2</sub> on *P. viridis* heart rate, and concluded that when mussels experiencing normal dissolved O<sub>2</sub> conditions (4.9 – 6.4 mg.L<sup>-1</sup> dissolved O<sub>2</sub>) were exposed to low dissolved O<sub>2</sub> conditions such as  $0.8 \pm 0.07$  mg.L<sup>-1</sup> dissolved O<sub>2</sub>, significant bradycardia is induced. However, heart rates increased as soon as the water was re-oxygenated to normal dissolved O<sub>2</sub> conditions. Dissolved O<sub>2</sub> levels in this study area did not fall below 5.5 mg.L<sup>-1</sup> dissolved O<sub>2</sub> during both seasons (Table 10), hence it can be assumed that low dissolved O<sub>2</sub> conditions would have had minimum, if any, influences on mussel cardiac activity.

Furthermore, the seawater and tissue metal concentrations found at Stations 1 – 3 strongly suggest the cardiac activity of the transplanted mussels were more influenced by the presence of anthropogenic contaminants in the environment than physico-chemical characteristics of the study area. Thus, the variations in cardiac activity for *P. perna* suggest that stations in Durban harbour are strongly affected by inputs from the rivers and stormwater drains that empty into the bay, as well as dry dock activities.

#### *Lysosomal membrane stability*

Reduced lysosomal membrane stability from mussels exposed to anthropogenic contaminants have been reported by numerous studies (Galloway *et al.*, 2002; Lowe *et al.*, 1995; Marigomez *et al.*, 1998; Matozza *et al.*, 2001; Moore *et al.*, 2006; Nicholson, 2001; Viarengo *et al.*, 2000; Wedderburn *et al.*, 2000). These findings are consistent with the results of this study. At the end of the transplant period of both sampling periods, stations from Durban harbour exhibited a

distinct reduction in lysosomal membrane stability. In contrast, mussels transplanted at Park Rynie did not show a uniform reduction in lysosomal membrane stability. This station provided the healthiest specimens with little reduction in lysosomal membrane stability. Similar results were obtained by Lowe *et al.* (1995); Nicholson, (1999b), Wedderburn *et al.* (2000), Kagley *et al.* (2003) and Petrovic *et al.* (2004), whereby reference and unpolluted stations exhibited significantly longer lysosomal membrane stability than stations positioned near industrial and urban areas. The stations position at the inner reaches of the harbour (Stations 1 – 2) provided contrasting results to Park Rynie as the lysosomal membrane stability showed a strong decline over time, which are consistent with the results obtained from the cardiac activity assays at Stations 1 and 2, which also displayed distinct declines over time.

During the rainy summer months of December and January, the bay could be exposed to fluctuating salinities and temperatures, particularly at Station 1. Ringwood *et al.* (1998) found that oysters, *Crassostrea virginica*, accustomed to a salinity range of 25 – 28‰, exhibited no significant differences in cellular biomarker responses, including lysosomal membrane instability when exposed to salinities ranging from 10 – 30‰. Moore *et al.* (1980) also did extensive work on the structure of lysosomes from the mussel *M. edulis* and its response to variations in salinity. The results were in agreement with the findings of Ringwood *et al.* (1998) in that there were no differences in lysosome function when salinity was reduced from 30 – 15‰. In contrast, Bayne *et al.* (1981) demonstrated that although an increase in salinity has a considerable influence on lysosomal membrane stability, the effects caused are reversible during acclimation and return to normal salinity; and Hauton *et al.* (1998) found that lysosomes from the oyster *Ostrea edulis* exhibited significantly reduced lysosomal membrane stability when exposed to a drop in salinity from 32 – 19‰ and below. Nicholson (2002) showed that lysosomes from *P. viridis* exposed to salinities at 25‰ and below, displayed a significant reduction in lysosomal membrane stability. The study used a rapid exposure approach; however, if the mussels were to be gradually acclimated to the lowered salinities, the degree of the lysosome dysfunction may have been reduced. The salinity ranges tested in these previous studies represented extremes which are unlikely to be observed in the field, and extend beyond those conditions normally experienced in Durban Harbour (Table 10). In the event of an excessive influx of freshwater into the bay, the effect on lysosomal membrane stability would be transient, and return to normal once normal salinity conditions of the bay returned. It can therefore be assumed that the effects of salinity fluctuation on lysosomal membrane stability during the transplant study would have been minimal.

According to Moore (1976) and Nicholson (2002), the observed reduction of lysosomal membrane stability at elevated temperatures could be due to the denaturing of lysosome enzymes, thus affecting the permeability of the organelles' membrane. The resulting elevation of metabolic activity may also induce an increase in protein and organelle processes which would necessitate an increase in lysosome hydrolases. Moore (1976) demonstrated that lysosomes from *M. edulis* undergo severe thermal stress and exhibit significantly reduced lysosomal membrane stability when exposed to high temperatures. Kagley *et al.* (2003) also found significantly reduced lysosomal membrane stability at different times during the year, however attributes such responses to energy depletion brought on by gonad development and spawning rather than influences of temperature. Domouhtsidou *et al.* (2004) found significantly higher lysosomal membrane stability from *M. galloprovincialis* sampled in colder months compared to those sampled in warmer months. However, the study also attributed the variation in seasonal results to an increased availability of food during the colder period, as well as the possible negative effect of spawning and gonad maturation on lysosomal membrane stability at the onset of the warmer period.

Studies by Wang *et al.* (2006) and Zhang *et al.* (2006) investigated the effects of gradual and rapid water temperature changes to lysosomal membrane stability, as well the reversal of the temperature experiments. Zhang *et al.* (2006) investigated the effects of both rapid and gradual water temperature changes on lysosomal membrane stability from oysters *Crassostrea gigas*. When the oysters were rapidly exposed to temperatures 10°C above and below the control temperature, lysosomes displayed a significant reduction in membrane stability within 3 hours of the temperature change. Wang *et al.* (2006) performed the same test design on abalone *Haliotis rubra*, with a 9°C temperature increment, and found similar results within 3 – 6 hours of the temperature change. Both species from the above studies showed similar lysosomal membrane stability times from animals exposed to the final altered temperatures and those documented from animals acclimated to the same temperatures.

On the other hand, oysters exposed to a gradual 2°C.day<sup>-1</sup> change in temperature showed that at 11°C and 19°C the lysosomal membrane stability times differed significantly from those animals acclimated at 15°C (Zhang *et al.*, 2006). And abalone exposed to a gradual 1°C.day<sup>-1</sup> and then followed by a 2°C.day<sup>-1</sup>, indicated that temperatures below 15°C and above 17°C induces significantly reduced lysosomal membrane stability (Wang *et al.*, 2006). Nonetheless, both studies found that irrespective of the manner of exposure (i.e. gradual or rapid), the lysosome membrane stabilities at the final temperature were the same. The studies also



investigated the reverse effect on lysosomal membrane stability by acclimating oysters and abalone to lower and higher salinities and then exposing them to the control salinity. These results showed that the lysosomal membrane destabilisation time is quicker than its stabilisation or recovery time, for both species.

Camus *et al.* (2000) postulated that when exposed to low temperatures, cellular membranes adjust to the stress by altering their structural nature to keep enzyme kinetic properties stable. The increased concentration of unsaturated lipids in mussels habituated to low temperature conditions help to maintain the viscosity of membrane components, and may influence the instability of lysosome membranes. Camus *et al.* (2000) also found the lysosomes from *M. edulis* exhibited significantly reduced lysosomal membrane stability at temperatures below 10°C. Hutton *et al.* (1998) found that lysosomes from the oyster *Ostrea edulis* similarly exhibited significantly reduced lysosomal membrane stability the temperature was dropped to 10°C

In the current study, the mean lysosomal membrane stability from the reference station (Park Rynie) were  $170 \pm 3$  min and  $180 \pm 5$  min in January and July respectively, with the corresponding water temperature ranges of 24 – 25°C and 19 – 21°C (Table 10). A field study by Pereira *et al.* (2007), which also employed lysosomal membrane stability from *P. perna* as a biomarker of marine pollution, found summer water temperatures to range from 29 – 30°C and winter water temperatures to range from 18.0 – 21.5°C. In view of the fact that the average winter water temperatures observed in both studies are comparable and the average summer water temperatures observed in this study do not reach as stressful conditions as those reported by Pereira *et al.* (2007), similar or higher lysosomal membrane stability can be expected. However, the mean lysosomal membrane stability found in the study by Pereira *et al.* (2007) contrasted with the present results. The mean lysosomal membrane stability documented in the study were  $40 \pm 13.83$  min during a summer month and  $34.0 \pm 11.42$  min during a winter month (Pereira *et al.*, 2007). Besides displaying contrasting lysosomal membrane stability during warm and cold months, the lysosomal membrane stability reported are much lower than those recorded in this study. According to Dailianis *et al.* (2003), Zhang *et al.* (2006) and Francioni *et al.* (2007), such differences in lysosomal membrane stability could be attributed to the following reasons:

1. the health status of the organisms used;
2. minor differences existing in the experimental procedures used to determine lysosomal membrane stability times;

3. the different lysosomal membrane stability times from the mussels are genetically characteristic of the area of origin.

In contrast, a field study by Dailianis *et al.* (2003) found no effects attributed to water temperature on the lysosomal membrane stability of the mussel *M. galloprovincialis* during warm and cold months, and concluded that the cellular biomarker was minimally affected by natural fluctuations in physico-chemical water parameters. An investigation by Nicholson (2002) found that lysosomes from *P. viridis* displayed reduced lysosomal membrane stability at temperatures at 29°C and above; however such extremes in temperature regimes used in the study are unlikely to occur in the field. The average temperatures experienced annually in the study area of this study fall between 19 – 25°C (Table 10), which are not as extreme as those temperatures tested in previous studies. Hence, it can be assumed that any effects induced by temperature fluctuations would have been minimal. With regards to the possible effects of low dissolved oxygen on lysosomal membrane stability, Nicholson (2002) found that lysosomes from *P. viridis* acclimated to normal dissolved O<sub>2</sub> conditions (4.9 – 6.4 mg.L<sup>-1</sup> dissolved O<sub>2</sub>), did not show significant differences in lysosomal membrane stability when exposed to low dissolved O<sub>2</sub> conditions (0.6 – 1.1 mg.L<sup>-1</sup> dissolved O<sub>2</sub>). According to the study, lysosomes have the ability to maintain lysosomal membrane stability during extended periods of low dissolved O<sub>2</sub> conditions via anaerobic metabolism. In this study however, dissolved O<sub>2</sub> conditions did not fall below 5.5 mg.L<sup>-1</sup> dissolved O<sub>2</sub> during both seasons (Table 10), hence it can be assumed that this physico-chemical parameter would have minimum, if any, influences on lysosomal membrane stability.

*Body condition index*

Body condition index is regarded as an accurate and useful bioindicator that provides information on the general health condition of mussels, and has successfully been employed in numerous studies (Avery *et al.*, 1996; Lundebye *et al.*, 1997; Nicholson, 1999b; Orban *et al.*, 2002; Galloway *et al.*, 2002; Smolders *et al.*, 2003; Amiard *et al.*, 2004). Studies by Nicholson (1999a,b), Mauri and Baraldi (2003), Avery *et al.* (1996) and Bodin *et al.* (2004) all documented significantly low condition indices from polluted sites than the reference or unpolluted sites. The spatial results of this study are consistent with these findings as the most polluted stations in Durban harbour (Stations 1 and 2) displayed the lowest indices and Station 5 and Park Rynie showed less variation and higher indices over the transplant periods. Body condition indices obtained from the January transplanted mussels exhibited much stronger body condition indices than those obtained in July. In this study, Durban harbour was shown to be

exposed to excessive contaminants in the summer sampling months, and all biomarker tests discussed thus far conform to this trend by displaying depressed biomarker responses during this transplant period. These results are also in contrast with studies that have documented a decrease in indices with an increase in pollutant loads (Nicholson; 1999a,b; Mauri and Baraldi, 2003; Avery *et al.*, 1996; Bodin *et al.*, 2004).

