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Accumulation and toxicity of metals in oysters (*Striostrea margaritacea)* from the South African South Coast

by

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DECLARATION

I, Michelle Yvonne Slabber, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

The current status of metal pollution off the South African South coast is not well known. This study was the first to be undertaken in many years using *Striostrea margaritacea* as a subject species. The aims of the study were to determine the degree of metal contamination in the water, sediments, oyster tissues and oyster shells at sites selected in Witsand, Wilderness and Goukamma, as well as to establish if *Striostrea margaritacea* qualifies as a successful biomonitor when using lysosomal destabilization as a tool. Seasonal variations between sites were also considered. Other objectives, such as the potential of a Marine Protected Area (MPA) as a control site and the necessity of a monitoring program along the south coast were also included.

Sites were sampled seasonally for one year at spring low tides. Ten oysters were collected from each individual site upon each visit. The Neutral Red Retention Time (NRRT) assay was used to determine lysosomal membrane integrity of oyster haemocytes, whereafter oysters were sacrificed for metal analyses. Metals that were analysed are aluminium (Al), copper (Cu), zinc (Zn) and iron (Fe). Metal analyses were done using an Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP- AES). All statistical analyses were performed using ANOVA on Ranks to determine if there were significant differences between sites and between sampling occasions.

Aluminium concentrations found in the water column at all sites were considered as low. Iron, zinc and copper concentrations within the water column can on the other hand be considered as high when comparisons are drawn with other studies and data sets. Sediment concentrations for all the metals within the present study were considered to be low when compared to other studies and guidelines. There were not many significant differences recorded between sites and no seasonal patterns were present.

Within the tissues of the oysters, the metal ranges are considered to be low when compared to other studies. No definite conclusion about the contamination status of the oyster tissue could be drawn due to the lack of comparative literature. A field study in conjunction with a laboratory experiment should yield more reliable

results. There were also no seasonal trends present and very few differences between sites. The bioaccumulation factors were considered as being low with a few exceptions where they were moderate when oyster tissue data was compared to water and sediment data.

Concentrations for Al, Zn and Cu in the shells could be considered low when comparisons are drawn, with the exception of Fe that was found to be high. The bioaccumulation factors were considered to be low when oyster shell concentrations were compared to water and sediment data. There were also no seasonal trends present and a prolonged sampling period is suggested to further investigate these findings.

When a comparison was drawn between the tissue and shell data a clear pattern was evident. Al and Fe concentrations were highest within the shell where as Zn and Cu concentrations were highest within the tissues of the animal. The theory of mineralization is supported by these findings where bivalves will use their shell as a reservoir for micronutrients and other substances.

The NRRT assay revealed that lysosomal membrane destabilization had occurred and that the animals appeared to be stressed for the duration of the sampling period. Site 3, within the MPA, had the longest retention time. The retention times that were recorded were short when compared to other studies. This assay did however show potential as a basic monitoring tool from which more thorough investigations can be initiated.

In conclusion, the study sites along the south coast of South Africa does not seem to be contaminated by Al, Zn, Cu or Fe when data is compared to international and local water quality guidelines, sediment quality guidelines and other studies. Also, as concentrations between sites did not differ greatly, it is inconclusive whether or not the MPA can be used as a reliable references site for *in situ* studies. More vigorous and lengthy studies should be undertaken to contribute to current knowledge of our indigenous species, *Striostrea margaritacea* and to aid in the development of better management of this resource as well as an ongoing monitoring programme.

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- Figure 2.1.2 – produced by Tony van der Buis (2009)

“Look deep into nature and then you will understand everything better” - **Albert Einstein**

DEDICATION

This thesis is dedicated to:

Van Alphen Bence Slabber

(01/11/1945 – 28/08/2012)

There are no words for what you contributed to my life.
Although you did not see my thesis come to fruition, know that you were by my side every step of the way, now and always

Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning. – *Albert Einstein*

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GLOSSARY

Terms/Acronyms/Abbreviations	Definition/Explanation
MPA	Marine Protected Area
Al	Aluminium
Cu	Copper
Zn	Zinc
Fe	Iron
Km	Kilometer (Measurement of distance)
SD	Standard Deviation
NRRT	Neutral Red Retention Time
BCF	Bio–concentration factor
Lysosomes	Membrane-bound spherical organelles that contain enzymes called acid hydrolases, which are capable of digesting organic molecules, such as fats, proteins, and polysaccharides, under acidic conditions (Miller & Harley,1992).
Biomarker	Consists of biochemical and/or physiological changes in organisms exposed to contaminants, and thus represent initial responses to environmental perturbations and contamination (Roy *et al*., 1996; Damiens *et al*., 2004).
Biomonitor	Defined as species which accumulate trace contaminants in their tissues, revealing essentially that fraction in the environment which may be of direct ecotoxicological relevance, i.e. the bioavailable chemical forms (Blackmore *et al.*, 1998; Paez-Osuna *et al*., 2002).
Bioaccumulation	Bioaccumulation can be defined, in short, as the accumulation of a variety of compounds within living cell tissues (Amaral *et al*., 2005).

CHAPTER 1

Introduction and Literature Review

1.1 Introduction

The causes, the monitoring and the control of marine pollution, particularly hydrocarbon and metal pollution, have become an important research topic in recent years. It has long been recognized that the continuing and increasing release of urban wastes into the marine environment affects the composition of waters and sediments and may also have an effect on the flora and fauna. This could prove toxic not only to marine life but can also have an adverse effect on the consumption of oceanic resources by humans (Watling & Watling, 1976). Darracott (1977) discussed the potential for biological monitors in the South African marine environment on the basis of the reported use of related species. The results of laboratory experiments on the accumulation of various elements have led to the belief that the Cape oyster, *Striostrea margaritacea* is a suitable bioindicator organism (Watling & Watling, 1982).

The current status of the metal pollution off the South African South coast is not well known. There is also no information available about the current degree of metal bioaccumulation by feral oysters along the coast and the degree of toxicity of these metals to the oysters. The toxicity of these metals to feral oysters could be measured in terms of biomarkers (Hauton *et al.,* 1998; Ringwood *et al.,* 1998; 2002). Lysosomal destabilization is one of the most extensively used biomarkers within the marine mollusc group and has proven to be highly successful as a biomarker for pollutant exposures, especially in mussels (Wedderburn *et al.,* 2000), and potentially in oysters too (Hauton *et al.,* 1998; Ringwood *et al.,* 2002).

Striostrea margaritacea is a hardy, fast-growing oyster, which is harvested along the South coast of South Africa (Watling, 1983). Currently, all oyster mariculture facilities in South Africa are compelled to screen oysters for metal contamination. Oysters that

are taken from the wild are not screened (G. Maharaj[1], *pers comm*. 2008) and could be toxic to humans if consumed. The results of the current study may be used to instate an ongoing monitoring program for the species, *Striostrea margaritacea*. It is important to have an effective monitoring system whereby any development in estuarine areas can be assessed for its degree of pollution.

Three study sites were selected along the South African South coast for the purpose and duration of the study. Oysters were harvested from the different sites seasonally. Haemocytes were extracted from the various oysters and tested with the neutral red retention time assay (NRRT) to reveal the degree of lysosomal destabilization. Furthermore, oysters were then analyzed for their aluminium, iron, zinc and copper concentrations. The metals that were used and tested in the study were identified and chosen through a primary screening. Ambient data such as the temperature, pH and salinity of the water and metal concentrations in the sediment and water were included to assist in the interpretation of the data and to contribute to the overall assessment of the health of the ecosystem in question.

[1] Mrs Genevive Maharaj. Marine Scientist II, Department of Fisheries and Forestry (DAFF)

1.2 Literature review

1.2.1 Marine pollution

Marine pollution can be defined as the introduction by humans, directly or indirectly, of substances or energy into the marine environment that cause harm to living resources, are hazardous to human health, hinder marine activities, or impair the quality of sea-water or coastal amenities (Attwood, 2000). An estimated 80% of marine pollution is emitted from land-based sources. The oceans of our planet constitute the ultimate sink for many of the anthropogenically produced chemicals and compounds. Such chemicals include halogenated hydrocarbons (eg. dioxins, hexachlorobenzene), an array of pesticides (eg. DDT, dieldrin, mirex), a wide spectrum of polycyclic compounds (particularly polycyclic aromatic hydrocarbons [PAHs]), and a number of metals (eg. lead, mercury, aluminum, zinc, copper) (Pritchard, 1993).

Aside from run-off from cultivated lands containing phosphates and nitrates from fertilizers, which in large quantities can be responsible for algal blooms, and organic wastes, oil spills and plastics, a variety of other sources of pollution have been identified which have a much more subtle visible appearance in the marine environment (Matthews, 2000). Among these, metals would be an appropriate example of subtle pollutants.

Although ecotoxicology is considered a relatively young science in South Africa, metal contamination of the coastal environment continues to attract the attention of environmental researchers locally and internationally. The main reason for this is the increasing metal input to the coastal environment from both rivers and non-point sources, especially in developing countries (Shulkin *et al.,* 2003). It has long been recognized that the continuing and increasing suburbanization and release of urban waste into the marine environment affects the composition of waters and sediments and may also have an effect on the flora and fauna (Watling & Watling, 1976). Other potential sources of metal pollution, such as recreational boating, harbours, antifouling

agents and agriculture are also being recognized and could be contributing to pollution in the aquatic environment (Walker *et al.,* 2006).

The potential for increased stress in marine organisms due to anthropogenic pressures associated with increasing development in the coastal zone (contaminant input and habitat alterations) demands careful monitoring of biological resources and development strategies to minimize the impact (Ringwood *et al.,* 1998).

1.2.2 Accumulation of metals in water and sediments in the marine environment

It has been recognized that the increasing release of anthropogenic waste into the marine environment affects the composition of water and sediments. Dassenakis *et al.* (1995) suggested that up to 90% of the suspended matter of rivers, which are the main carriers of metals, settles in estuaries and the coastal zone. Thus, the analysis of water and sediments, to assess the degree of metal contamination in marine environments, has become widespread practice throughout the world (Dassenakis *et al.,* 1995; Shulkin *et al.,* 2003; Blackmore & Wang, 2004; De Mora *et al.,* 2004).