As sensitive as condition indices are to anthropogenic contaminants, it must be noted that biotic and abiotic biotic factors such as seasonal changes, reproductive activity and nutritional health of the organisms can also influence mussel growth and health (Lundebye *et al.*, 1997). *Perna perna* display annual reproductive, growth and storage cycles that cause significant changes in soft tissue mass (Phillips, 1976a; Cain and Luoma, 1986; Soto *et al.*, 1995). Cain and Luoma (1986), Cossa *et al.* (1980) and Bodin *et al.* (2004) speculated that adult Mytilid specimens may increase their body masses by more than three times as a result of increased glycogen and lipid production in the gonads before spawning. Once the spawning period is over, the energy resources of these organisms are depleted and significant mass is lost. A study on the reproductive cycle of *P. perna* from KZN by van Erkom Schurink and Griffiths (1991) showed that the species used in this study spawns over winter months (particularly from late May – July) and is accompanied by a pronounced decrease in dry flesh mass. Thus this significant mass loss will strongly influence the calculation of the June and July condition indices. Mussels have also been found to expel water from their tissues via osmosis when exposed to extremely low salinities (Phillips, 1977), also resulting in significant wet mass loss. Although Station 1 may have been subjected to typical coastal conditions during wet seasons, where excess runoff frequently flushes into the harbour, the mean summer salinity range at this station did not vary greatly from the rest of the other stations. It can therefore be assumed that physico-chemical water parameters may have had a minimal effect on mussel body condition index, and that body condition index were more influenced by contaminants present in the bay.

#### *Some experimental design considerations for mussel transplant studies*

Active biomonitoring has numerous advantages besides providing a biological assessment of an area where native organisms either don't occur or occur in too small populations to be sampled (Salazar, 1997; Salazar, 1999; Salazar and Salazar, 1995, 2000; Smolders *et al.*, 2003; Bervoets *et al.*, 2004). Transplanting test specimens from an unpolluted site to a polluted site provides the opportunity to specify the exposure duration, as well as obtain a more acute account of the effects of pollutants on biomarker responses as responses from native organisms may be influenced by the organisms ability to adapt to its local conditions (Smolders *et al.*, 2003;

Bervoets *et al.*, 2004). However, some precautions or considerations must be taken into account during the experimental design of such studies:

1. The selected test organisms must be the same age or size, belong to a well established family of bioindicators, have extensive tests previously performed on its physiology (Phillips, 1976b).
2. Test organisms that will be used in transplant studies must be carefully removed from rocks by meticulously cutting off the byssal threads and carefully removing any epizoans from the shells in order to ensure that the well being of the organisms are not compromised (Honkoop *et al.*, 2003).
3. The constructed mussels bags or cages must be off non-toxic materials that will not corrode or dissolve, nor influence the behaviour or health of the test organisms. According to Salazar and Salazar (1995, 2000), materials such as glass, PVC, polyethylene and nylon are highly suggested for the construction of such holding devices.
4. During the design of the mussel bag or cage, the mesh size used must not only ensure free circulation of food and water, but also hold and protect the specimens efficiently from predators or other interfering organisms. The bags should be divided into sub-compartments to ensure minimal stress due to overcrowding, facilitate growth and valve movements and ensure all organisms have equal exposure to ambient conditions. Also compartments will allow for the convenient cataloguing of measurement data from specimens throughout the study duration. The bag size and number of compartments will be decided by the size and number of test organisms per compartment.
5. The type of bag deployment will be decided by the medium that will be tested. According to Salazar (1999), bags can be transplanted to the water column for the assessment of bioavailable contaminants present in the water column or bags can be deployed on sediment for the assessment of bioavailable contaminants present in sediment. These different types of media require different types of bag deployment. For water column deployment, bags may be attached onto existing structures like navigational bouys, piers or floating moorings (Mauri and Baraldi, 2003), or be secured by weights at the sea bottom and floats that suspend the bags to the desired depth (Borchardt *et al.*, 1988; Roesijadi *et al.*, 1984; Bervoets *et al.*, 2004). Bags for sediment deployment must be fixed to the sediment of the sea floor, and designed such that all compartments and specimens are exposed to the sediment. Studies by Phillips (1976b) and Salazar (1999) suggest multiple bags be used along a vertical

gradient in the water column in order to sample a greater vertical height. However all bags must be deployed at the same depth and either have equal periods of air exposure during low tides or be completely submerged at all times.

6. The number of transplanted organisms per bag and samples per replicate must be considered with the type and number of biomarker tests that will be assayed in mind. Furthermore, the loss of test organisms due to mortality, loss of bags or any other unforeseen event must also be considered.
7. The spatial range of transplant sites must be considered. The sampling of a site or area and 'hotspots' isolated within a site are different from each other and thus require different sampling protocols and dispersed spatial replicates. According to Salazar and Salazar (1995, 2000), replicate distances should be proportional to, and representative of, the locality that is being investigated. In this study, replicates for each station were deployed 2.5 meters apart due to the attachment of mussel bags onto existing navigational bouys that were not scheduled for maintenance removal, thus representing a hotspot rather than a larger contaminated area (Shugart *et al.*, 1992; Underwood, 1998).
8. Physico-chemical water parameters (salinity, temperature, pH and dissolved oxygen) must be monitored at each transplant site, as each of these parameters can influence biomarker responses.
9. The test duration must be determined by the biomarker type that will be assayed, as some biomarkers only display a change in responses after just 4 hours (Curtis *et al.*, 2000) and others much longer (Salazar, 1999; Salazar and Salazar, 1995, 2000). Transplant studies must also consider the effects of seasonal variations on biomarker assays and should therefore be planned to end before a new season starts. Studies that are planned to investigate the effects of seasonal variations however, must be undertaken over a minimum period of 3 years for seasonal comparisons to be statistically valid (Borchardt *et al.*, 1988). This is re-emphasised by Coull (1985), who also recommends any research of biological changes due to seasonal fluctuations be investigated by long-term sampling designs. In this study, transplant experiments were undertaken during winter months (June – July) and summer months (December – January), however the experiments comprised of only 1 year of data. According to Underwood (1998) replicate samples from a single seasonal cycle (over a 1 year cycle) are pseudoreplicates which provide invalid conclusions as the results from a single season could have been affected by an isolated event rather than natural variations in the environment and biological fluctuations. Although any statistical

comparison between the two seasons sampled is inconclusive, the findings of this study are supported by those of previous studies. Hence, despite the time scale limitations of the experimental design, the seasonal differences in biomarker responses from *P. perna* reflected fluctuations that should conform to data collected over a 3 year sampling period.

## **Conclusion**

This study showed that transplanted *P. perna* mussels could be used to assess metal bioavailability and biomarker effects at polluted stations in Durban harbour and unpolluted stations in Park Rynie. In conclusion, the various biomarker responses, seawater and soft tissue metal concentrations suggest that the environmental status of Durban harbour is heavily influenced by tidal exchange and contaminated freshwater and stormwater outputs entering the bay, as stations exposed to the contaminants entering the harbour via the rivers and stormwater drains, particularly Station 1, displayed the lowest biomarker responses and highest metal concentrations in tissue and water samples. The study also emphasises the sensitivity and usefulness of employing a suit of biomarkers that include both physiological and cytological responses to evaluate the biological health of a port, and its ability to identify localised ‘hotspots’ which are areas of concern with regards to marine pollution. The need for long-term seasonal monitoring of such biomarkers to provide an integrated approach to marine pollution biomonitoring is also highlighted, as well as to obtain a clear understanding of how seasonal fluctuations can affect biological responses from mussels. Recommendations for future transplant studies would be to apply a more comprehensive and long-term study in Durban harbour that would not only employ filter-feeding mussels as biomonitoring species, but also use deposit feeders to give an indication on the biological effects and bioavailability of contaminants trapped within sediment. This should also be complimented by a detailed sediment analysis for the different types of anthropogenic contaminants usually present in shallow marine environments. Another interesting study to be considered for the future would be to investigate the changes in biomarker responses from mussels transplanted from (1) a polluted area to an unpolluted area; (2) a polluted area to other polluted areas with different degrees of contamination; and (3) to compare biomarker responses from transplanted mussels and indigenous mussels.

## Chapter 5: Conclusion and recommendations for future studies

The use of oceans as a repository site for anthropogenic wastes has resulted in alterations of the marine ecosystems (Schachter and Serwer, 1971; Gross, 1978; Moldan, 1989; Fatoki and Mathabatha, 2001). Hence marine pollution and its effects on ecosystems have been recognised as an important global issue that need urgent attention. At present, South Africa's marine environment is threatened mostly by land derived sources of pollution, and habitat degradation (Begg, 1978; Moldan, 1995; Forbes *et al.*, 1996; Weerts and Cyrus, 2002), with estuaries and near-shore waters being the most vulnerable as poor water circulation and the inability to efficiently assimilate large waste loads (Taljaard *et al.*, 2006). Despite this, not much work has been undertaken on the pollution status along the country's coastline, particularly in KZN. The only detailed article on the KZN effluent discharge points was published in 1988 (Connell, 1988), thus clearly illustrating the urgency for further research on marine pollution in KZN, as well as on the latest international advances on marine pollution monitoring techniques.

### *Biomonitoring*

Biomonitoring is a recent advancement in the field of ecotoxicology and has drawn significant attention. In addition to providing a true reflection of the effects of anthropogenic contaminants on organisms, biomarkers serve as an early warning signal before irreversible damages at population or ecosystem levels can occur as they are determined at sub-organism levels (Moore *et al.*, 2006; Shugart *et al.*, 1992). Numerous past studies have employed these monitoring tools to identify spatial and temporal patterns of contamination in the marine environment (Rainbow, 1995). According to Lam and Rudolf (2003), the lack of funds, research, resources and skilled scientists are the main factors which deter the implementation of biomarkers in pollution monitoring programmes. Rapid, reliable, cost-effective and easy to use biomarker techniques are therefore in demand, particularly in countries such as South Africa where current marine pollution monitoring techniques are outdated. Active biomonitoring allows the exposure period of the test organisms to be controlled over space and time, and provides an accurate comparison between sites, particularly if some sites have either a limited or no population of the biomonitoring species (Salazar, 1997; Smolders *et al.*, 2003). This approach has been used in numerous biomonitoring studies, and proved to be successful in this study (refer to Chapter 4). A suite of biomarkers, accompanied with chemical analysis, are recommended for environmental monitoring programmes, as this integrated approach would provide a detailed account on the effects of anthropogenic pollutants on the biomonitoring organism. The multi-biomarker approach used in this study successfully utilises physiological, cytological and

biochemical responses from mussels to indicate environmental stress due to anthropogenic contamination.

*Perna perna as a bioindicator species*

This study aimed to show that biomarker responses from indigenous marine mussels can be successfully used as an ecotoxicological tool for marine pollution monitoring. Chapter 2 investigated the potential use of two mussel species as bio-indicator organisms by exposing the organisms to increasing Cu dosages. *Perna perna* and *B. semistriatus* both occur in tropical and sub-tropical regions, and are particularly prevalent along the eastern South African coastline (Ragagopal *et al.*, 2003). Although *P. perna* has been the subject of numerous experiments, little is known about the physiology of *B. semistriatus*. In the present study, *P. perna* was shown to be a suitable bio-indicator organism as the species exhibited a stronger sensitivity to increasing Cu treatments. In contrast, *B. semistriatus* did not show any distinct variation in biomarker response when exposed to low Cu concentrations for most biomarker assays. Further experiments on the biological responses of the species must be undertaken in future studies to gain detailed knowledge on the physiology of the organism before utilising the species as biomonitors in marine pollution monitoring programmes.

*Perna perna* occurs in densely populated communities throughout KZN rocky shores. Seeing as this species has a wide geographic distribution, their tolerance to varying environmental factors is evident. In addition, these mussels proved to be resilient to removal from their natural habitats and relocation into aquaria and another natural environment, as the majority of the mussels survived the entire duration of the laboratory and transplant experiments. Thus this species adapts well to new surroundings and were able to display distinct variations in biomarker responses to identify spatial gradients of contamination along the KZN coast, as well as localised areas of severe contamination in Durban harbour as seen in Chapters 3 and 4 respectively.