The concentrations of metals may also have an effect on the flora and fauna that could prove detrimental to marine life (Watling & Watling, 1976), as many sedentary marine species are filter–feeders, which implies that they obtain their food from filtering water, suspended sediment and food particles through their digestive tracts (Amiard *et al.,* 2007). Particle bound organic matter may be ingested by filter-feeders and any associated pollutants may be released in the gut, where after metals, initially bound to sediments/particles, may be incorporated into the organisms' through various uptake mechanisms by membranes (Amiard *et al.,* 1995*)*.

1.2.2.1 Water

It has been observed that the air-sea interface is a site at which pollutants may be concentrated (as much as 1000 times the concentration in the rest of the water column) (Pritchard, 1993). The sediment-water interface of a marine basin is an area where the greatest gradients in chemical and physical properties occur, and which can

in turn affect metal concentrations (Mdzeke, 2004). Also, in natural water systems, metals can be partitioned between different physical states such as free or complex, associated with colloids or with particles (Gue´guen *et al.,* 2004). Both the area of study (i.t.o depth) and the ionic state of metal particles should be considered when assessing water samples for possible metal contamination, as these factors have an effect on measurements.

Furthermore, metal analysis in natural waters requires the use of extremely careful procedures to avoid sample contamination (Santos-Echeandía *et al.,* 2009). It has been reported in the past that metal concentrations in water samples (especially samples from earlier years) were subject to a significant overestimation due to contamination (Kremling, 1983). As a result of the complexity of metal analysis, a limited number of complete and accurate metal contaminated water datasets are available, despite the well-known role of the coastal zone in the transfer of metals from land-based sources (e.g. rivers) to the oceans (Martin & Windom, 1991).

1.2.2.2 Sediment

Sediment contaminant analyses can document the presence of contaminants (Ringwood *et al.,* 1998). The determination of contaminant concentrations in sediments, which are likely to be detrimental to the surrounding biota (Ringwood *et al.,*1999 a) has grown significantly due to its useful application. Quantitatively, sediments represent the major compartment for metal storage in aquatic environments (Gagnon & Fisher, 1997). Besides the natural processes, additional influx of the solid wastes into the river sediments from the industrial activities and mining practices causes enrichment of the metal concentrations (Rath *et al.,* 2009). The amount of waste materials contributed to the aquatic system would vary depending upon the intensity of these activities. A number of studies regarding the development of sediment guidelines based on biological effects, and the general contribution of sediment data to metal contamination analysis have been published (Dassenakis *et al.,* 1995; Long *et al.,* 1995, 1998; Amiard *et al.,* 2007; Hannan *et al.,* 2009). There are no sediment guidelines for marine environments available that are focused on South Africa.

The value of water and sediment metal data is increased significantly by the simultaneous determination of metal concentrations in organisms (Luoma, 1989). By combining these data sets, the assessment of the bioavailability of metals is made possible and simultaneously delivers a broader understanding of the immediate environmental health status in terms of metal pollution (Amiard *et al.,* 2007).

1.2.3 Bioaccumulation and compartmentalization of metals in marine invertebrates

The evaluation of marine pollution levels is based not only on direct measurements of the abiotic/ambient components, but also on measurements of the abundance and bioaccumulation of metals in selected marine organisms (Catsiki & Florou, 2006).

Bioaccumulation can be defined, in short, as the accumulation of a variety of compounds within living tissues (Amaral *et al.,* 2005). The bioaccumulation of metals in the tissues and organs of marine bivalves has been broadly studied throughout the world. Bivalves such as oysters (Regoli & Orlando, 1994; Ke & Wang, 2001; Lincoln-Smith & Cooper, 2004; Amaral *et al.,* 2005; Damiens *et al.,* 2006; Richards & Chaloupka, 2009) and mussels (Anandraj *et al.,* 2002; Giarratano *et al.,* 2010) are some of the most commonly used organisms for this type of research. This area of study is deemed highly relevant to the field of ecotoxicology as it led to the adoption of the bioindicator concept for the environmental quality assessment (Catsiki & Florou, 2006).

The bio-concentration factor (BCF) is a tool used to reflect that some pollutants are assimilated by organisms to a greater extent than others (Walker *et al.,* 2006). It also gives an indication of the level of bioaccumulation. According to Dallinger (1993) and Walker *et al.* (2006) the BCF can be expressed as:

$$BCF = \frac{\text{Concentration of the chemical in the organism}}{\text{Concentration of the ambient environment}}$$

The bio-concentration factor and bioaccumulation often have a strong correlation (Walker *et al.,* 2006). As metals are not biodegradable and can not be broken down into less harmful components, the detoxification of metals by various organisms consist of hiding active metal ions within a protein such as metallothionein or depositing them in an insoluble from in intracellular granules for long–term storage or excretion in the faeces (Walker *et al.,* 2006). In a study conducted by Frias-Espericueta et al. (1999) it was concluded that different organs of the oyster *Crassostrea iridescens* accumulate/store different metals in various concentrations where some of the metals, such as zinc in the reproductive organs, are used in metabolic activities. Body concentrations are affected by seasonal and lifetime changes in body weight as the concentration of a trace metal in an organism will be affected by changes in body weight and in the proportions of various tissues, for example during growth, 'degrowth', starvation, accumulation/loss of gametes, food reserves (Rainbow *et al.,* 1990).

Furthermore, it has been established that metals such zinc and copper can substitute for the Ca^{2+} ion and so become incorporated in the calcium carbonate shell of bivalves (Tynan *et al.*, 2005), adopting the concept that the shells of molluscs can act as a deposition centre for excess metals. A study conducted by Al-Aasm et al. (1998) suggested that bivalve shells could be superior to soft tissues for monitoring metal pollution. A further study conducted by Pearce & Mann (2006) supports this theory by stating that the hard parts of aquatic organisms have the potential to record in their shells changes in the environmental conditions in which the organism lived.

Bioaccumulation by molluscs for various metals also vary seasonally in the tissues as well as the shells and can be attributed to factors such the quantity of metals in the water column (dissolved and/or particulate) and some biological parameters (e.g. reproductive cycle) (Frias-Espericueta *et al*.,1999). Seasonal data sets are not abundant/current within the literature for *Striostrea margaritacea* i.t.o seasonal trace metal concentrations within the shell.

1.2.4 Metal toxicity and the use of biomarkers in toxicity assessment

Although the total metal concentrations in sediments, water and organisms give a convenient measure of metal pollution and bioavailability, such measures do not necessarily predict the toxicity of these pollutants to aquatic organisms (Luoma, 1995; Amiard *et al.,* 2007). Metals can cause long-term effects on ecosystems even if their impact has no immediate visible influence in comparison to other pollutants. Effects on organisms can be traced to molecular and cellular responses, as pollution impact does not necessarily lead to observable effects on a population or ecosystem level (Catsiki & Florou, 2006).

The study of biological effects on organisms, caused by pollutants, is a fundamental approach to assessing the impact of anthropogenic disturbances and stresses (Dassenakis *et al.,* 1995; Regoli *et al.,* 1998). Despite the limitations (e.g. contamination of samples, lack of South African guidelines, and complexity of studies) there is a considerable body of evidence demonstrating that the exposure to chemicals (eg. metals, polyaromatic hydrocarbons and polychlorinated biphenyls), either singly or in combination, could be detrimental to animal health (Moore & Lowe, 1975; Lowe *et al.,* 1995; Shulkin *et al.,* 2003; de Mora *et al.,* 2004; Maduro *et al.,* 2006; Giarratano *et al.,* 2010).

Since the primary toxic effect of chemicals occurs at the cellular and biochemical level, studies have relied on the use of measures at these levels as a means of assessing whether or not exposure to pollutants had detrimental effects (Bayne *et al.,* 1988). In this respect, such alterations at the cellular level may represent early warning signals for rapid and sensitive detection of environmental disturbance (Moore, 1990), possibly associated with environmental pollutants. Even though investigations can be carried out at different levels of biological organization (from molecules and cells to communities and ecosystems), cellular and biochemical responses to environmental stress can often be detected before a more integrated toxicity becomes apparent in the physiology of the whole organism (Regoli *et al.,* 1998) or in the environment as a whole. From the view of the ecotoxicologist, predicting adverse effects is made even more difficult in that chemical pollutants seldom occur in isolation in the environment

but are present in a mixture or “cocktail” (Lowe *et al.,* 1995). Consequently, cause and effect relationships are usually extremely difficult to establish.

The tools and analytical capabilities for documenting the contaminants in marine habitats and organisms are well developed (Ringwood *et al.,* 1998). Cellular and molecular indices are prompt, sensitive, easily measurable and quantitatively common to different organisms (Etxeberria *et al.,* 1994). They also deliver the most potential for identifying individuals and/or populations that have been affected by contaminants and are experiencing chronic or periodical stress. According to Etxeberria *et al*. (1994), a stress response can be defined as a measurable alteration of a functional steady state, which is caused by an environmental factor and which renders the individual (or the population) more vulnerable to further environmental changes. This alteration from the normal state, if left unchecked, may progress to severe effects at the ecosystem level (Ringwood *et al.,* 1998). In order to measure the stress response, a variety of indices have been developed at different levels of biological organization.

An understanding of the mechanisms by which pollutants exert their toxicity is a prerequisite to establishing causality. It is then possible to predict how the presence of other chemicals/pollutants and changes in abiotic and biotic factors modify toxicity. One way to achieve this is through biomarkers that respond to chemical challenges and which are used to explore the mechanisms involved in contaminant-induced alterations in cell structure and function (Lowe *et al.,* 1995).

Biomarkers are generally accepted as useful tools in monitoring programs for assessment of the impact on marine organisms as a result of pollutants and anthropogenic activities (Regoli *et al.,* 1998). Depledge & Kure (1994) pointed out that whilst biomarkers indicative of exposure to pollutants are useful, those revealing adverse effects experienced by the organism are more ecologically relevant.

In contrast to the simple measurement of contaminants accumulating in body tissues, biomarkers can offer more complete and biologically more relevant information on the potential impacts of contaminants on the health of organisms (Van der Oost *et al.,*

1996). The use of biomarkers offers opportunities for a fast and sensitive detection of chemical stresses within organisms (Van Gestel & Van Brummelen, 1996).

1.2.5 Lysosomal membrane destabilization

A large body of literature exists on the use of different kinds of biomarkers in molluscs, particularly in mussels (Moore & Lowe, 1977; Lowe, 1988; Lowe & Clarke, 1989; Hole *et al.,* 1993; Wedderburn *et al.,* 2000). One such biomarker is lysosomal membrane destabilization, which has been used successfully as a biomarker of stress from pollutant exposure in marine mollusc species, such as mussels (Etxeberria *et al.,* 1994; Krishnakumar *et al.,* 1994; Grundy *et al.,* 1996; Pipe *et al.,* 1997; Viarengo *et al.,* 2000; Da Ros *et al.,* 2002; Mamaca *et al.,* 2005), oysters (Hauton *et al.,* 1998; Ringwood *et al.,* 1998; 2002; Zhang & Li, 2006; Zhang *et al.,* 2006); periwinkles (Vega *et al.,* 1989) and scallops (Regoli *et al.,* 1998).

Lysosomes have the ability to concentrate a wide range of environmental contaminants, including metals, thereby representing an important cellular compartment for metal detoxification (Lowe *et al.,* 1995; Moore *et al.,* 1988; Viarengo *et al.,* 2000). Subsequently, if the intracellular accumulation of metals is extreme, these organelles become a preferential target for metal toxic effects. Exposure to continuous environmental stressors can contribute to deleterious structural changes in the lysosomal membrane (Cho & Jeong, 2005). Blood cells, or haemocytes, which are generally easy to obtain without harming the host, offer a sensitive but robust lysosome-rich model cell type that can be studied (Lowe *et al.,* 1995). Lysosomal membrane destabilization responses may function as valuable early warning signals, one of the cell's first responders to perturbations, or indicators of chronic damage (Ringwood *et al.,* 1998).

To assess lysosomal membrane destabilization, the Neutral Red Retention Time (NRRT) assay that utilizes live cells has been developed (Ringwood *et al.,* 1998). Researchers who developed the neutral red uptake (NRU) assay noted that stressed cells did not take up as much of the supravital neutral red dye (3 – amino – 7 -

dimethylamino – 2 methylphenzine hydrochloride) as did control cells and concluded that this was due to a decrease in cellular membrane stability. The NRU assay was subsequently modified into what has become known as the neutral red retention time (NRRT) assay. After a defined period of dye loading, the loss of dye from the cells was recorded and it was noted that stressed cells lost their dye at a faster rate than control cells (Hauton *et al.,* 1998). The acid environment of lysosomes is maintained by the membrane Mg^{2+} ATPase dependent H^{+} ion proton pump (Lowe *et al.,* 1992). The NRRT assay reflects the efflux of the lysosomal contents into the cytosol following damage to the membrane and possible impairment of the H^{+} ion pump (Lowe *et al.,* 1992, 1995). The NRRT assay is quick, easy and cost effective and therefore highly successful in toxicity assessment studies (Snyman *et al.,* 2000, 2002).

The NRRT assay, using molluscs as models, has been used widely in the field of ecotoxicology and environmental monitoring, for example: (1) contaminant exposure on mussel digestive tissue (Hauton *et al.,* 1998; Lowe, 1988; Wedderburn *et al.,* 2000); (2) copper exposure on haemocytes of snails (Snyman *et al.,* 2000, 2002; Sarkar *et al.,* 2008); (3) contaminant exposure on haemocytes of mussels (Pipe *et al.,* 1997, Fang *et al.,* 2010, Giarratano *et al.,* 2010) and oysters (Ringwood *et al.,* 1999; Moraga *et al.,* 2005, Richards & Chaloupka, 2009, Chou *et al.,* 2010) to name but a few.

A study undertaken by Ringwood *et al*. (1998) indicated that lysosomal membrane destabilization responses in oysters are potentially valuable biomarkers of pollutant stress.

1.2.6. Statement of the research problem

The use of marine organisms such as mussels, scallops, oysters and periwinkle, as biomonitors, is well documented in the literature (e.g. Watling & Watling, 1982; Watling, 1983; Weeks, 1995; Meiller & Bradley, 2002; Damiens *et al.,* 2004). The continuation and increased implementation of “Mussel Watch” type programmes confirms the interest in metal concentrations in molluscs specifically (Cantillo, 1998).

Molluscs serve as sentinels of chemical contamination (O'Conner & Lauenstein, 2005) and are used extensively in global research. The sedentary mode of life of filter-feeding bivalves means that they are particularly vulnerable to both acute and chronic exposure to contaminants, thus rendering them useful as environmental indicators (Grundy *et al.,* 1996). Many are sessile and they are sufficiently robust that they can be collected in areas where less hardy species may be absent (Farrington *et al.,* 1983). It has also been verified that aquatic species that frequent coastal waters are especially vulnerable to pollution because of the tendency of pollutants to accumulate in the estuaries (Sarkar *et al.,* 1994). Estuaries provide critical feeding, spawning and nursery habitats for numerous species, including bivalves (Ringwood *et al.,* 1998). They are some of the most important aquatic habitats for marine and freshwater species in terms of breeding, development and nursing grounds (Lamberth *et al.,* 2008). Rivers/estuaries can be considered as a source of input of anthropogenic and natural sources of pollution into the environment as a result of runoff from either terrestrial sources or processes such as the weathering rocks (McLeod & Wing, 2008).

Maintaining good marine environmental quality is crucial for several socio-economic reasons such as food consumption and export (de Mora *et al.,* 2004). Oysters are widely distributed along the world's coasts and many of the *Striostrea* species have already been used as a sentinel species in several environmental studies (e.g. Hauton *et al.,* 1998; Ringwood *et al.,* 1998; 1999; 2002; Amaral *et al.,* 2005). According to Al-Aasm et al. (1998), organisms that qualify as bio-monitoring species must be: (1) relatively long-lived; (2) sedentary; (3) simple to collect and handle; (4) widely distributed, common and accessible; (5) able to withstand a high metal concentration without suffering mortality, and (6) available throughout the year. A brief overview of oyster biology has been provided in Chapter 2, section 2.1., to provide a better understanding of the subject species and its possible implications as a bio-monitoring species.

In South Africa, laboratory studies by Watling & Watling (1976; 1982; 1983) have shown that *Striostrea margaritacea* (previously known as *Crassostrea margaritacea*) may be a suitable indicator organism for the South African marine environment.

However, since these publications, no studies were done using *Striostrea* from South African coastlines. Consequently, there is no present literature on the health status of the feral oyster *Striostrea margaritacea,* and its current degree of metal contamination on the Southern coast of South Africa.

1.2.7 Research objectives

1. To determine the degree of metal contamination in the water and sediments at sites selected in Witsand, Wilderness and Goukamma.

2. To determine the degree of metal bioaccumulation in the wild oyster, *Striostrea margaritacea*.

3. To determine the main site for metal storage in the wild oyster, *Striostrea margaritacea*, in terms of shell and tissue comparisons.

4. To determine if there is seasonal variability in the metal concentrations at the three study sites and in metal bioaccumulation by the oyster tissues.

5. To determine the toxicity of selected metals to the oysters, by using a biomarker (lysosome membrane destabilization) as tool. The biomarker was tested using the neutral red retention time assay.

6. To establish if *Striostrea margaritacea* qualifies as a successful biomonitor.

7. To determine if the Goukamma Marine Protected Area (MPA) can be used in field studies as control sites by doing an *in situ* study.

8. To establish if there is a need for a monitoring programme for metal contamination at the selected study sites.

CHAPTER 2

Methods and Materials

2.1 Basic oyster biology

Oyster biology, with relevance to this study, including basic anatomy, shell composition, feeding strategies and distribution are presented below:

2.1.1 Basic anatomy

According to Eble & Scro, (1996), oysters are soft bodied animals that are protected by two shells that are attached to one another by means of a hinge. The adducter muscle is situated in the posterior region of the body, with its primary function, including keeping the shell closed (Bougrier *et al*., 1985). When the adducter muscle is relaxed, the shell will gape, exposing the tissue of the animal. The internal organs are covered with a fleshy fold of tissue called the mantle or pallium (Eble & Scro, 1996). The mantle is always in contact with the valves (shell) although it is not attached (Morrison, 1993).

Most oysters posses an open-type circulatory system with a systemic heart that pumps hemolymph into a network of arteries and ultimately open into sinuses (Eble, 1996). The pericardial coelom is a thin walled chamber that lies between the visceral mass and the adducter muscle (Hawkins *et al*., 1980). As a fluid filled cavity, It is a specialized multifunction region of the coelom that protects the systemic heart and also serves to reduce the sudden surge of hemolymph that fills the ventricle when the adducter muscle contracts (Elston, 1980).

The gills of the oyster consist of four folds (demibranchs) of tissue suspended from the visceral mass and occupy much of the ventral and ventro-posterior portions of the mantle cavity (Eble, 1996). The two gills together constitute the largest organ of the oyster's body and functions as a site for food acquisition and gaseous exchange (respiration) (Newell & Langdon, 1996), creating water currents and moving food particles to the labial palps for further sorting (Eble & Scro, 1996).

2.1.2 Shell composition

The term bivalve can be roughly translated into “two valves”. Oysters, being classified under the group Bivalvia, have two shells that are attached to one another by means of a hinge (Carriker, 1996). This author describes the formation of the shell in bivalves to be a complex process with many different variables and processes involved. For the purpose of this study it is of importance to note that the shell is made up of a Calcium Carbonate ($CaCO_3$) matrix and is excreted from the internal mantel layer continuously (Carriker, 1978).

2.1.3 Feeding strategy

Striostrea margaritacea are classified as filter feeders, meaning that they remove particles suspended in the water column for feeding (Newell & Langdon, 1996). Such particulate food is often quantitatively and qualitatively variable in estuarine waters and is composed of complex mixtures of a wide range of living micro-organisms, detritus and inorganic matter (Beninger *et al*., 1991).

The digestive system consists of a mouth, esophagus, stomach, style sac, digestive gland, intestine, rectum and anus. Before food particles enter the mouth they are sorted by the labial palps, which are specialized organs known to control the total amount of food ingested (Newell & Langdon, 1996). Ingested food particles are transported through the mouth and short esophagus into the stomach (Purchon, 1987). Ward et al. (1994) observed that particles in stomachs of living oysters appear to be suspended in non-viscous mucus. Ingested materials are held in the gut and subjected to digestive processes and absorbed, after which the remains are moved along the mid–gut to the rectum and expelled through the anus as faeces (Langdon & Newell, 1996).