Changes in physiological and cytological biomarkers from *P. perna* were evident during the summer and winter transplant months as found in Chapter 4. It was concluded that these differences in response were due to two factors. The first is the excessive amount of contaminants entering the bay during the wet season, and thus affecting the biomarker responses accordingly. The second is the effects of spawning on the physiology of *P. perna*. It is therefore recommended that detailed investigations are conducted on the effects of the different reproductive stages and other physiological factors such as size, sex and age on biomarker



responses. In this study, such variables were minimised by ensuring mussels of a similar size were used, and all specimens were obtained during a short collection from the field, from similar tidal habitats. Studies on the effects of environmental factors such as salinity and temperature extremes, air exposure and the availability of food on biomarker responses should also be further investigated.

### *Cardiac activity as a bioindicator of marine pollution*

Cardiac activity as a biomarker proved to be a good indicator of contaminant exposure as *P. perna* and *B. semistriatus* displayed a decline in heart rates as the Cu dosages were increased (refer to Chapter 2). This finding is consistent with those of previous studies (Widdows, 1973; Sabourin and Tullis, 1981; Nicholson, 1999a,b; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Galloway, 2002; Abessa *et al.*, 2005). In Chapter 3, mussels from heavily industrialised and polluted sites along the KZN coast reflected depressed heart rates compared to the cardiac activities obtained from relatively un-urbanised areas. Cardiac activity recorded from *P. perna* sampled at Durban, Umkomaas and Isipingo exhibited significantly decreased heart rates compared to mussels obtained from Park Rynie and Zinkwazi, which are regarded as relatively pristine sites. The results of Chapter 3 are supported by similar studies that document depressed heart rates from polluted sites (Nicholson, 1999a; Curtis *et al.*, 2000; Curtis *et al.*, 2001; Galloway, 2002). Chapter 4 examined the cardiac activities from *P. perna* transplanted in Durban harbour during summer and winter months. Distinct bradycardia was observed at several stations within the harbour, particularly those positioned at the inner most and heavily polluted regions. This result strongly indicates that the water quality and well-being of organisms in the harbour are strongly influenced by contaminants entering the bay via rivers and stormwater drains that empty into the harbour. In contrast, the rest of the transplant stations in Durban harbour and the reference site (Park Rynie) did not show such large fluctuations in mussel heart rates. Again, these results are consistent to previous results in similar studies, and to the rest of the results of this study, which demonstrate that mussels exposed to anthropogenic contaminants exhibit bradycardia. Thus the utilisation of this biomarker as an ecotoxicological tool is highlighted.

### *Lysosomal membrane stability as a biomarker*

Domouhtsidou *et al.* (2004) stated that although extremes in environmental factors such as salinity and temperature may influence lysosomal membrane stability, the damages inflicted to the lysosomal membrane by contaminant exposure are much more severe. This was clearly demonstrated in this study. As shown in Chapter 2, the increasing Cu dosages caused a distinct

decline in lysosomal membrane stability from both *P. perna* and *B. semistriatus*. Interestingly *P. perna* obtained from the polluted site (Isipingo Beach) exhibited greater lysosomal membrane stability than those obtained from mussels sampled from park Rynie. It was concluded that lysosomes from Isipingo Beach mussels had a higher tolerance level to the Cu concentrations, which is attributed to the organisms' adaptation to elevated pollutant exposure. The lysosomal membrane stability recorded from *B. semistriatus* showed that the species was able to tolerate the effects of the lowest Cu treatments; however the lysosomal membranes were rendered dysfunctional when the species were exposed to the higher Cu treatments. In Chapter 3, lysosomal membrane stability obtained from *P. perna* collected from polluted sites along the KZN coastline exhibited significantly reduced stability times compared to unpolluted sites; thus identifying a geographic trend of localised contamination of the marine environment. Similar results were obtained from previous studies that compared mussel lysosomal stabilities from polluted and unpolluted sites (Lowe *et al.*, 1995; Nasci *et al.*, 1999; Wedderburn *et al.*, 2000; Nicholson, 2001; Riveros *et al.*, 2002; Domouhtsidou *et al.*, 2004; Petrovic *et al.*, 2004; Abessa *et al.*, 2005). In Chapter 4, significantly reduced lysosomal membrane stability from *P. perna* transplanted to heavily polluted sites were observed, particularly during the summer transplant month at the inner most area of the harbour which receives freshwater inflow. As with the depressed heart rates recorded at the same station, it was concluded that these biomarker results were due to an excessive quantity of contaminants being flushed into the harbour by the summer rains. Hence lysosomal membrane stability can be regarded as a sensitive and reliable biomarker.

#### *Body condition index of P. perna as an indication of polluted environments*

Body condition index has been successfully used in numerous studies to show the effects of contaminant exposure on the health condition of marine organisms (Lundebye *et al.*, 1997; Nicholson, 1999b; Orban *et al.*, 2002; Galloway *et al.*, 2002; Smolders *et al.*, 2003; Amiard *et al.*, 2004). However as seen in Chapter 2, the condition indices of *P. perna* and *B. semistriatus* failed to exhibit any difference in their body indices between the increasing Cu treatments. It was deduced that the experimental period was too brief to retard somatic growth rates, and that this biomarker should only be used for long-term monitoring programmes. Nevertheless, Chapters 3 and 4 revealed significant differences in the health conditions of mussels between sites along the KZN coastline and Durban harbour respectively. Low body condition indices of marine mussels sampled from polluted regions are well documented by previous articles (Avery *et al.*, 1996; Nicholson, 1999a,b; Mauri and Baraldi, 2003; Bodin *et al.*, 2004), and support the findings of this study as the lowest indices from *P. perna* along the

coastline were recorded from Umkomaas and Isipingo, the most polluted sites sampled in Chapter 3. The highest body condition indices from *P. perna* were found at Park Rynie and Zinkwazi. These findings are consistent with the results of the heart rate and lysosomal membrane stability biomarker tests at the above sites, thus validating the results of the condition indices. In Chapter 4, mussels from the most polluted stations in Durban harbour displayed the lowest body condition indices, whereas those from less polluted and reference stations exhibited healthier body condition indices. An interesting contrast was found between the condition indices of *P. perna* from the summer and winter transplant months. Regardless of Durban harbour experiencing an excessive flush of contaminants during the summer months and the other biomarkers responding accordingly, the body condition indices of mussels found during these months indicated healthier mussel conditions than those of winter transplant months. It was concluded that seeing as the spawning period of *P. perna* occurs in late winter, the considerable body mass loss due to this event heavily influenced the body condition indices. This highlights the significant effects that biotic and abiotic factors such as seasonal changes, reproductive activity and nutritional health of the organisms can have on body condition indices. Thus a future detailed study on such influences is needed; nonetheless, this biomarker can provide important information on the health of mussels if used in long-term studies.

*The use of malate dehydrogenase enzyme activity as a suitable biomarker response*

MDH has been successfully used in previous studies to assess the metabolic rate of marine invertebrates (Lushchak *et al.*, 1997; Fahraeus-Van Ree and Payne, 1999; Orbea *et al.*, 1999; Dahlhoff *et al.*, 2001; Dahlhoff *et al.*, 2002; Luk'yanova, 2006). However, in this study significant inhibition in MDH enzyme activity from mussel exposed to low Cu treatments was not evident; thus concluding that very low metal dosages combined with a short exposure period cannot affect MDH enzyme activity in *P. perna* and *B. semistriatus* (refer to Chapter 2), hence the biomarker was not employed in any further investigations in this study. Nevertheless the two mussel species displayed a decline in enzyme activity with increasing Cu treatments, which is supported by the findings of Pellerin-Massicotte and Pelletier (1987). It must be noted that factors such as temperature and salinity extremes, hypoxia and food availability can have an effect on enzyme activity (Dahlhoff, 2004); hence it is strongly recommended that should MDH activity be used in monitoring programmes, it must be complimented by a suit of biomarkers to support the results of the biomarker response.

*Heavy metal bioaccumulation in P. perna*

Bivalves are extensively used in global biomonitoring programmes, particularly marine mussels as they have the unique ability to accumulate heavy metals within their body tissue (Goldberg, 1986; Rainbow, 1995; Boening, 1997; Nasci *et al.*, 1999; Ravera, 2001; Smolders *et al.*, 2003; Domouhtsidou *et al.*, 2004). In this study, the metal content of *P. perna* body tissue proved to be a good reflection of the amount of metal contaminants that are bioavailable to marine organisms, as seen in Chapter 3. Significant correlations between mussel tissue and sediment metal concentrations suggest that *P. perna* is an effective metal bioaccumulator species, particularly for metals Cd, Pb, Zn, Cr and Fe. Additionally, the metal analysis of *P. perna* soft tissues and sediment, as well as the adverse biomarker responses indicated by significant bradycardia, reduced lysosomal membrane stability and poor condition indices from mussels, identified a pollution gradient along the KZN coastline. Durban, Isipingo and Umkomaas were singled out as being the most contaminated sites along the coast, and Zinkwazi and Park Rynie as the most unpolluted. Seeing as the most contaminated sites identified in this study support the majority of the province's chief industries, it stands to reason that the highest metal concentrations in *P. perna* tissue would be found at these sites; however tissue metals Fe and Ni did not show a pronounced geographic trend as did the rest of the tissue metal concentrations. The sediment metal concentration of these two metals, together with the rest of the heavy metals analysed, did however conform to the same trend displayed by the *P. perna* tissue concentrations. Hence, the metal analysis of both *P. perna* soft tissues and sediment sampled along the KZN coast validates the biomarker responses recorded at the different sites. In Chapter 4, similar spatial trends were observed in metal tissue and seawater concentrations. Stations located nearest to the freshwater inputs and stormwater drains displayed the highest metal concentrations for most metals. Nevertheless, high concentrations of metals Zn, Pb and Cr also displayed high concentrations at those stations exposed to a continuous exchange of fresh seawater during the summer transplant months. It was concluded that this was attributed to the summer rains flushing excessive pollutants from the city into the bay.

With regards to the *P. perna* tissue concentrations from the different transplant months, the highest tissue concentrations were observed during summer. As discussed in Chapter 4, a dilution effect of metal contaminants by mussel soft tissue in summer months have been previously documented; as well as the loss of significant body mass during winter spawning which results in a concentration of heavy metals in tissues. However, in this study, metal bioaccumulation of transplanted *P. perna* were found to be more influenced by the presence of excessive contaminants in the seawater than seasonal reproductive activities. Nevertheless, it is

recommended that the complex biological process that occurs during spawning must be further investigated as it can have a significant effect on metal bioaccumulation. It is also recommended that all biomarker studies be accompanied by chemical analysis. Although this may increase the cost of implementation, a routine chemical analysis will provide a detailed account on which and how much heavy metals are attributing to biomarker results.

### *Key issues of experimental design*

Each chapter discusses important experimental design considerations. Chapter 2 reiterates the importance of proper experimental planning and execution. The basic structure of an experimental design is outlined, as well as the importance of having adequate sampling procedures, suitable sample sizes, appropriate controls and using accurate experimental units for statistical analysis. The consequences of not having random, independent replicates are discussed, as well the incorrect use of statistics on such data. Chapter 3 highlights some key protocols that must be considered for *in-situ* sampling and comparisons of results from different locations. The practical recommendations listed would minimise the influences of environmental factors from different locations on biomarker responses from mussels, thereby reducing the variability between results. Chapter 4 outlines some practical considerations for the experimental design of active biomonitoring studies using marine mussels. The most important being to ensure the health of the specimen during removal and relocation, as any compromise to the organisms' health will provide inaccurate biomarker results. Secondly, the number of transplanted organisms and replicates used must be considered together with the biomarkers that will be investigated. Thirdly, the spatial range of transplant sites must be considered, and replicate distances should be proportionate and representative of the locality that is being investigated. The rest of the considerations discusses in detail practical issues of cage construction and deployment.