2.1.4 Distribution

Striostrea margaritacea, also known as the Cape rock oyster, can be found from False Bay to Mozambique, occurring predominantly on the open coast but also penetrate mouths of estuaries with a vertical position on the shore from extremely low tide to a depth of 5 meters (Robinson *et al.,* 2005).

2.2 Study Sites

For the purpose of this study, estuarine environments were used, as, a) it is the habitat of the subject species, *Striostrea margaritacea,* b) it serves as a gateway from the terrestrial water supply to the oceans, i.e. land-based runoff accumulates and enters the ocean here, and c) it is an invaluable habitat structure that should be studied in depth to gather information and present a possible conservation strategy via monitoring programmes.

Study sites were chosen through the process of a primary screening. Various sites along the south coast were sampled in the primary screening process. These samples were prepared and analysed in the laboratory and the results were used to aid in the selection of adequate sites. Each site had to meet a specific set of prerequisites namely:

- Location in terms of accessibility
- River input into oceanic systems
- Anthropogenic influence and influx
- Activities along the rivers
- Oyster availability

Three study sites were chosen (Figure 2.2.1) namely: Wilderness (S34°00.091', E22°37.372') [Site 1]; Witsand (S34° 23.915'; E20° 50.785') [Site 2] and Goukamma Marine Protected Area (MPA) [Site 3] (S34°03.860', E22°54.671').

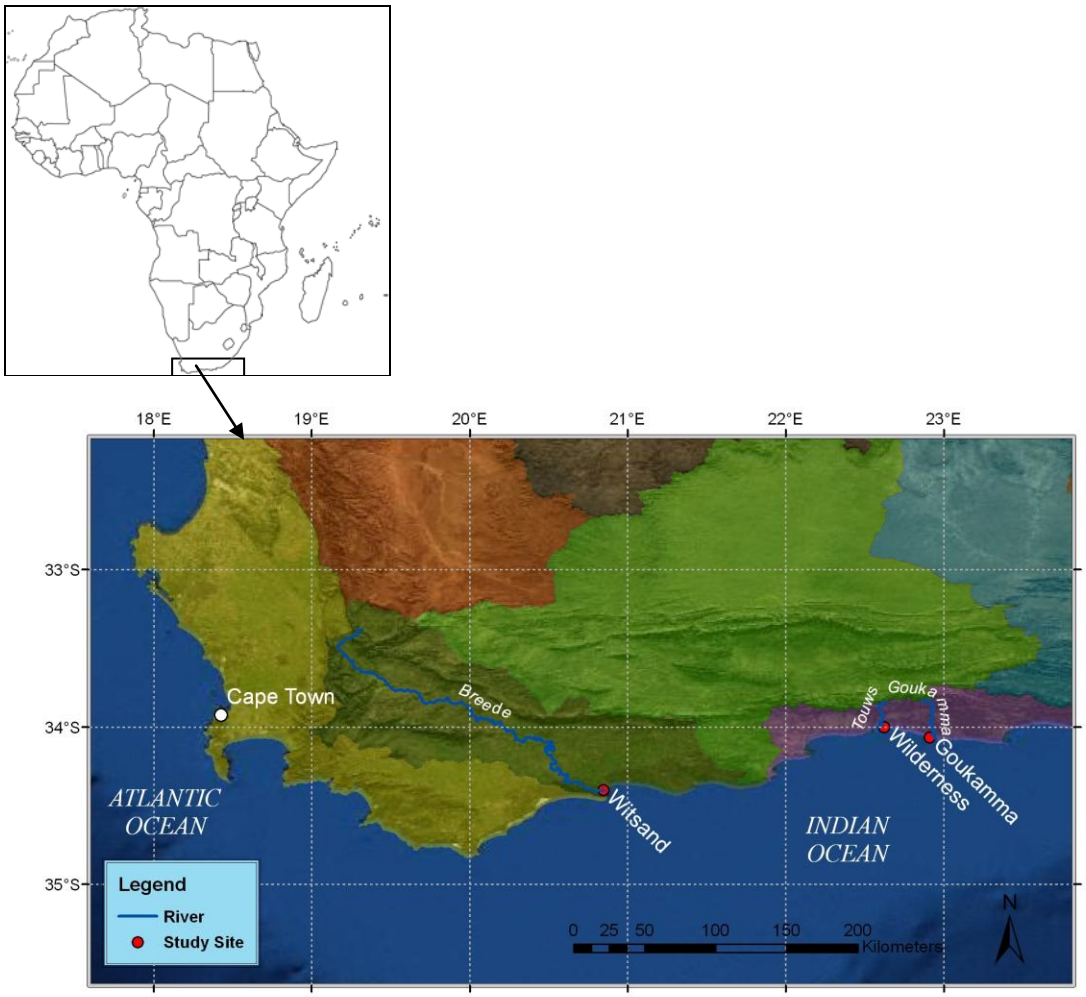

Figure 2.2.1 Map displaying the three study sites used in the study namely Witsand (S34° 23.915'; E20° 50.785'), Wilderness (S34°00.091', E22°37.372') and Goukamma (MPA) (S34°03.860', E22°54.671'). Figure produced by Fiona Cuff (2009).

2.2.1 Wilderness

Wilderness is situated on the South Coast of South Africa and is an integral part of the Garden Route. It is a popular holiday destination, especially in the summer months.

The dominant river system in the area is the Touws River. It originates in the Outeniekwa mountain range and winds its way towards the Wilderness Lagoon where it eventually empties into the Indian Ocean. The Rondevlei, Bo-langvlei and Island Lake are all connected to the Wilderness Lagoon via the Serpentine River and thus contribute their input into this system (personal observation as interpreted from official maps). There are numerous buildings and ruins around the Wilderness Lagoon as well as the Touws River. It is a highly developed area with constant new additions. A mine dump is situated a few kilometers away from the Touws River north of the lagoon. The Wilderness Nature Reserve is adjacent to this aquatic system and offers leisure activities such as hiking trails and fishing to the increasing influx of tourists. Recreational activities in the lagoon include fishing, bait collection and canoeing. Guests are allowed to use motorized boats, but only at idle speed (personal observation). Figure 2.2.2 indicates the collection site of oysters in the Wilderness area.

The mouth of the Touws River was open from the 11th November 2008 to the 21st of December 2008 (N. Grootendorst; *pers comm*., 2008[2]) and did not open naturally again for the duration of this study (Table 2.1). The opening of the river mouth coincided with the floods that plagued this area in the summer of 2008.

2.2.2 Witsand

Witsand is situated 300km from Cape Town and is a popular tourist destination. Its unique aquatic system offers a variety of recreational activities along the Breede River all year round (Lamberth *et al.,* 2008).

[2] Mrs Nellie Grootendorst. Wilderness, Section manger, SANParks

Figure 2.2.2 Satellite image of the Wilderness study area including the mouth of the Touws River. Collection site of oysters indicated by red arrow. (Source: Google Earth)

The Breede River is 332km long from its source near Ceres to where it enters the Indian Ocean in Sebastian Bay and falls within the warm-temperate Agulhas bio-geographical region (Lamberth *et al.,* 2008). The banks of the Breede River are predominantly occupied by cultivated lands and huts. These cultivated lands include orchards and vineyards (personal observation as interpreted from official maps).

Fifty kilometers upstream, between Infanta and Swellendam, the river changes into a rapid flowing estuary that separates Witsand and Infanta. The Breede River estuary is one of the largest estuaries in South Africa and offers recreational activities such as motorized water sports and fishing. These activities decrease during the winter months but are abundant in summer, with the exception to fishing, which is common all year around (personal observation, 2008, 2009). Figure 2.2.3 indicates the collection site of oysters in the Witsand area.

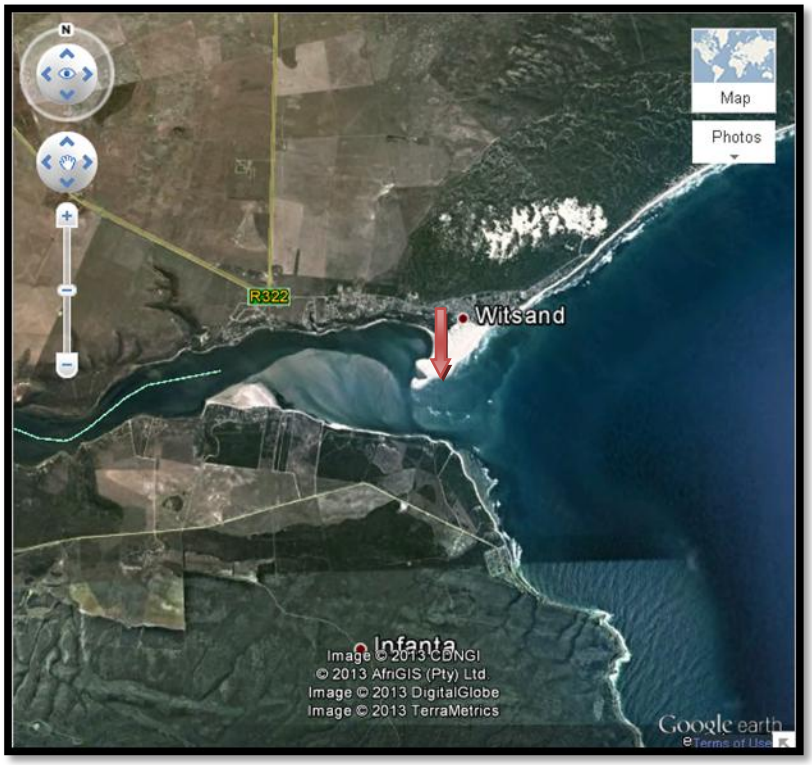

Figure 2.2.3 Satellite image of the Witsand study area including the Breede River estuary. Collection site of oysters indicated by red arrow. (Source: Google Earth)

In November 2008 and June 2009, Witsand experienced heavy rainfall that led to floods and a high volume of freshwater influx into the estuarine environment *(*K. Erasmus. *pers. comm.,* 2009[3]*).*

2.2.3 Goukamma Marine Protected Area (MPA)

The Goukamma Nature Reserve is situated 20km west of Knysna and stretches eastward for 18km. The Marine Protected Area (MPA) in the reserve includes the coastline and extends 1 Nautical Mile (NM) (1 NM = 1.852km) seawards (Figure 2.2.4). Regulations state that no vessels, motorized or other, may enter the MPA's oceanic boundary for fishing or other purposes. Shore based angling is allowed with the appropriate permit issued by Marine and Coastal Management (now Department of Agriculture, Forestry and Fisheries).