### **Concluding remarks**

This study shows that a suite of simple, rapid, reliable and cheap biomarkers from native brown mussels are useful ecotoxicological tools; and if employed in marine pollution monitoring programs, can identify geographical gradients and localised areas of heavy metal contamination. *Perna perna* proved to be an appropriate biomonitoring species in the study, and the biomarker responses obtained in the various experiments demonstrated the organism's ability to provide a true reflection of the pollution status of an environment. The biomarkers used provided consistent results and supported each others findings; however it is emphasised that these biomarkers can be influenced by both environmental and biological factors, particularly body

condition indices and MDH activities. Therefore these biomarkers must be used with these considerations. Studies that are recommended for the future include:

1. Detailed investigations on the influences of biotic factors such as age, sex, size, reproductive activity, nutritional health of the organism, and abiotic factors which include thermal and salinity extremes, seasonal changes and exposure to air, on biomarker results.
2. The use of different biomarkers from *P. perna* must be investigated, particularly biomarkers from lower organism levels such as molecular biomarkers, as well as the combination of population and community studies, to obtain an integrated approach to assessing the effects of anthropogenic contaminants on marine ecosystems.
3. The effects of a combination of contaminants must further be studied in extensive laboratory studies to investigate such interactions in biomarker responses.
4. The effects of low concentrations of contaminants in long-term laboratory studies must also be determined as natural populations of organisms are more likely to be continually exposed to mild levels of pollutants rather than short-term exposure to extremely high contaminant levels.
5. A detailed study on seasonal variations in biomarkers from *P. perna* sampled from the KZN coastline.
6. An extensive long-term study in Durban harbour that would use filter-feeding and deposit feeding biomonitor species to give an indication on the biological effects and bioavailability of contaminants trapped within sediment.
7. Transplant studies that include the changes in biomarker responses from mussels transplanted from (1) a polluted area to an unpolluted area; (2) a polluted area to other polluted areas with different degrees of contamination; and (3) to compare biomarker responses from transplanted mussels and indigenous mussels.

## REFERENCES

- Abessa, D. M. S., Zaroni, L. P., de Sousa, C. P. M., Gasparro, M. R., Pereira, C. D. S., Rachid, B. R. F., Depledge, M. H. and King, R. S. 2005. Physiological and cellular responses in two populations of the mussel *Perna perna* collected at different sites from the coast of São Paulo, Brazil. *Brazilian Archives of Biology and Technology* 48(2): 217–225.
- Abuodha, P. A. W. and J. G. Kairo. 2001. Human-induced stresses on mangrove swamps along the Kenyan coast. *Hydrobiologia* 458: 255 – 265.
- Almeida, E. A., de Almeida Marques, S., Klitzke, C. F., Dias Bainy, A. C., de Medeiros, M. H. G., Di Mascio, P. and de Melo Loureiro, A. P., 2003. DNA damage in digestive gland and mantle tissue of the mussel *Perna perna*. *Comparative Biochemistry and Physiology* 135(C): 295–303.
- Alongi, D. M. 2002. Present state and future of the world's mangrove forests. *Environmental Conservation* 29(3): 331 – 349.
- Alves, S. R. C., Severino, P. C., Ibbotson, D. P., Silva, A. Z., Lopes, F. R. A. S., Saenz, L. A. and Bainy, A.C. D. 2002. Effects of furan in the brown mussel *Perna perna* and in the mangrove oyster *Crassostrea rhizophorae*. *Marine Environmental Research* 54(3-5): 241–245.
- Amiard, J. C., Bacheley, H., Barillé, A. L., Barillé L., Geffard A. and Himery N. 2004. Temporal changes in nickel and vanadium concentrations and in condition index and metallothionein levels in three species of molluscs following the "Erika" oil spill. *Aquatic Living Resources* 17: 281–288.
- Amiard-Triquet, T., Berthet, C. and Amiard, J. C. 1986. Contribution to the ecotoxicological study of cadmium, lead, copper and zinc in the mussel, *Mytilus edulis*. 2. Experimental study. *Marine Biology* 92: 7–13.
- Anandraj, A., Marshall, D. J., Gregory, M. A. and McClurg, T. P. 2002. Metal accumulation, filtration and O<sub>2</sub> uptake rates in the mussel *Perna perna* (Mollusca: Bivalvia) exposed to

## References

- $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ . *Comparative Biochemistry and Physiology* 132(C): 355–363.
- Anderson, V., Maage, A. and Johannessen, P. J. 1996. Heavy metals in blue mussels (*Mytilus edulis*) in the Bergen Harbour area, Western Norway. *Bulletin of Environmental Contamination and Toxicology* 57: 589–596.
- Atkins, P. W., Clugston, M. J., Frazer, M. J. and Jones, R. A. Y. 1988. *Chemistry principles and applications*. Pages 363 – 386. Longman Inc., New York. 1988.
- Avery, E. L., Dunstan, R. H. and Nell, J. A. 1996. The detection of pollutant impact in marine environments: condition index, oxidative DNA damage, and their associations with metal bioaccumulation in the Sydney rock oyster *Saccostrea commercialis*. *Archives of Environmental Contamination and Toxicology* 31: 192–198.
- Bainy, A. C. D., Almeida, E. A., Mueller, I. C., Ventura, E. C. and Medeiros, I. D. 2000. Biochemical responses in farmed mussel *Perna perna* transplanted to contaminated sites on Santa Catarina Island, Brazil. *Marine Environmental Research* 50: 411–416.
- Bahmet, I. N., Beger, V. J. and Halaman, V. V. 2005. The effect of salinity change on the heart rate of *Mytilus edulis* specimens from different ecological zones. *Journal of Experimental Marine Biology and Ecology* 318: 121–126.
- Bakhmet, I. N. and Khalaman, V. V. 2006. Heart rate variation patterns in some representatives of Bivalvia. *Biology Bulletin* 33(3): 276–280.
- Baldwin S., Deaker M. and Maher, W. 1994. Low volume microwave digestion of marine biological tissues for the measurement of trace elements. *Analyst* 119: 1701–1704.
- Baraj, B., Niencheski, L. F. and Corradi, C. 2004. Trace Metal Content Trend of Mussel *Perna perna* (Linnaeus, 1758) from the Atlantic Coast of Southern Brazil. *Water, Air and Soil Pollution* 145(1-4): 205–214.
- Barracco, M. A., Medeiros, I. D. and Moreira F. M. 1999. Some haemato-immunological parameters in the mussel *Perna perna*. *Fish and Shellfish Immunology* 9: 387–404.



## References

- Batterman, S., Chernyak, S., Gounden, Y. and Matooane, M. R. 2007. Concentrations of persistent organic pollutants in ambient air in Durban, South Africa. *Organohalogen Compounds* 68: 1–6.
- Bayne, B. L., Moore, M. N. and Koehn, R. K. 1981. Lysosomes and the response by *Mytilus edulis* L. to an increase in salinity. *Marine Biology Letters* 2: 193–204.
- Begg, G. 1978. *The Estuaries of Natal*. Natal Town and Regional Planning Report 41.
- Beiras, R. Fernandez, N., Bellas, J., Besada, V., Gonzalez-Quijano, A. and Nunes, T. 2003. Integrative assessment of marine pollution in Galician estuaries using sediment chemistry, mussel bioaccumulation, and embryo-larval toxicity bioassays. *Chemosphere* 52(7): 1209–1224.
- Berger, V. J. and Kharazova, A. D. 1997. Mechanisms of salinity adaptations in marine molluscs. *Hydrobiologia* 355: 115–125.
- Bervoets, L., Voets, J., Chu, S., Covaci, A., Schepens, P. and Blust, R. 2004. Comparison of accumulation of micropollutants between indigenous and transplanted Zebra mussels (*Dreissena polymorpha*). *Environmental Toxicity and Chemistry* 23(8): 1973–1983.
- Biseswar, R., Moodley, G. K. and Naidoo, A. D. 2002. Note on an invasion of intertidal zoanthid colonies by a chaetopterid polychaete at Park Rynie Beach, KwaZulu-Natal. *South Africa Journal of Marine Science* 24: 371–374.
- Blackmore, G. and Wang, W-X. 2003. Comparison of metal accumulation in mussels at different local and global scales. *Environmental Toxicology and Chemistry* 22(2): 388–395.
- Blair, A. 2006. *Studies on the environmental impact of Sappi Saiccor effluent on the KZN south coast: surveys made in 2006*. CSIR Report No. ENV-D-C-2006-012.
- Boalch, R; Chan, S. and Taylor, D. 1981. Seasonal Variation in the Trace Metal Content of *Mytilus edulis*. *Marine Pollution Bulletin* 12(8): 276–280.

## References

- Bodin, N., Burgeot, T., Stanisière, J. Y., Bocquenè, G. Menard, D., Minier, C. Boutet, I., Amat, A., Cherel, Y, and Budzinski, H. 2004. Seasonal variations of a battery of biomarkers and physiological indices for the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. *Comparative Biochemistry and Physiology* 138(C): 411–427.
- Boening, D. W. 1997. An evaluation of bivalves as biomonitors of heavy metals pollution in marine waters. *Environmental Monitoring and Assessment* 55: 459–470.
- Bootsma, N., Macey, D. J., Webb, J. and Talbot, V. 1990. Use of carbonyl iron to induce iron loading in the mussel *Mytilus edulis*. *Bulletin of Environmental Contamination and Toxicology* 44(2): 205–209.
- Borchardt, T., Burchert, S., Hablitzel, H., Karbe, L. and Zeitner, R. 1988. Trace metal concentrations in mussels: comparison between estuarine, coastal and offshore regions in the south eastern North Sea from 1983 to 1986. *Marine Ecology Progress Series* 42: 17–31.
- Braby, C. E. and Somero, G. N. 2006. Following the heart: temperature and salinity effects on heart rate in native and invasive species of blue mussels (genus *Mytilus*). *Journal of Experimental Biology* 209: 2554–2566.
- Branch, G., Griffiths, C. L., Branch, M. L. and Beckley, L. E. 1994. *Two Oceans: A Guide to the Marine Life of Southern Africa*. First edition. Pages 114–116. David Philip Publishers, South Africa. 1994.
- Brown, R. J., Galloway, T. S., Lowe, D., Browne, M. A., Dissanayake, A. Jones, M. B. and Depledge, M. H. 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. *Aquatic Toxicology* 66: 267–278.
- Bryan, G. W. 1971. The effects of heavy metals (other than mercury) on marine and estuarine organisms. *Proceedings of the Royal Society of London. Series B, Biological Sciences* 177: 389–410.
- Cain, D. J. and Luoma, S. N. 1986. Effect of seasonally changing tissue weight on trace metal concentrations in the bivalve *Macoma balthica* in San Francisco Bay. *Marine Ecology Progress Series* 28: 209–217.

## References

- Camus, L., Grosvik, B. E., Borseth, J. F., Jones, M. B. and Depledge, M. H. 2000. Stability of lysosomal and cell membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures. *Marine Environmental Research* 50: 325–329.
- Chapman, M. G. 2000. Poor design of behavioural experiments gets poor results: examples from intertidal habitats. *Journal of Experimental Marine Biology and Ecology* 250: 77–95.
- Chase, M. E., Jones, S. h., Hennigar, P., Sowles, J., Harding, G. C. H., Wells, P. G., Krahforst, C., Coombs, K., Crawford, R., Pederson, J. and Taylor, M. 2001. Gulf watch: monitoring spatial and temporal patterns of trace metal and organic contaminants in the Gulf of Maine (1991–1997) with the blue mussel, *Mytilus edulis* L. *Marine Pollution Bulletin* 42(6): 491–505.
- Chong, K. and Wang, W-X. 2000. Bioavailability of sediment-bound Cd, Cr and Zn to the green mussel *Perna viridis* and the Manila clam *Ruditapes philippinarum*. *Journal of Experimental Marine Biology and Ecology* 255: 75–92.
- Chong, K. and Wang, W-X. 2001. Comparative studies on the biokinetics of Cd, Cr and Zn in green mussel *Perna viridis* and the Manila clam *Ruditapes philippinarum*. *Environmental Pollution* 115: 107–121.
- Chung, K. S. and Acuna, A. 1981. Upper temperature tolerance limit of mussel, *Perna perna*. *Bulletin of the Japanese Society of Scientific Fisheries* 47(3): 441.
- Clark, R. B. 2001. *Marine Pollution*. Fifth edition. Pages 1–231. Oxford University Press Inc., New York. 2001.
- Connell, A. D. 1988. Pollution and effluent disposal off Natal. In: *Lecture Notes on Coastal and Estuarine Studies*. Schumann, E. H. (ed). Springer-Verlag, Germany. Pages 226–251.
- Connell, A. D. 2001. Pelagic eggs of marine fishes from Park Rynie, KwaZulu-Natal, South Africa: seasonal spawning patterns of the three most common species. *African Zoology* 36(2): 197–204.

## References

- Cossa, D., Bourget, E., Pouliot, D., Piuze, J. and Chanut, J. P. 1980. Geographical and seasonal variations in the relationship between trace metal content and body weight in *Mytilus edulis*. *Marine Biology* 58: 7–14.
- Costa, M. Paiva, E. and Moreira, I. 2000. Total mercury in *Perna perna* mussels from Guanabara Bay - 10 years later. *The Science of the Total Environment* 261(1–3): 69–73.
- Cottenie, K. and De Meester, L. 2003. Comment to Oksanen (2001): reconciling Oksanen (2001) and Hurlbert (1984). *Oikos* 100(2): 395–396.
- Coull, B. C. 1985. The use of long-term biological data to generate testable hypothesis. *Estuaries* 8(2A): 84–92.
- Creel, L. 2003. *Ripple effects: population and coastal regions*. In: *Measure Communication*. Washington, D.C.: Population Reference Bureau, USA. Available on [website http://www.prb.org](http://www.prb.org). Date accessed: 07-09-2006.
- Cunningham, M. A., Ho, L., Nguyen, D. T., Gillilan, R. E. and Bash, P. A. 1997. Simulation of the enzyme reaction mechanism of malate dehydrogenase. *Biochemistry* 36: 4800–4816.
- Curtis, T. M., Williamson, R. and Depledge, M. H. 2000. Simultaneous, long-term monitoring of valve and cardiac activity in the blue mussel *Mytilus edulis* exposed to copper. *Marine Biology* 136: 837–846.
- Curtis, T. M., Williamson, R. and Depledge, M. H. 2001. The initial mode of action of copper on the cardiac physiology of the blue mussel, *Mytilus edulis*. *Aquatic Toxicology* 52: 29–38.
- Cyrus, D. P. and Forbes, A. T. 1996. Preliminary results on the role of KwaZulu-Natal harbours as nursery grounds for juveniles of selected marine organisms which utilize estuaries. *South African Journal of Wildlife Research* 26(1): 26–31.
- Dafre, A.L., Dias Medeiros, I., Müller, I.C., Ventura, E.C. and Dias Bainy, A.C., 2004. Antioxidant enzymes and thiol/disulfide status in the digestive gland of the brown mussel *Perna perna* exposed to lead and paraquat. *Chemical and Biological Interactions* 149, 97–105.

## References

- Dahlhoff, E. P. and Menge, B. A. 1996. Influence of phytoplankton concentration and wave exposure on the ecophysiology of *Mytilus californianus*. *Marine Ecology Progress Series* 144: 97–107.
- Dahlhoff, E. P. 2004. Biochemical indicators of stress and metabolism: applications for marine ecological studies. *Annual Review of Physiology* 66: 183–207.
- Dahlhoff, E. P., Buckley, B. A. and Menge, B. A. 2001. Physiology of the rocky intertidal predator *Nucella ostrina* along an environmental stress gradient. *Ecology* 82(10): 2816–2829.
- Dahlhoff, E. P., Stillman, J. H. and Menge, B. A. 2002. Physiology community ecology: variation in metabolic activity of ecologically important rocky intertidal invertebrates along environmental gradients. *Integrated and Comprehensive Biology* 42: 862–871.
- Dailianis, S., Domouhtsidou, G. P., Raftopoulou, E., Kaloyianni, M., and Dimitriadis, V. K. 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. *Marine Environmental Research* 56: 443–470.
- Davenport, J. and Redpath, K. J. 1984. Copper and the mussel *Mytilus edulis* L. In: *Toxin, drugs and pollutants in marine animals*. Bollis, L., Zadunaisky, J., Gilles, R. (eds). Springer-Verlag, Berlin. Pages 176–189.
- Davies, I. M. and Pirie, J. M. 1980. Evaluation of a mussel watch project for heavy metals in Scottish coastal waters. *Marine Biology* 57: 87–93.
- De Boer, W. F. 2002. The rise and fall of the mangrove forests in Maputo Bay, Mozambique. *Wetlands Ecology and Management* 10: 313–322.
- De Pirro, M. and Marshall, D. J. 2005. Phylogenetic differences in cardiac activity, metal accumulation and mortality of limpets exposed to copper: A prosobranch–pulmonate comparison. *Journal of Experimental Marine Biology and Ecology* 322(1): 29–37.

- De Vooy, C. G. N. 1991. Anaerobic metabolism in sublittoral living *Mytilus galloprovincialis* in the mediterranean—IV. Role of amino acids in adaptation to low salinities during anaerobiosis and aerobiosis. *Comparative Biochemistry and Physiology Part A: Physiology* 100(2): 423–431.
- Depledge, M. H. and Anderson, B. B. 1990. A computer-aided physiological monitoring system for continuous, long-term recordings of cardiac activity in selected invertebrates. *Comparative Biochemistry and Physiology* 96(A): 474–477.
- Diab, R., Prause, A. and Bencherif, H. 2002. Analysis of SO<sub>2</sub> pollution in the South Durban industrial basin. *South African Journal of Science* 98: 543–546.
- Dimitriadis, V. K., Domouhtsidou, G. P. and Raftopoulou, E. 2003. Localization of Hg and Pb in the palps, the digestive gland and the gills in *Mytilus galloprovincialis* (L.) using autometallography and X-ray microanalysis. *Environmental Pollution* 125(3): 345–353.
- Domouhtsidou, G. P. and Dimitriadis, V. K. 2000. Ultrastructure localization of heavy metals (Hg, Ag, Pb and Cu) in gills and digestive gland of mussels, *Mytilus galloprovincialis* (L.). 2000. *Archives of Environmental Contamination and Toxicology* 38: 472–478.
- Domouhtsidou, G. P., Dailianis, S., Kaloyianni, M. and Dimitriadis, V. K. 2004. Lysosomal membrane stability and metallothionein content in *Mytilus galloprovincialis* (L.) as biomarkers. Combination with trace metal concentrations. *Marine Pollution Bulletin* 48: 574–588.
- Eberhardt, L. L. and Thomas, J. M. 1991. Designing environmental field studies. *Ecological Monographs* 61(1): 53–73.
- Fåhræus-Van Ree, G. E. and Payne, J. F. 1999. Enzyme cytochemical responses of mussels (*Mytilus edulis*) to resin acid constituents of pulp mill effluents. *Bulletin of Environmental Contamination and Toxicology* 63: 430–437.
- Farrington, J. W., Goldberg, E. D., Risebrough, R. W., Martin, J. H. and Bowen, V. T. 1983. U.S. “Mussel Watch” 1976–1978: An Overview of the Trace-Metal, DDE, PCB, Hydrocarbon, and Artificial Radionuclide Data. *Environmental Science and Technology* 17: 490–496.

## References

- Fatoki, O. S. and Mathabatha, S. 2001. An assessment of heavy metal pollution in the East London and Port Elizabeth harbours. *Water SA* 27(2): 233–240.
- Ferreira, R. P. and Salomao, L.C. 2000. The ionic basis of cardiac activity in the bivalve mollusc *Perna perna*. *Journal of Experimental Marine Biology and Ecology* 249(11): 1–12.
- Fisher, N. S., Teyssie, J. L., Fowler, S. W. and Wang, W-X. 1996. Accumulation and Retention of Metals in Mussels from Food and Water: A Comparison under Field and Laboratory Conditions. *Environmental Science and Technology* 30(11): 3232–3242.
- Forbes, A. T., Demetriades, N. T. and Cyrus, D. P. 1996. Biological significance of harbours as coastal habitats in KwaZulu-Natal, South Africa. *Aquatic Conservation: Marine and Freshwater Ecosystems* 6: 331–341.
- Fossi, M. C., Casini, S., Marsili, L., Neri, G., Mori, G., Ancora, S., Moscatelli, A., Ausili, A. and Notarbartolo-di-Sciara, G. 2002. Biomarkers for endocrine disruptors in three species of Mediterranean large pelagic fish. *Marine Environmental Research* 54(3–5): 667–671.
- Francioni, E., Wagener, A. L. R., Scotfield, A. L., Depledge, M. H. and Cavalier, B. 2007. Evaluation of the mussel *Perna perna* as a biomonitor of polycyclic aromatic hydrocarbon (PAH) exposure and effects. *Marine Pollution Bulletin* 54: 329–338.
- Francioni, E., Wagener, A. L., Calixto, R. C. and Bastos, G. C. 2004. Evaluation of *Perna perna* (Linne, 1758) as a tool to monitoring trace metals contamination in estuarine and coastal waters of Rio de Janeiro, Brazil. *Journal of the Brazilian Chemical Society* 15(1): 103–110.
- Galloway, T. S., Sanger, R. C., Smith, K. L., Fillman, G., Ford, T. E. and Depledge, M. H. 2002. Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays. *Environmental Science and Technology* 36: 2219–2226.
- Goldberg, E. D. 1986. The mussel watch concept. *Environmental Monitoring and Assessment* 7: 91–103.
- Goward, C. R. and Nicholls, D. J. 1994. Malate dehydrogenase: A model for structure, evolution, and catalysis. *Protein Science* 3: 1883–1888.

## References

- Gregory, M. A., George, R. C., Marshall, D. J., Anandraj, A. and McClurg, T. P. 1999. The Effects of Mercury Exposure on the Surface Morphology of Gill Filaments in *Perna perna* (Mollusca: Bivalvia). *Marine Pollution Bulletin* 39(1-12): 116–121.
- Gregory, M. A., Marshall, D. J., George, R. C., Anandraj, A. and McClurg, T. P. 2002. Correlations between metal uptake in the soft tissue of *Perna perna* and gill filament pathology after exposure to mercury. *Marine Pollution Bulletin* 45: 114–125.
- Grobler, D. F., Badenhorst, J. E. and Kempster, P.L. 1996. PCBs, chlorinated hydrocarbon pesticides and chlorophenols in the Isipingo Estuary, Natal, Republic of South Africa. *Marine Pollution Bulletin* 32(7): 572–575.
- Gross, M. G. 1978. Effects of waste disposal operations in estuaries and the coastal ocean. *Annual Reviews of Earth Planetary and Science* 6: 127–143.
- Handy, R. D., Galloway, T. S. and Depledge, M. H. 2003. A proposal for the use of biomarkers for the assessment of chronic pollution and in regulatory toxicology. *Ecotoxicology* 12: 331–343.
- Harris, S. A. and Cyrus, D. P. 2000. Comparison of larval fish assemblages in three large estuarine systems, KZN, South Africa. *Marine Biology* 137: 527–541.
- Hauton, C., Hawkins, L. E. and Hutchinson, S. 1998. The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L). *Comparative Biochemistry and Physiology Part B* 119: 619–623.
- Heffner, R. A., Butler, M. J. and Reilly, C. K. 1996. Pseudoreplication revisited. *Ecology* 77(8): 2558–2562.
- Hennig, HF-KO. 1984. *The future of metal determination in pollution studies*. South African National Research Institute of Oceanology Report.
- Hicks, D. W. and McMahon, R. F. 2002. Respiratory responses to temperature and hypoxia in the nonindigenous Brown Mussel, *Perna perna* (Bivalvia: Mytilidae), from the Gulf of Mexico. *Journal of Experimental Marine Biology and Ecology* 277: 61–78.