[3] Mr. Kosie Erasmus. Oyster Permit holder/resident within Witsand

The Goukamma River flows through the nature reserve and empties into the Indian Ocean. The Rooi River, Platbos River and various other smaller rivers originate in the Outeniekwa mountains and filter down into the Homtini River, which then becomes the Goukamma River. The banks of these rivers are mostly situated within the surrounding nature reserves, namely the Goukamma Nature Reserve and the Jubilee Creek Nature Reserve, with very few cultivated areas bordering them (personal observation as interpreted from official maps). Figure 2.2.5 indicates the collection site of oysters in the Goukamma MPA.

The mouth of the Goukamma estuary was open for most of 2008, closing in August for three weeks only (K. Spencer, *pers comm*. 2009[4]). The mouth closed on 22 December 2008 and only reopened again in July 2009 (Table 2.1).

[4] Mr Keith Spencer, Manager of Goukamma Nature Reserve, CapeNature

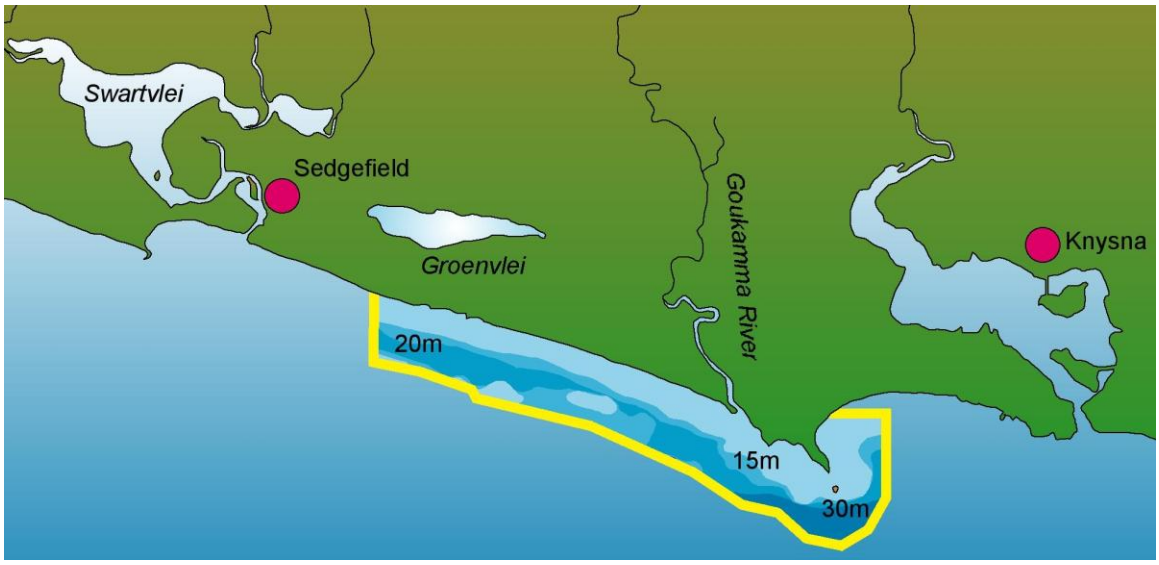

Figure 2.2.4 The Goukamma Marine Protected Area (MPA)'s boundary seaward, including the bathymetry of the area. Figure produced by Tony van der Buis (2009)

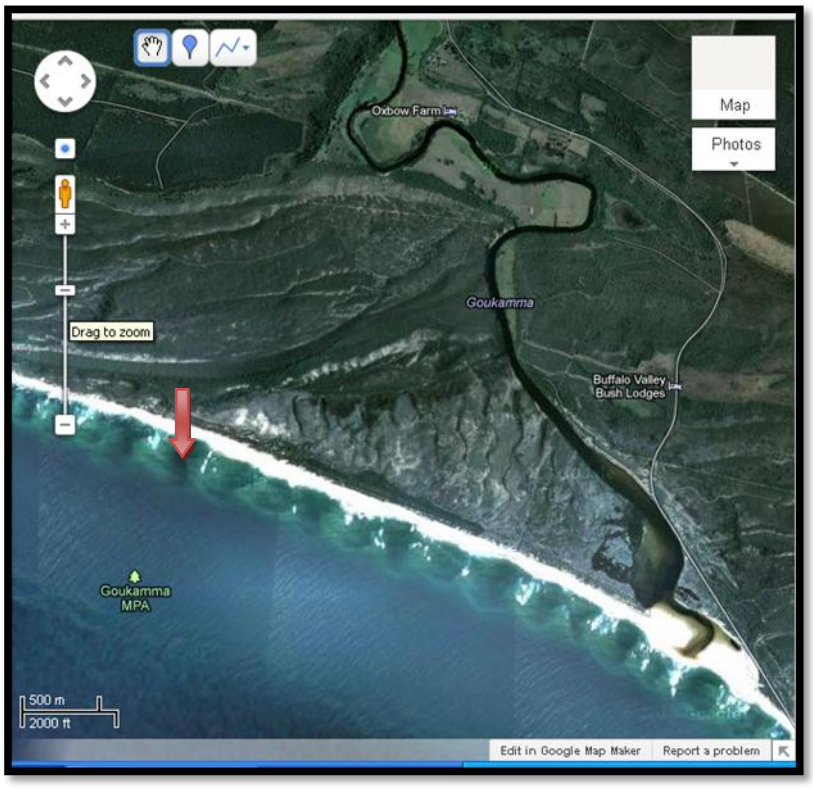

Figure 2.2.5 Satellite image of the Goukamma (MPA) study area including the Goukamma river. Collection site of oysters indicated by red arrow. (Source: Google earth)

Table 2.1: The open/closed status of the estuary mouth at each of the three study sites from January 2008 to June 2009. If the Estuary/River mouth was open, i.e. a flowthrough into the ocean, this is indicated with *; if it was closed, this is indicated by a blank space.

	Jan-08	**Feb-08**	**Mar-08**	**Apr-08**	**May-08**	**Jun-08**	**Jul-08**	**Aug-08**	**Sep-08**	**Oct-08**	**Nov-08**	**Dec-08**	**Jan-09**	**Feb-09**	**Mar-09**	**Apr-09**	**May-09**	**Jun-09**
Breede (Witsand)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Touws (Wilderness)											*	*						
Goukamma (Goukamma Nature Reserve)	*	*	*	*	*	*	*		*	*	*	*						*

2.3 Sample collection

All samples (oyster, water and sediment) were collected quarterly, during the spring low tide, over the course of one year, from June 2008 to March 2009. The sampling frequency was adapted to coincide with seasonal changes, thus representing the four seasons: June = Winter; September = Spring; December = Summer; March = Autumn.

Ten oysters were collected from each individual site upon each visit. No gender distinction was made in this study and consequently the sampled animals were a mixture of male and female. Mean length and diameter are displayed in Table 2.2 and were measured using a Venire Caliper. Sample acquisition in Witsand and Wilderness was done through shore based dives. Equipment that was utilized included a diving mask, snorkel, fins, collection bag and crowbar. Equipment and methods were in compliance with the South African Marine Living Resources Act, 1998 (Act no. 18 of 1998). At the site within the Goukamma MPA, only an oysterknife was used to remove the oysters from the rocks that were exposed during the low tide. Oysters were transported in individual 20L buckets with water from their original habitats. Water was aerated by means of an airstone, plastic tubing and pump.

Table 2.2: Mean lengths and diameters of the collective oyster samples taken throughout the sampling period. N = 40 (the total sample size per site over the entire sampling period). SD = Standard Deviation.

Study site	**Length (Mean)(±SD) (cm)**	**Diameter (Mean)(±SD) (cm)**
Wilderness	7.01 (±0.59)	5.73 (±0.40)
Witsand	7.86 (±0.42)	6.29(±0.43)
Goukamma (MPA)	6.63 (±0.60)	5.61(±0.46)

Six water and six sediment samples were taken seasonally at each site. Polytop containers were used for the collection and transport of these samples. Samples were refrigerated and transported back to the laboratory. Furthermore, the temperature, pH and salinity of the ambient water were measured during each sampling occasion. An alcohol-based thermometer was used to measure water temperature, a HANNA pH-

EC-TDS handheld pH meter to measure the pH and an ATAGO handheld refractometer to measure the salinity.

2.4 Neutral Red Retention Time (NRRT) Assay

Oysters were kept in aerated buckets for a period of 24 hours after collection to acclimatize and avoid additional stress after collection. Thereafter oysters were opened with the aid of an oysterknife. It was noted for each sample whether it was in a state of spawning or not (Table 2.3). This is suspected to have a effect on the level of stress of the animal (Perdue *et al.,* 1981). Haemolymph used for the NRRT assay was collected from each individual oyster's pericardium using an adaptation of the method described by Svendsen and Weeks (1995) and Snyman *et al*. (2000). Haemocytes were harvested by gently prying open the oysters' valves, inserting a 1ml needle through the pericardium and withdrawing 20µl haemolymph from the "pericardium" into a 1ml sterile syringe containing an equal volume of temperature- adjusted bivalve Ringer (0.53g NaCl + 0.107g KCl + 0.13g $CaCl_2$ dissolved in 1L of distilled water). Two drops were placed upon a microscope slide and 20µl neutral red working solution added to each drop. The working solution was prepared by mixing 2.5ml Ringer and 10µl neutral red stock solution (20mg neutral red powder dissolved in 1ml DMSO). To avoid crystallization of the nonpolar neutral red in the aqueous Ringer solution, the working solution was renewed every hour during the measuring period (Weeks & Svendsen, 1996). After the microscope slide was covered with a glass cover slip, it was placed into a humidity chamber for 30 seconds before being analyzed. This was done to aid in the adhesive effect of cells to the slide.

As described by Snyman *et al*. (2000), the total number of haemocytes, as well as the number of stained haemocytes in the haemolymph sample were counted under a light microscope at 2-minute intervals. After each observation interval, the slide was returned to the humidity chamber for a further 2-minute period to minimize exposure to ambient conditions. Only the most abundant cell type, namely, the smaller, hyaline, agranular haemocytes (Auffret, 1988) with pseudopodia, were counted. The point

(expressed in minutes) at which 50% or more of the total number of haemocytes were stained was expressed as the neutral red retention time.

2.5 Sample preparation for metal analysis

Oyster soft tissue, Shells and Sediments

Oysters were frozen in individually labeled bags after NRRT measurements. Metal analysis was done according to the methods described by Snyman *et al*. (2000). Samples were dried in an oven for 72h at 60°C. Thereafter, oyster soft tissue were separated from the shells and ground with the aid of a mortar and pestle to obtain a ±0.3g subsample of whole tissue and a ±0.3g subsample of shell, respectively. A ±0.5g subsample was used for the sediment samples. Samples were digested in 10ml 55% nitric acid at a temperature of 40^{o}C for a period of 1h, after which the temperature was increased to 120^{o}C for 3h. After cooling, samples were filtered through 90mm Whatman filter paper and then again through 0.45µm cellulose nitrate membrane filter paper using a syringe. Finally, the samples were diluted to 100ml with distilled water.

Water

As in the method described in Shuping (2008), 10 ml of each water sample was used for the digestion process and 5ml 55% nitric acid was added. The rest of the digestion and filtration process was the same as described for oyster soft tissue, shells and sediments.

2.6 Metal analysis

Metals that were analysed are aluminium (Al), copper (Cu), zinc (Zn) and iron (Fe). These metals were selected from a potential of nine metals. A primary screening was undertaken (refer to section 2.1), and the results indicated that the selected metals were the most abundant in the areas that were to be used as study sites. The remaining metals, namely Cadmium (Cd), Lead (Pb), Cobalt (Co), Nickel (Ni),

Manganese (Mn) and Chromium (Cr) were mostly below detectable levels in the water, sediment and oysters.

Table 2.3: The percentage of spawning oysters, collected from each site, during each sampling occasion. N=10 per sampling site, per collection.

Sites	Spawning %	Not Spawning %
June (Winter)		
Site 1	0	100
Site 2	0	100
Site 3	30	70
Sep (Spring)		
Site 1	0	100
Site 2	0	100
Site 3	15	85
Dec (Summer)		
Site 1	60	40
Site 2	0	100
Site 3	20	80
Mar (Autumn)		
Site 1	30	70
Site 2	30	70
Site 3	60	40

Samples were analyzed using an Inductively Coupled Plasma - Atomic Emission Spectrometer (ICP- AES) at Stellenbosch University. Data were manipulated using the formulae **a**) [(ICP value - Blank) X 100] / Mass (g) = mg/kg for tissues, shells and sediment and **b**) [(ICP value - Blank) X dilution factor] = mg/l for water.

2.7 The bio-concentration factor (BCF)

As previously mentioned in Chapter 1, the bio-concentration factor (BCF) is a tool used to reflect that some pollutants are assimilated by organisms to a greater extent than others (Walker *et al.,* 2006). It also gives an indication of the level of bioaccumulation by the organisms with reference to the surrounding environment. According to Dallinger (1993) and Walker *et al.* (2006) the BCF can be expressed as:

$$BCF = \frac{\text{Concentration of the chemical in the organism}}{\text{Concentration of the ambient environment}}$$

Results for the BFCs' of this study are reflected in Tables 3.4.1, 3.4.2 and 3.4.3 for the shells and tissue samples (as fractions of the water and sediment concentrations). Mean concentrations were used in the analyses. Environmental concentrations that were recorded as 0 were not used in calculations and are represented as "can not be calculated (CBC). Both water and sediment concentrations were used for calculating BCFs' as both environmental factors are in contact with the animal (refer to section 2.1)

2.8 Statistical analysis

All statistical analyses were performed using Sigmastat 3.5 software. The Shapiro-Wilk normality test was conducted for all data sets and post hoc tests were conducted pending the outcome of the distribution. Median values were used for analysis as data was not normally distributed (Townend, 2002).

A non–parametric Kruskal–Wallis One-Way ANOVA on Ranks test was done to test for significant differences between sites and between sampling occasions (seasons) as most of the data sets were not normally distributed. Seasons were compared consecutively to one another i.e Winter (June) was compared to Spring (September), Spring (September) was compared to Summer (December) and Summer (December) was compared to Autumn (March). This was done to determine if there were trends in

the seasonal changes. Townend (2002) stated that when using the Kruskal–Wallis test, a P-value > 0.05 can be considered as not significant and a P-value < 0.05 indicates a significant difference. A P-value of <0.001 indicated that differences between treatment groups were significantly greater than would be expected by chance. If significant differences were detected amongst data sets, the Tukey test was used as a post–hoc test to specify differences.

Comparisons that were drawn between tissue and shell data within seasons were determined by means of the Mann-Whitney *U*-test for non-parametric data, as the two sets of data were not normally distributed. The Mann-Whitney U-test helped to analyze the specific sample pairs for significant differences (Townend, 2002).

CHAPTER 3

Results

3.1 Physico-chemical parameters

The temperature, salinity and pH measured in the ambient water for the duration of the study period is displayed in Table 3.1.

Table 3.1: Physico-chemical measurements (temperature, salinity and pH) taken from site 1, 2 and 3 for the duration of the study.

Sampling Dates	*Environmental Factor*	Site 1	Site 2	Site 3
June '08	**Temperature (^{o}C)**	21	23	20
Winter	**Salinity (ppm)**	35	27	35
	Ph	7.9	7.9	7.9
September '08	**Temperature (^{o}C)**	18	17	18
Spring	**Salinity (ppm)**	38.5	29.5	39
	pH	7.9	8	8
December '08	**Temperature (^{o}C)**	22	24	19.5
Summer	**Salinity (ppm)**	35	33.5	37
	pH	7.7	8	7.7
March '09	**Temperature (^{o}C)**	24.5	23.5	23.5
Autumn	**Salinity (ppm)**	34.5	34	34
	pH	8	8	8

3.2 Metal Analysis

Mean aluminium, zinc, copper and iron measured in water, sediment, oyster tissues and shells for the duration of the study period, are displayed in the sections below. The H statistic (h), degrees of freedom (d.f) and p values (p) have also been included for all Kriskal – Wallis One – Way ANOVA on Ranks tests for statistical differences. Appendix A includes all H-statistics (h), degrees of freedom (d.f) and p-values (p) for aluminium, zinc, copper and iron in tables A1 – 4 respectively.

3.2.1 Aluminium

3.2.1.1 Water

The mean aluminium concentrations measured in the water samples for the duration of the study period are displayed in Figure 3.2.1 and Table 3.2.1.

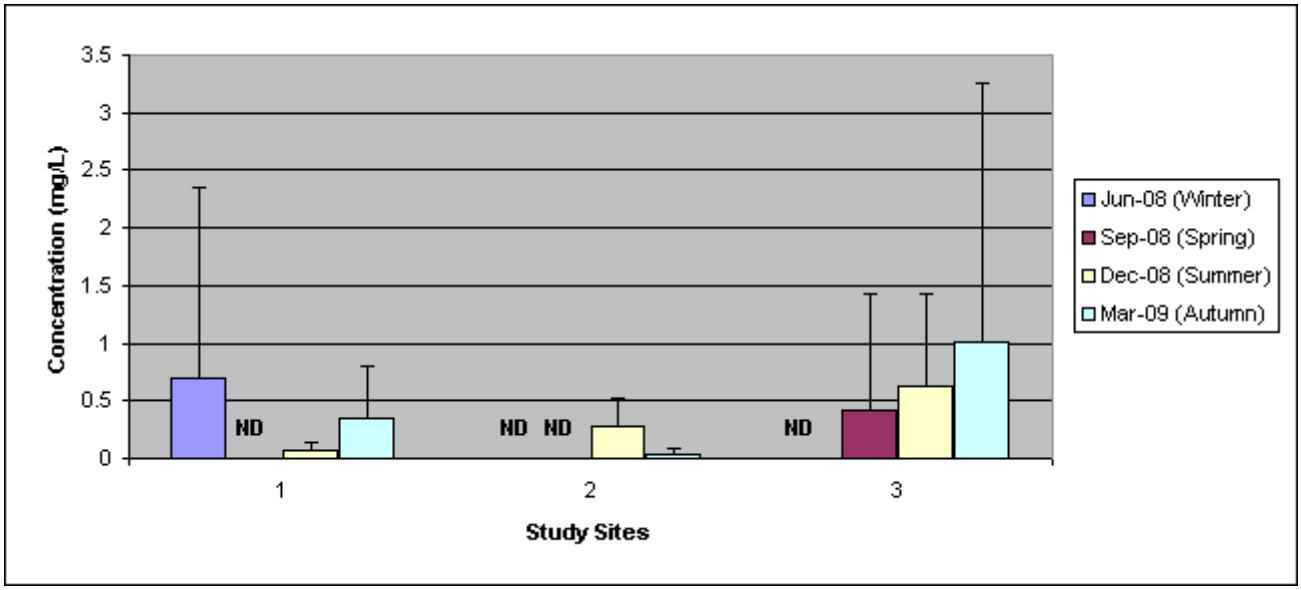

Figure 3.2.1 The mean (±SD) concentrations (mg/L) of aluminium in water from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72) with intervals for each season. ND = not detectable.

Comparisons of seasonal metal concentrations

Significant differences ($p<0.05$) were recorded in water aluminium concentrations between seasons per site. Pairwise multiple comparisons indicated that the aluminium concentrations in the water, increased significantly ($p<0.05$) from spring (Sep 2008) to summer (Dec 2008), at sampling site 2 ($h=13.802$, d.f: 3, $p= 0.003$).

Comparisons of metal concentrations between sites

There were no significant differences ($p>0.05$) in water aluminium concentrations between any of the sites during any of the sampling occasions.

3.2.1.2 Sediments

The mean aluminium concentrations measured in the sediment samples for the duration of the study period are displayed in Figure 3.2.2 and Table 3.2.1.