## References

- Honkoop, P. J. C., Bayne, B. L., Underwood, A. J. and Svensson, S. 2003. Appropriate experimental design for transplanting mussels (*Mytilus sp.*) in analyses of environmental stress: an example in Sydney Harbour (Australia). *Journal of Experimental Marine Biology and Ecology* 297(2): 253–268.
- Horowitz, A. J. 1991. *A primer on Sediment-Trace Element Chemistry*. Second edition. Pages 1–136. Chelsea, Michigan, Lewis Publishers Inc., USA. 1991.
- Hummel, H., Modderman, R., Amiard-Triquet, C., Rainglet, F., van Duijn, Y., Herssevoort, M., de Jong, J., Bogaards, R., Bachelet, G., Desprez, M., Marchand, J., Svand, B., Amiard, J. C., Rybarczyk, H. and de Wolf, L. 1997. A comparative study on the relation between copper and condition in marine bivalves and the relation with copper in the sediment. *Aquatic Toxicology* 38(1-3): 165–181.
- Hurlbert, S. 1984. Pseudoreplication and the design of ecological field experiments. *Ecological Monographs* 54(2): 187–211.
- Hurlbert, S. 2004. On misinterpretations of pseudoreplication and related matters: a reply to Oksanen. *Oikos* 104(3): 591–596.
- Irato, P., Santovito, G., Cassini, A., Piccinni, E. and Albergoni, V. 2003. Metal accumulation and binding protein induction in *Mytilus galloprovincialis*, *Scapharca inaequivalvis*, and *Tapes philippinarum* from the Lagoon of Venice. *Archives of Environmental Contamination and Toxicology* 44: 476–484.
- Janssen, H. H. and Scholz, N. 1979. Uptake and cellular distribution of cadmium in *Mytilus edulis*. *Marine Biology* 55(2): 1432–1793.
- Joiris, C. R., Holsbeek, L. and Otchere, F. A. 2000. Mercury in the Bivalves *Crassostrea tulipa* and *Perna perna* from Ghana. *Marine Pollution Bulletin* 40(5): 457–460.
- Kagley, A. N., Snider, R. G., Krishnakumar, P. K. and Casillas, E. 2003. Assessment of seasonal variability of cytochemical responses to contaminant exposure in the blue mussel *Mytilus edulis* (Complex). *Archives of Environmental Contamination and Toxicology* 44: 43–52.

## References

- Kalicharran, S. and Diab, R. 1993. Proposals for rehabilitation and management of Isipingo lagoon and estuary, South Africa. *Environmental Management* 17(6): 759–764.
- Ke, C. and Wang, W-X. 2002. Trace metal ingestion and assimilation by the green mussel *Perna viridis* in a phytoplankton and sediment mixture. *Marine Biology* 140(2): 327–335.
- Kennish, M. J. 2002. Environmental threats and environmental future of estuaries. *Environmental Conservation* 29: 78–107.
- Khessiba, A., Hoarau, P., Gnassia-Barelli, M., Aissa, P. and Romeo, M. 2001. Biochemical response of the mussel *Mytilus galloprovincialis* from Bizerta (Tunisia) to chemical pollutant exposure. *Archives of Environmental Contamination and Toxicology* 40: 222–229.
- Kilburn, R. and Rippey, E. 1982. *Sea shells of Southern Africa*. Cadell, E. M. (ed). First edition, Pages 158–159. Macmillan South Africa, 1982.
- Kozlov, M. V. 2007. Improper sampling design and pseudoreplicate analysis: conclusions by Veličković (2004) questioned. *Hereditas* 144: 43–44.
- Lagadic, L., Caquet, T. and Ramade, F. 1994. The role of biomarkers in environmental assessment (5). Invertebrate populations and communities. *Ecotoxicology* 3: 193–208.
- Lam, P. K. S. and Rudolf, S. S. W. 2003. *Use of biomarkers in environmental monitoring*. STAP (Scientific and Technical Advisory Panel) Workshop on the use of bioindicators, biomarkers and analytical methods for the analysis of POPs in developing countries (December 2003). Centre for Coastal Pollution and Conversation, City University of Hong Kong.
- Lapointe, B. E and M. W. Clark. 1992. Nutrient inputs from the watershed and coastal eutrophication in the Florida Keys. *Estuaries* 15 (4): 465 – 476.
- Latouche, D. and Mix, M. C. 1981. Seasonal variation in soft tissue weights and trace metal burdens in the bay mussel, *Mytilus edulis*. *Bulletin of Environmental Contamination and Toxicology* 27(1): 821–828.

## References

- Lawrence, D. R. and Scott, G. I. 1982. The determination and use of condition index of oysters. *Estuaries* 5(1): 23–27.
- Lionetto, M. G., Caricato, R. Giordano, M. E., Pascariello, M. F., Marinosci, L. and Schettino, T. 2003. Integrated use of biomarkers (acetyl-cholinesterase and antioxidant enzymes activities) in *Mytilus galloprovincialis* and *Mullus barbatus* in an Italian coastal marine area. *Marine Pollution Bulletin* 46(3): 324–330.
- Lobel, P. B. 1986. Role of the kidney in determining the whole soft tissue zinc concentration of individual mussels (*Mytilus edulis*). *Marine Biology* 92(3): 335–3359.
- Lowe, D. M., Fossato, V. U. and Depledge, M. H. 1995. Contaminant-induced lysosomal membrane damage in blood cells of mussel *Mytilus galloprovincialis* from the Venice Lagoon: an *in vitro* study. *Marine Ecology Progress Series* 129: 189–196.
- Lowe, T. P. and Day, D. D. 2002. Metal concentrations in Zebra mussels and sediments from embayments and riverine environments of Eastern Lake Erie, South Lake Ontario, and the Niagara River. *Archives of Environmental Contamination and Toxicology* 43: 301–308.
- Lui, J. H. and Kueh, C. S. W. 2005. Biomonitoring of heavy metals and trace organics using the intertidal mussel *Perna viridis* in Hong Kong coastal waters. *Marine Pollution Bulletin* 51: 857–875.
- Luk'yanova, O. N. 2006. Molecular biomarkers of energy metabolism in mussels under anthropogenic pollution of Peter the Great Bay, the Sea of Japan. *Russian Journal of Ecology* 37(3): 205–209.
- Lundebye, A. K., Langston, W. J. and Depledge, M. H. 1997. Stress proteins and condition index as biomarkers of tributyltin exposure and effect in mussels. *Ecotoxicology* 6: 127–136.
- Lund-Thomsen, P. 2005. Corporate accountability in South Africa: the role of community mobilizing in environmental governance. *International Affairs* 81(3): 619–633.

## References

- Lushchak, V. I., Bahnjukova, T. V. and Spichenkov, A. V. 1997. Modification of pyruvate kinase and lactate dehydrogenase in foot muscle of the sea mussel *Mytilus galloprovincialis* under anaerobioses and recovery. *Brazilian Journal of Medical and Biological Research* 30(3): 381–385.
- Macleod, D. C. 1982. Sludge Disposal to Sea in South Africa. Sludge Disposal to Sea in South Africa. *Water Science Technology* 14(3): 93–107.
- Mardon, D. and Stretch, D. 2004. Comparative assessment of water quality at Durban beaches according to local and international guidelines. *Water SA* 30(3): 317–324.
- Marigomez, I., Kortabitarte, M. and Dussart, G. B. J. 1998. Tissue-level biomarkers in sentinel slugs as cost-effective tools to assess metal pollution in soils. *Archives of Environmental Contamination and Toxicology* 34: 167–176.
- Marigomez, I., Soto, M., Cajaraville, M. P., Angulo, E. and Giamberini, L. 2002. Cellular and subcellular distribution of metals in molluscs. *Microscopy Research and Technique* 56: 358–392.
- Marshall, D. J., Peter, R. and Chown, S. L. 2004. Regulated bradycardia in the pulmonate limpet *Siphonaria* (Gastropoda: Mollusca) during pollutant exposure: implication for biomarker studies. *Comparative Biochemistry and Physiology* 139(A): 309–316.
- Marshall, D. J. and Rajkumar, A. 2003. Imposex in the indigenous *Nassarius kraussianus* (Mollusca: Neogastropoda) from South African harbours. *Marine Pollution Bulletin* 46(9): 1150–1155.
- Matooane, L. and Diab, R. D. 2001. Air pollution carrying capacity in the South Durban industrial basin. *South African Journal of Science* 97: 450–453.
- Matozzo, V., Ballarin, L., Pampanin, D. M. and Marin, M. G. 2001. Effects of copper and cadmium exposure on functional responses of haemocytes in the clam, *Tapes philippinarum*. *Archives of Environmental Contamination and Toxicology* 41: 163–170.

## References

- Mauri, M. and Baraldi, E. 2003. Heavy metal bioaccumulation in *Mytilus galloprovincialis*: a transplantation experiment in Venice lagoon. *Chemistry and Ecology* 19(2-3): 79–90.
- McClurg, T. P., Parsons, G. A., Pillay, S. and Newman, B. K. 2006. *Sea disposal of sewage: environmental surveys in the Durban outfalls region – surveys made in 2006*. CSIR Report No. CSIR/NRE/PW/2006.
- McGeer, J. C., Brix, K. V., Skeaff, J. M., Deforest, D. K., Brigham, S. I., Adams, W. J. and Green, A. Inverse relationship between bioconcentration factor and exposure concentration for metals: implications for hazard assessment of metals in the aquatic environment. *Environmental Toxicology and Chemistry* 22(5): 1017–1037.
- McGregor, P. K. 2000. Playback experiments: design and analysis. *Acta Ethologica* 3:3–8.
- Millar, R. B. and Anderson, M. J. 2004. Remedied for pseudoreplication. *Fisheries Research* 70: 397–407.
- Misheer, N., Kindness, A. and Jonnalagadda, S. B. 2006a. Elemental uptake by seaweed, *Plocamium corallorhiza* along the KZN coastline, South Africa. *Journal of Environmental Science and Health* 41(A): 1037–1048.
- Misheer, N., Kindness, A. and Jonnalagadda, S. B. 2006b. Seaweeds along KZN coast of South Africa-4: Element uptake by edible seaweed *Caulerpa racemosa* (sea grapes) and the arsenic speciation. *Journal of Environmental Science and Health* 41(A): 1217–1233.
- Misheer, N., Kindness, A. and Jonnalagadda, S. B. 2006c. Elemental distribution in seaweed, *Gelidium abbottiorum* along the KZN coastline, South Africa. *Journal of Environmental Science and Health* 41(A): 1639–1653.
- Mizrahi, L. and Aчитuv, Y. 1989. Effect of heavy metals ions on enzyme activity in the mediterranean mussel, *Donax trunculus*. *Bulletin for Environmental Contamination and Toxicology* 42: 854–859.

- Moldan, A. G. S. 1995. Marine pollution. In: *Oceans of life of Southern Africa*. Payne, A. I. L. and Crawford, R. J. M. (eds). Second edition. Pages 41–49. Vlaeberg Publishers, South Africa, 1995.
- Moore, M. N. 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel, *Mytilus edulis*, and changes induced by thermal stress. *Cell and Tissue Research* 175: 297–287.
- Moore, M. N., Allan, J. I. and McVeigh, A. 2006. Environmental prognostics: an integrated model supporting lysosomal stress response as predicted biomarkers of animal health status. *Marine Environmental Research* 61: 278–304.
- Moore, M. N., Koehn, R. K. and Bayne, B. L. 1980. Leucine aminopeptidase (aminopeptidase-l), n-acetyl- $\beta$ -hexosaminidase and lysosomes in the mussel, *Mytilus edulis* L., in response to salinity changes. *Journal of Experimental Zoology* 214: 239–249.
- Moore, S. W. 1999. The subterranean estuary: a reaction zone of ground water and sea water. *Marine Chemistry* 65(1-2): 111–125.
- Mundry, R. and Sommer, C. 2007. Discriminate function analysis with non-independent data: consequences and an alternative. *Animal Behaviour* 74: 965–976.
- Musset, L. 2006. *Current approaches in the statistical analysis of ecotoxicology data: guidance to application*. OECD Environment Health and Safety Publications: Series on Testing and Assessment No. 54. Report no. ENV/JM/MONO(2006)18.
- Muyssen, B. T. A., Brix, K. V., DeForest, D. K. and Janssen, C. R. 2004. Nickel essentiality and homeostasis in aquatic organisms. *Environmental Reviews* 12(2): 113–131.
- Narbonne, J. F., Clerandau, A. C., Daubeze, M., Narbonne, J., Champeau, O. and Garrigues, P. 2005. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in Mediterranean coasts and temporal trends. *Biomarkers* 10(1): 58–71.