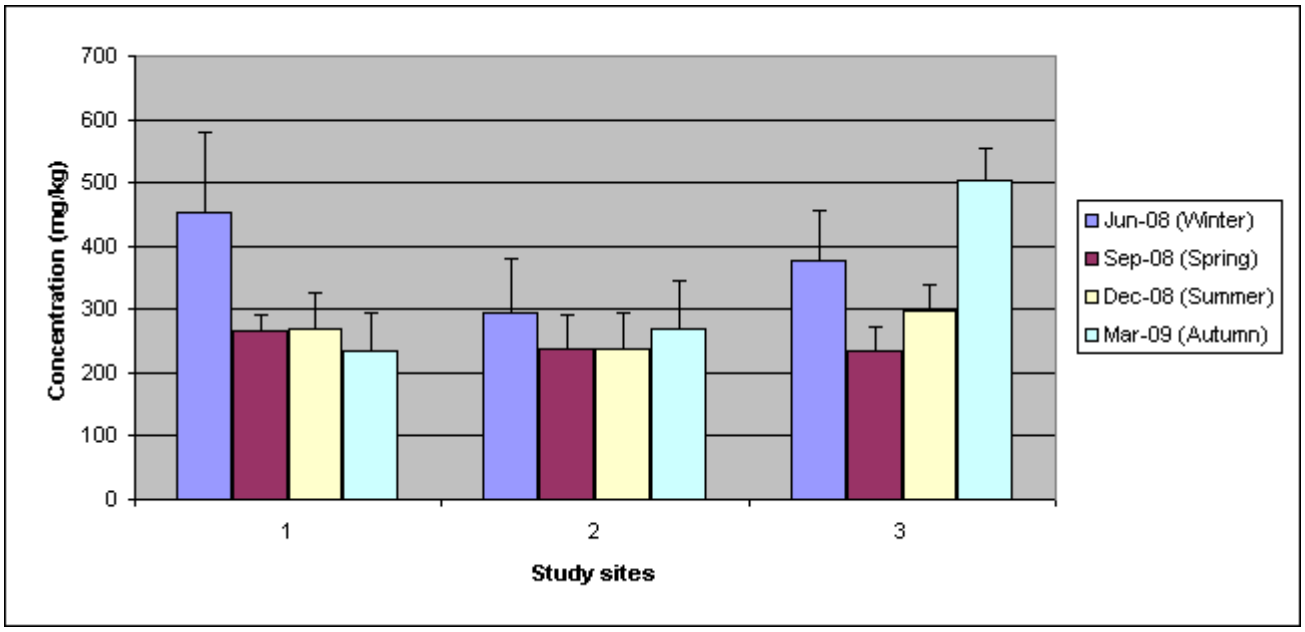

Figure.3.2.2 The mean (±SD) concentrations (mg/kg) of aluminium in the sediment from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72), with intervals for each season.

Comparisons of seasonal metal concentrations

Significant differences ($p<0.001$) were recorded in sediment aluminium concentrations between seasons per site All pairwise multiple comparisons indicated that the aluminium concentrations in the sediment, increased significantly ($p<0.001$) from summer (Dec 2008) to autumn (Mar 2009) and significantly decreased ($p< 0.001$) from winter (Jun 2008) to spring (Sep 2008), at sampling site 3 ($h=18.373$, d.f: 3, $p= <0.001$). No other statistical differences were recorded.

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicated that the aluminium concentrations measured in the sediments significantly differed between sampling sites 1 and 3 ($p<0.05$) and also between sampling sites 2 and 3 on the autumn ($h=11.661$, d.f: 2,

p=0.003) sampling occasion, where sampling site 3 had the highest mean. No other statistical differences were recorded.

3.2.1.3 Oyster tissue

The mean aluminium concentrations measured in the oyster tissue samples for the duration of the study period are displayed in Figure 3.2.3 and Table 3.2.1.

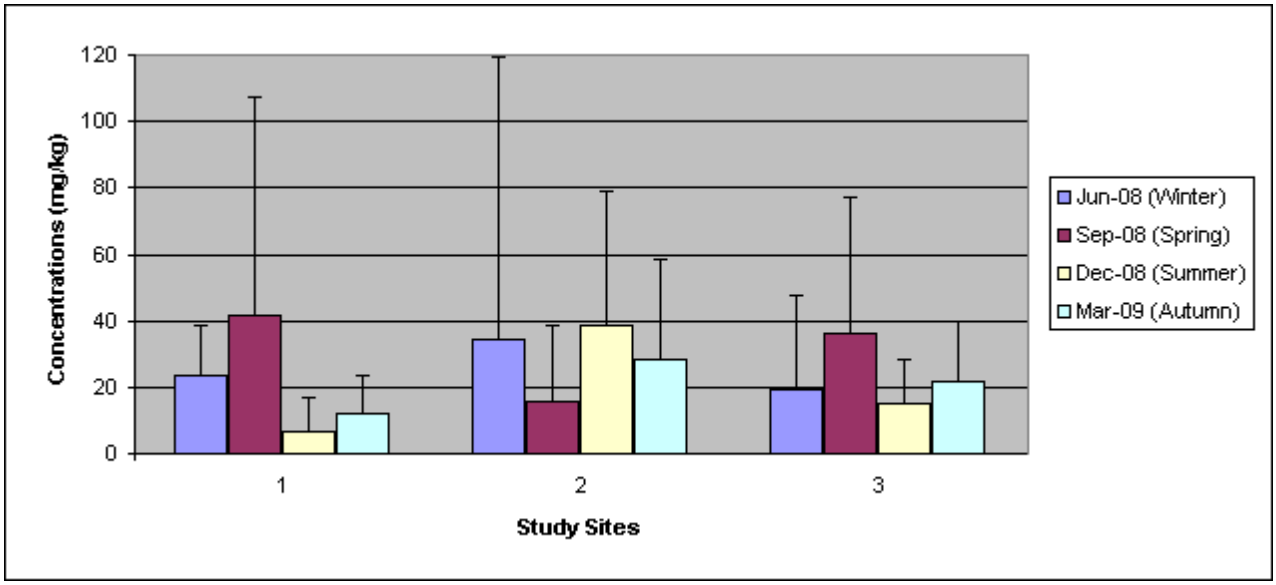

Figure 3.2.3 The mean (±SD) concentrations (mg/kg) of aluminium in the tissues of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season.

Comparisons of seasonal metal concentrations

No significant differences (p>0.05) were recorded in tissue aluminium concentrations between seasons, per site, in the course of the sampling period.

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicated that the aluminium concentrations measured in the oyster tissues significantly differed (p<0.05) between sampling sites 1 and 2 on the summer sampling occasion (h=6.598, d.f: 2, p=0.037), where sampling site 2 had the highest mean. No other statistical differences were recorded.

3.2.1.4 Shells

The mean aluminium concentrations measured in the shell samples for the duration of the study period are displayed in Figure 3.2.4 and Table 3.2.1.

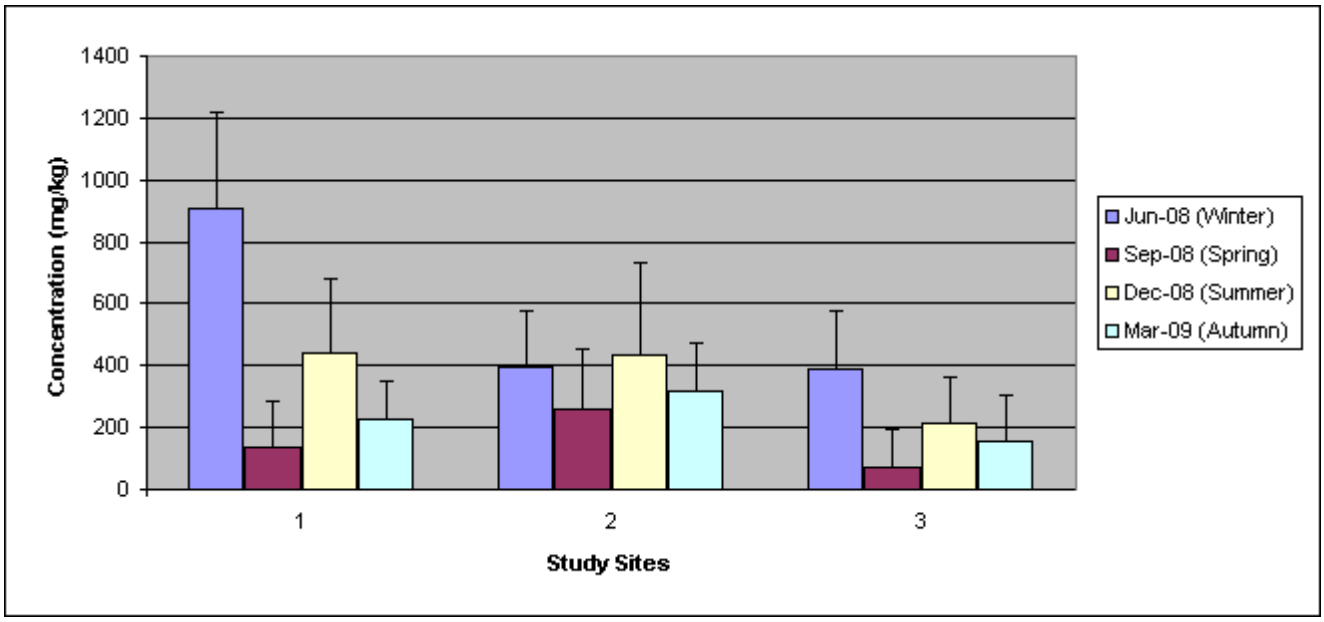

Figure 3.2.4 The mean (±SD) concentrations (mg/kg) of aluminium measured in the shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the aluminium concentrations in the shells of oysters from sampling sites 1 ($h=25.021$, d.f: 3, $p<0.001$) and 3 ($h=17.666$, d.f: 3, $p<0.001$), decreased significantly ($p<0.001$) from winter (Jun 2008) to spring (Sep 2008). Also, pairwise multiple comparisons indicated that from summer (Dec 2008) to autumn (Mar 2009), there was a statistically significant decrease at sampling site 3 ($h=17.666$, d.f: 3, $p<0.001$). No other statistical differences were recorded.