## References

- Narbonne, J. F., Daube, M., Clerandau, C. and Garrigues, P. 1999. Scale of classification based on biochemical markers in mussels: application to pollution monitoring in European coasts. *Biomarkers* 4(6): 415–424.
- Nasci, C., Da Ros, L., Campesan, G., Van Vleet, E. S., Salizzato, M., Sperti, L. and Pavoni, B. 1999. Clam transplantation and stress-related biomarkers as useful tools for assessing water quality in coastal environments. *Marine Pollution Bulletin* 39: 255–260.
- Neufeld, D. S. and Wright, S. H. 1996. Response of cell volume in *Mytilus* gill to acute salinity change. *The Journal of Experimental Biology* 199: 473–484.
- Newell, R. C., Maughan, D. W., Seiderer, L. J., Trett, M. W. and Nicholson, C. G. 1991. Assessing the impact of liquid wastes discharge in the sea. A case study off Amanzimtoti, near Durban, Natal, South Africa. *South African Journal of Marine Science* 10: 383–396.
- Nicholson, S. and Lam, P. K. S. 2005. Pollution monitoring in Southeast Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). *Environmental International* 31(1): 121–132.
- Nicholson, S. 1999a. Cardiac and lysosomal responses to periodic copper in the mussel *Perna viridis* (Bivalvia: Mytilidae). *Marine Pollution Bulletin* 38(12): 1157–1162.
- Nicholson, S. 1999b. Cytological and physiological biomarker responses from green mussels, *Perna viridis* (L.) transplanted to contaminated sites in Hong Kong coastal waters. *Marine Pollution Bulletin* 39(1-12): 261–268.
- Nicholson, S. 2001. Ecocytological and toxicological responses to copper in *Perna viridis* (L.) (Bivalvia: Mytilidae) haemocyte lysosomal membranes. *Chemosphere* 45: 399–407.
- Nicholson, S. 2002. Ecophysiological aspects of cardiac activity in the subtropical mussel *Perna viridis* (L.) (Bivalvia: Mytilidae). *Journal of Experimental Marine Biology and Ecology* 267: 207–222.

- Nicholson, S. 2003. Lysosomal membrane stability, phagocytosis and tolerance to emersion in the mussel *Perna viridis* (L.) (Bivalvia: Mytilidae) following exposure to acute, sublethal, copper. *Chemosphere* 52: 1147–1151.
- Nigro, M., Falleni, A., Del Barga, I., Scarcelli, V., Lucchesi, P., Regoli, F. and Frenzilli, G. 2006. Cellular biomarkers for monitoring estuarine environments: transplanted versus native mussels. *Aquatic Toxicology* 77: 339–347.
- Nurick, R. and Johnson, V. 1998. Towards community based indicators for monitoring quality of life and the impact of industry in south Durban. *Environment and Urbanization* 10(1): 233–250.
- O' Donoghue, S. and Marshall, D. J. 2003. Marine pollution research in South Africa: a status report. *South African Journal of Science* 99: 349–356.
- Oelofse, S. H., Viljoen, P., Taljaard, S. and Botes, W. A. M. 2004. Discharge of water containing waste emanating from land to the marine environment: A water quality management perspective. *Water SA* 30(5): 56–60.
- Oksanen, L. 2001. Logic of experiments in ecology: is pseudoreplication a pseudoissue? *Oikos* 94: 27–38.
- Orban, E., Di Lena, G., Nevigato, T., Casini, I., Marzetti, A. and Caproni, R. 2002. Seasonal changes in meat content, condition index and chemical composition of mussels (*Mytilus galloprovincialis*) cultured in two different Italian sites. *Food Chemistry* 77(1): 57–65.
- Orbea, A., Marigómez, I. Fernández, C., Tarazona, J. V., Cancio, I and Cajarville, M. P. 1999. Structure of peroxisomes and activity of the marker enzyme catalase in digestive epithelial cells in relation to PAH content of mussels from two Basque estuaries (Bay of Biscay): seasonal and site-specific variations. *Archives of Environmental Contamination and Toxicology* 36: 158–166.
- Otchere, F. A. 2005. Organochlorines (PCBs and pesticides) in the bivalves *Anadara Senilis senilis*, *Crassostrea tulipa* and *Perna perna* from the lagoons of Ghana. *Science of the Total Environment* 348(1-3): 102–114.



## References

- Park, S.-J., Cotter, P. A. and Gunsalus, R. P. 1995. Regulation of malate dehydrogenase (mdh) gene expression in *Escherichia coli* in response to oxygen, carbon, and heme availability. *Journal of Bacteriology* 177(22): 6652–6656.
- Parlak, H., Katalay, S. and Buyukisik, B. 2004. Accumulation and Loss of Chromium by Mussels (*M. galloprovincialis*). *Bulletin of Environmental Contamination and Toxicology* 62(3): 286–292.
- Parsons, G. 2002. *Sediment assessment programme for the Port of Durban, Trace metal survey*. CSIR Report No. CSIR/NRE/PW/2002.
- Pellerin-Massicot, J. and Pelletier, E. 1987. Evaluation of sublethal effects of pollutants with biochemical indicators. *Oceans* 19: 1550–1554.
- Pempkowiak, J., Sikora, A. and Biernacka, E. 1999. Speciation of heavy metals in marine sediments vs their bioaccumulation by mussels. *Chemosphere* 39(2): 313–321.
- Pereira, C. D. S., Abessa, D. M. D. S., Bainy, A. C. D., Zarni, L. P., Gasparro, M. R., Bicego, M. C., Taniguchi, S., Furley, T. H. and De Sousa, E. C. P. M. 2007. Integrated assessment of multilevel biomarker responses and chemical analysis in mussels from São Sebastião, São Paulo, Brazil. *Environmental Toxicology and Chemistry* 26(3): 462–469.
- Petrović, S., Semenčić, L., Ozretić, B. and Ozretić, M. 2004. Seasonal variations of physiological and cellular biomarkers and their use in the biomonitoring of north adriatic coastal waters (Croatia). *Marine Pollution Bulletin* 49(9-10): 713–720.
- Phillips, D. J. H. 1976a. The common mussel *Mytilus edulis* as an indicator of pollution by zinc, cadmium, lead and copper. I. Effects of environmental variables on uptake of metals. *Marine Biology* 38(1): 59–69.
- Phillips, D. J. H. 1976b. The common mussel *Mytilus edulis* as an indicator of pollution by zinc, cadmium, lead and copper. II. Relationship of metals in the mussel to those discharged by industry. *Marine Biology* 38(1): 71–80.

## References

- Phillips, D. J. H. 1977. Effects of salinity on the net uptake of zinc by the common mussel *Mytilus edulis*. *Marine Biology* 41(1): 79–88.
- Phillips, D. J. H. 1985. Organochlorines and trace metals in green-lipped mussels *Perna viridis* from Hong Kong waters: a test of indicator ability. *Marine Ecology Progress Series* 21: 251–258.
- Pickens, P. E. 1965. Heart rate of mussels as a function of latitude, intertidal height, and acclimation temperature. *Physiology and Zoology* 38: 390–405.
- Pillay, S. 2003. Water quality monitoring programme for the Port of Durban 2002–2003. CSIR Report No. ENV-2003.
- Pillay, S. 2006a. Tongati estuary intermediate reserve determination: water and sediment quality report 2006. CSIR Report No. D/MER/Tongati Mdolti/Tongati 2006.
- Pillay, S. 2006b. Mdloti estuary intermediate reserve determination: water and sediment quality report 2006. CSIR Report No. D/MER/Tongati Mdolti/Mdolti 2006.
- Pillay, S. 2007. Water quality monitoring programme for the Port of Durban. CSIR Report No. CSIR/NRE/PW/ER/2007.
- Rainbow, P. S. 1995. Biomonitoring of heavy metal availability in the marine environment. *Marine Pollution Bulletin* 31(4-12): 183–192.
- Rainbow, P. S. 2002. Trace metal concentrations in aquatic invertebrates: why and so what? *Environmental Pollution* 120: 497–507.
- Rainbow, P. S., Wolowicz, M., Fialkowski, W., Smith, B. D. and Sokolowski, A. 2000. Biomonitoring of trace metals in the gulf of Gdansk, using mussels (*Mytilus trossulus*) and barnacles (*Balanus improvisus*). *Water Research* 34(6): 1823–1829.
- Rajagopal, S., Venugopalan, V. P., Van de Velde, G. and Jenner, H. A. 2003. Responses of fouling brown mussel, *Perna perna* (L.), to chlorine. *Archives of Environmental Contamination and Toxicology* 44: 369–376.

- Ravera, O. 2001. Monitoring of the aquatic environment by species accumulator of pollutants: a review. *Journal of Limnology* 60(1): 63–78.
- Regoli, F. and Orlando, E. 1994. Seasonal variation of trace metal concentrations in the digestive gland of the mediterranean mussel *Mytilus galloprovincialis*: comparison between a polluted and a non-polluted site. *Archives of Environmental Contamination and Toxicology* 27: 36–43.
- Regoli, F. 1998. Trace metals and antioxidants enzymes in gills and digestive gland of the mediterranean mussel *Mytilus galloprovincialis*. *Archives of Environmental Contamination and Toxicology* 34: 48–63.
- Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D. and Nigro, M. 2004. Time-coarse variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquatic Toxicology* 68: 167–178.
- Regoli, F., Hummel, H., Amiard-Triquet, C., Larroux, C. and Sukhotin, A. 1998. Trace metals and variations of antitoxidant enzymes in artie bivalve populations. *Archives of Environmental Contamination and Toxicology* 35: 594–601.
- Reinfelder, J. R., Wang, W-X., Luoma, S. N. and Fisher, N. S. 1997. Assimilation efficiencies and turnover rates of trace elements in marine bivalves: a comparison of oysters, clams and mussels. *Marine Biology* 129: 443–452.
- Resgalla, C., de Souza Brazil, E. and Salomao, L. C. 2007. The effect of temperature and salinity on the physiological rates of the mussel *Perna perna* (Linnaeus 1758). *Brazilian Archives of Biology and Technology* 50(3): 543–556.
- Ringwood, A. H., Connors, D. E. and Hoguet, J. 1998. Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassostrea virginica*. *Marine Ecology Progress Series* 166: 163–171.

## References

- Ringwood, A. H., Connors, D. E. and Keppler, C. J. 1999. Cellular responses of oysters, *Crassostrea virginica*, to metal-contaminated sediments. *Marine Environmental Research* 48(4-5): 427–437.
- Rittschof, D. and McCellan-Green, P. 2005. Molluscs as multi-disciplinary models in environment toxicology. *Marine Pollution Bulletin* 50(4): 369–375.
- Riveros, A., Zúñiga, M., Hernandez, A. and Camano, A. 2002. Cellular biomarkers in native and transplanted populations of the mussel *Perumytilus purpuratus* in the intertidal zones of San Jorge Bay, Antofagasta, Chile. *Archives of Environmental Contamination and Toxicology* 42: 303–312.
- Roditi, H. A. and Fisher, N. S. 1999. Rates and routes of trace element uptake in Zebra mussels. *Limnology and Oceanography* 44(7): 1730–1749.
- Roesijadi, G., Young, J. S., Drum, A. S. and Gurtisen, J. M. 1984. Behavior of trace metals in *Mytilus edulis* during a reciprocal transplant field experiment. *Marine Ecology Progress Series* 18: 155–170.
- Sabourin, T. D. and Tullis, R. E. 1981. Effect of three hydrocarbons on respiration and heart rates of the mussel, *Mytilus californianus*. *Bulletin for Environmental Contamination and Toxicology* 26: 729–736.
- Salazar, M. H. 1999. Caged mussel monitoring pilot study - ballast water treatment facility. *Applied Biomonitoring*. WA 98034. (425) 823–3905.
- Salazar, M. H. and Salazar, S. M. 1995. In situ bioassays using transplanted mussels: I. Estimating chemical exposure and bioeffects with bioaccumulation and growth. *In: Environmental Toxicology and Risk Assessment*. Hughes, J. S., Biddinger, G. R. and Mones, E. (eds.) ASTM STP 1218, American Society for Testing and Materials. Third Volume, Pages 216–241. Philadelphia, 1995.
- Salazar, M. H. and Salazar, S. M. 2000. Standard guide for conducting field bioassays with marine, estuarine & freshwater bivalves. *Applied Biomonitoring*. Kirkland, WA 98034. (425) 823–3905.

- Salazar, M. H. 1997. Critical evaluation of bivalve molluscs as a biomonitoring tool for the mining industry in Canada. *In: Technical evaluation of molluscs as a biomonitoring tool for the CMI CANMET-Aquatic Effects Technology Evaluation (AETE) Programme*. Stewart and Malley, Project 2.3.1. Part 2. Pages 164–248. 1997.
- Schachter, O. and Serwer, D. 1971. Marine pollution problems and remedies. *The American Journal of International Law* 65(1): 84–111.
- Schleyer, M. H., Heikoop, J. M. and Risk, M. J. 2006. A benthic survey of Aliwal Shoal and assessment of the effects of a wood pulp effluent on the reef. *Marine Pollution Bulletin* 52: 503–514.
- Scholz, N. 1980. Accumulation, loss and molecular distribution of cadmium in *Mytilus edulis*. *Helgoland Marine Research* 33(1-4): 68–78.
- Shugart, L. E., McCarthy, J. F. and Halbrook, R. S. 1992. Biological markers of environmental and ecological contamination: An overview. *Risk Analysis* 12(3): 353–360.
- Sidoumou, Z., Gnassia-Barelli, M., Siau, Y., Morton, V. and Romeo, M. 2006. Heavy metal concentrations in molluscs from the Senegal coast. *Environment International* 32(3): 384–387.
- Siko, M. 1996. South Africa's Maritime Interest and Responsibilities. *In: African Security Review* Vol. 5 No. 2. 1996.
- Smith, J. R. 1985. Copper exposure and ciliary function in gill tissue of *Mytilus californianus*. *Bulletin of Environmental Contamination and Toxicology* 35: 556–563.
- Smolders, R., Bervoets, L., Wepener, V. and Blust, R. 2003. A conceptual framework for using mussels as biomonitors in whole effluent toxicity. *Human and Ecological Risk Assessment* 9(3): 741–760.
- Sokolowski, A., Bawazir, A. S. and Wolowicz, M. 2004. Trace metals in the brown mussel *Perna perna* from the coastal waters off Yemen (Gulf of Aden): how concentrations are

- affected by weight, sex, and seasonal cycle. *Archives of Environmental Contamination and Toxicology* 46: 67–80.
- Soto, M., Kortabitarte, M. and Marigómez, I. 1995. Bioavailable heavy metals in estuarine waters as assessed by metal/shell-weight indices in sentinel mussels *Mytilus galloprovincialis*. *Marine Ecology Progress* 125: 127–136.
- Stewart-Oaten, A., Murdoch, W. W. and Parker, K. R. 1986. Environmental Impact Assessment: "Pseudoreplication" in Time? *Ecology* 67(4): 929–940.
- Stickle, W. B and Sabourin, T. D. 1979. Effects of salinity on the respiration and heart rate of the common mussel, *Mytilus edulis* L., and the Black chiton, *Katherina tunicata* (Wood). *Journal of Experimental Marine Biology and Ecology* 41: 257–268.
- Stucchi-Zucchi, A. and Salomao, L. C. 1998. The ionic basis of membrane potentials and adaptation to hyposmotic stress in *Perna perna*, an osmoconforming mollusc. *Comparative Biochemistry and Physiology* 121(A): 143–148.
- Sumich, J. L. 1992. *An introduction to the biology of marine life*. Kane, K. (eds). Fifth edition. Pages 395 – 407. Wm. C. Brown Publishers, USA. 1992.
- Sze, P. W. C. and Lee, S. Y. 2000. Effects of chronic copper exposure on the green mussel *Perna viridis*. *Marine Biology* 137: 379–392.
- Szefer, P. Fowler, S. W., Ikuta, K. I., Osuna, F. P., Ali, A. A., Kim, B. S., Fernandez, H. M., Belzunce, M. J., Guterstam, B., Kunzendorf, H., Wolowicz, M., Hummel, H. and Deslous-Paoli, M. 2006. A comparative assessment of heavy metal accumulation in soft parts and byssus of mussels from sub arctic, temperate, subtropical and tropical marine environments. *Environmental Pollution* 139: 70–78.
- Szefer, P., Geldon, J., Ali, A. A., Bawazir, A. and Sad, M. 1997a. Distribution and association of trace metals in soft tissue and byssus of mollusc *Perna perna* from the gulf of Aden, Yemen. *Environment International* 23: 53–61.

- Szefer, P., Ikuta, K., Kushiya, S., Szefer, K., Frelek, K. and Geldon, J. 1997b. Distribution and association of trace metals in soft tissue and byssus of *Mytilus edulis* from the east coast of Kyushu Island, Japan. *Archives of Environmental Contamination and Toxicology* 32: 184–190.
- Taljaard, S. 2006. *Baseline assessment of sources and management of land-based marine pollution in the Benguela Current Large Marine Ecosystems (BCLME) region*. CSIR unpublished report: BCLME Project BEHP/LBMP/03/01.
- Taljaard, S., Botes, W. A. M., Oelofse, S. H. H. and Viljoen, P. 2006. Operational policy for disposal of land-derived wastewater to the marine environment of South Africa. *Water SA* 32(4): 527–533.
- Thomas, C. M., Perissinotto, R. and Kibirige, I. 2005. Phytoplankton biomass and size structure in two South African eutrophic, temporarily open/closed estuaries. *Estuarine, Coastal and Shelf Science* 65: 223–238.
- Tsangaris, C.; Moate, R.; Nikolaidou, A. and Papathanassiou, E. 1997. *Heavy metals in subcellular organelles in the kidney and digestive gland of mussels Mytilus galloprovincialis from Larymna Bay, northern Evoikos*. 5th Hellenic Symposium on Oceanography and Fisheries. Kavala, Greece, April 15–18, 1997. Volume 2. Fisheries, Aquaculture, Inland Waters.
- Turner, R. K., Subak, S and Adger, W. N. 1996. Pressures, trends, and impacts in coastal zones: interactions between socioeconomics and natural systems. *Environmental Management* 20(2): 159–173.
- Underwood, A. J. 1998. *Experiments in ecology: their logical design and interpretation using analysis of variance*. Cambridge University Press, UK. 1998.
- Van Alstine, J. 2007. *Institutions for the environmental governance of multinational corporations: the SAPREF oil refinery in Durban, South Africa*. Paper presented on Earth System Governance: Theories and Strategies for Sustainability at the Amsterdam Conference on the Human Dimensions of Global Environmental Change, 24–26 May 2007.

## References

- Department of Geography and Environment - London School of Economics and Political Science.
- Van Erkom Schurink, C. and Griffiths, C. L. 1991. A comparison of reproductive cycles and reproductive output in four southern African mussel species. *Marine Ecology Progress Series* 76: 123–134.
- Van Hattum, B., Van Straalen, N. M. and Govers, H. A. J. 1996. Trace metals in populations of freshwater isopods: influence of biotic and abiotic variables. *Archives of Environmental Contamination and Toxicology* 31: 303–318.
- Vandermeulen, J. H. 1996. Environmental trends of ports and harbours: implications for planning and management. *Maritime Policy and Management* 23(1): 55–66.
- Velickovic, M. V. 2007. Sampling designs, pseudoreplication and a good practice in modern science: a response to Mikhail V. Kozlov desultoriness, and recommendations to environmental scientists. *Hereditas* 144: 45–47.
- Vermeulen, L. and Wepener, V. 1999. Spatial and temporal variations of metals in Richards Bay Harbour (RBH), South Africa. *Marine Pollution Bulletin* 39: 304–307.
- Viarengo, A., Marro, A., Marchi, B. and Burlando, B. 2000. Single and combined effects of heavy metals and hormones on lysosomes of haemolymph cells from the mussel *Mytilus galloprovincialis*. *Marine Biology* 137: 907–912.
- Walsh, K. M. and O'Halloran, J. 1998. Accumulation of chromium by a population of mussels (*Mytilus edulis*) exposed to leather tannery effluent. *Environmental Toxicology and Chemistry* 17: 1429–1438.
- Wang, T., Li, X., Bott, K., Song, L., Clarke, S and Zhao, W. 2006. Effects of water temperature on the lysosomal membrane stability in haemocytes of blacklip abalone, *Haliotis rubra* (Leach). *Journal of Shellfish Research* 25(3): 935–940.
- Wang, W-X. and Dei, R. C. H. 1999. Factors affecting trace element uptake in the black mussel *Septifer virgatus*. *Marine Ecology Progress Series* 186: 161–172.



## References

- Wang, W-X. and Fisher, N. S. 1999. Delineating metal accumulation pathways for marine invertebrates. *The Science of the Total Environment* 237(238): 459–472.
- Wedderburn, J., McFadzen, I. Sanger, R. C., Bessley, A., Heath, C., Hornsby, M. and Lowe, D. 2000. The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. *Marine Pollution Bulletin* 40(3): 257–267.
- Weerts, S. P. and Cyrus, D. P. 2002. Occurrence of young and small-sized fishes in different habitats within a subtropical South African estuary and adjacent harbour. *Marine and Freshwater Research* 53: 447–456.
- Wells, P. G. 1999. Biomonitoring the health of coastal marine ecosystems – The roles and challengers of microscale toxicity tests. *Marine Pollution Bulletin* 39(1-12): 39–47.
- Wells, P.G., Depledge, M. H., Butler, J. N., Manock, J. J. and Knap, A. H. 2001. Rapid toxicity assessment and biomonitoring of marine contaminants – Exploiting the potential of rapid biomarker assays and microscale toxicity tests. *Marine Pollution Bulletin* 42(10): 799–804.
- Wepener, V. and Vermeulen, L. 2005. A note on the concentrations and bioavailability of selected metals in sediments of Richards Bay Harbour, South Africa. *Water SA* 31: 589–595.
- Whitefield, A. K. 1997. Fish conversation in South African estuaries. *Aquatic Conservation: Marine and freshwater ecosystems* 7: 1–11.
- Widdows, J. 1973. Effect of temperature and food uptake on the heart beat, ventilation rate and oxygen uptake of *Mytilus edulis*. *Marine Biology* 20: 269–276.
- Widdows, J. and Donkin, P. 1989. The application of combined tissue residue chemistry and physiological measurements of mussels (*Mytilus edulis*) for the assessment of environmental pollution. *Hydrobiologia* 188: 455–461.
- Wiens, J. A. and Parker, K. R. 1995. Analyzing the effects of accidental environmental impacts: approaches and assumptions. *Ecological Applications* 5(4): 1069–1083.

## References

- Yap, C. K., Ismail, A. Tan, S. G. and Omar, H. 2002. Correlations between speciation of Cd, Cu, Pb and Zn in sediment and their concentrations in total soft tissue of green-lipped mussel *Perna viridis* from the west coast of Peninsular Malaysia. *Environment International* 28: 117–126.
- Young, G. A. 1985. Byssus-thread formation by the mussel *Mytilus edulis*: Effects of environmental factors. *Marine Ecology Progress Series* 24(3): 261–271.
- Young, T-H. and Somero, G. N. 1993. Effects of feeding and food deprivation on oxygen consumption, muscle protein concentration and activities of energy metabolism enzymes in muscle and brain of shallow-living (*Scorpaena guttata*) and deep-living (*Sebastolobus alascanu*) scorpaenid fishes. *Journal of Experimental Biology* 181: 213–232.
- Zaroogian, G. E. and Johnson, M. 1984. Nickel uptake and loss in the bivalves *Crassostrea virginica* and *Mytilus edulis*. *Archives of Environmental Contamination and Toxicology* 13(4): 411–418.
- Zhang, Z., Li, X., Vandeppeer, M. and Zhao, W. 2006. Effects of water temperature and air exposure on the lysosomal membrane stability of hemocytes in pacific oysters, *Crassostrea gigas* (Thunberg). *Aquaculture* 256: 502–509.