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicated that the aluminium concentrations measured in the oyster shells in winter ($h=13.985$, d.f: 2, $p<0.001$) and summer ($h=7.463$, d.f: 2, $p=0.024$) were significantly higher ($p<0.05$) in oysters from site 1, compared to site 3. Also, pairwise multiple comparisons indicate that the aluminium

concentrations in oyster shells from sampling sites 1 and 2 differed significantly ($p<0.001$) in the winter ($h=13.985$, d.f: 2, $p<0.001$) sampling occasion, where site 1 had the highest mean. Aluminium concentrations in oyster shells from sampling sites 2 were the highest in the spring ($h=11.279$, d.f: 2, $p=0.004$) sampling occasion where a significant difference ($p<0.05$) was measured between site 2 and 3.

No other statistical differences were recorded.

Table 3.2.1: The mean (±SD) concentrations of aluminium for water, sediment, oyster tissue and shells; collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

	Water (mg/L)		***Sediment (mg/kg)***		***Tissues (mg/kg)***		***Shells (mg/kg)***	
Sites and sampling occasion	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
June								
1	^{A} 0.71^{a}	1.64	^{A} 453.52^{a}	125.68	^{A} 23.46^{a}	15.30	^{A} 908.48^{a}	307.36
2	^{A} 0.00^{a}	0.00	^{A} 295.46^{a}	84.73	^{A} 34.24^{a}	84.87	^{A} 395.30^{b}	180.79
3	^{A} 0.00^{a}	0.00	^{A} 376.64^{a}	79.56	^{A} 19.27^{a}	28.17	^{A} 388.66^{b}	187.02
Sep								
1	^{A}0.00^{a}	0.00	^{A} 267.04^{a}	23.51	^{A} 41.88^{a}	65.29	^{B} 133.70^{a}	154.66
2	^{A} 0.00^{a}	0.00	^{A} 237.19^{a}	55.78	^{A} 15.74^{a}	22.81	^{A} 256.65^{a}	199.17
3	^{A} 0.00^{a}	0.00	^{B} 233.40^{a}	37.89	^{A} 36.07^{a}	41.40	^{B} 69.80^{a}	123.26
Dec								
1	^{A} 0.08^{a}	0.07	^{A} 269.00^{a}	57.83	^{A} 6.85^{a}	10.00	^{B} 439.31^{a}	244.41
2	^{B} 0.28^{a}	0.23	^{A} 236.16^{a}	59.45	^{A} 38.43^{b}	40.36	^{A} 436.79^{a}	296.75
3	^{A} 0.63^{a}	0.80	^{B} 299.14^{a}	38.23	^{A} 15.29^{a}	12.83	^{B} 212.65^{b}	151.47
Mar								
1	^{A} 0.34^{a}	0.46	^{A} 234.28^{a}	61.35	^{A} 12.24^{a}	10.98	^{B} 226.02^{a}	122.97
2	^{B} 0.04^{a}	0.06	^{A} 267.68^{a}	76.93	^{A} 28.26^{a}	30.50	^{A} 319.17^{a}	153.90
3	^{A} 1.02^{a}	2.24	^{C} 502.28^{b}	50.75	^{A} 21.44^{a}	18.33	^{C} 152.94^{a}	148.72

* Statistical significant differences between sites per season are indicated by lower case letters (a,b) on the right hand side of mean values. Significant differences between seasons per site are indicated by capital letters (A, B, C) on the left hand side of mean values. Seasons were compared consecutively.

3.2.2 Zinc

3.2.2.1 Water

The mean zinc concentrations measured in the water samples for the duration of the study period are displayed in Figure 3.2.5 and Table 3.2.2.

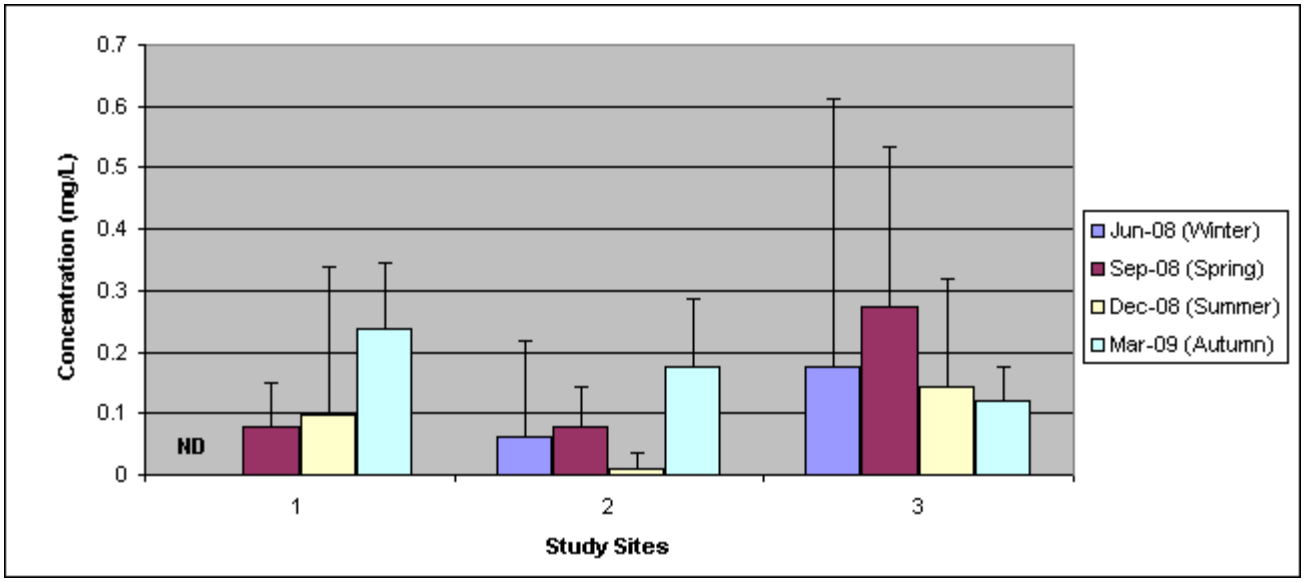

Figure 3.2.5. The mean (±SD) concentrations (mg/L) of zinc in water from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72) with intervals for each season. ND = not detectable.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the zinc concentrations in the water, increased significantly ($p<0.05$) from summer (Dec 2008) to autumn (Mar 2009) at sampling sites 1 ($h=15.525$, d.f: 3, $p=0.001$) and 2 ($h=10.490$, d.f: 3, $p=0.015$). No other statistical differences were recorded.

Comparisons of metal concentrations between sites

There were no significant differences ($p>0.05$) in water zinc concentrations between any of the sites during the sampling period.

3.2.2.2 Sediments

The mean zinc concentrations measured in the sediment samples for the duration of the study period are displayed in Figure 3.2.6 and Table 3.2.2.

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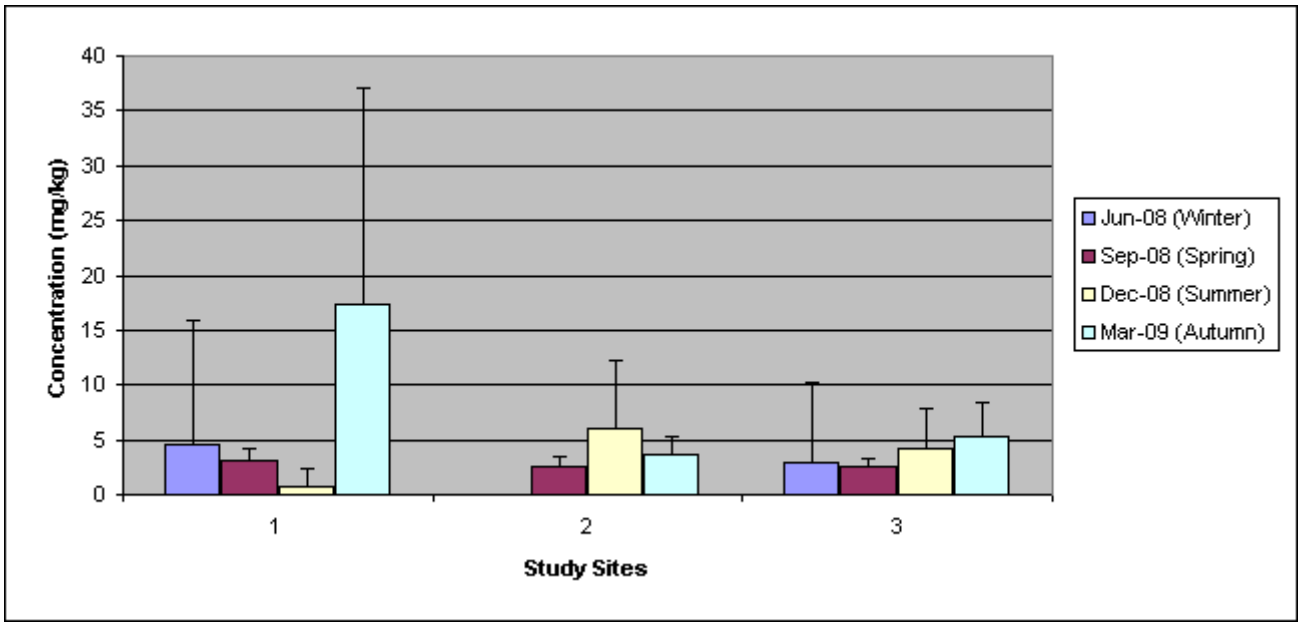

Figure 3.2.6 The mean (±SD) concentrations (mg/kg) of zinc in the sediment from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72), with intervals for each season.

Comparisons of seasonal metal concentrations

Pairwise multiple comparisons indicated that the zinc concentrations in the sediment, increased significantly ($p < 0.05$) from summer (Dec 2009) to autumn (Mar 2009) at sampling site 1 ($h=13.091$, d.f: 3, $p=0.004$)

Comparisons of metal concentrations between sites

There were no significant differences ($p>0.05$) in sediment zinc concentrations between any of the sites during the sampling period.

3.2.2.3 Oyster tissue

The mean zinc concentrations measured in the oyster tissue samples for the duration of the study period are displayed in Figure 3.2.7 and Table 3.2.2.

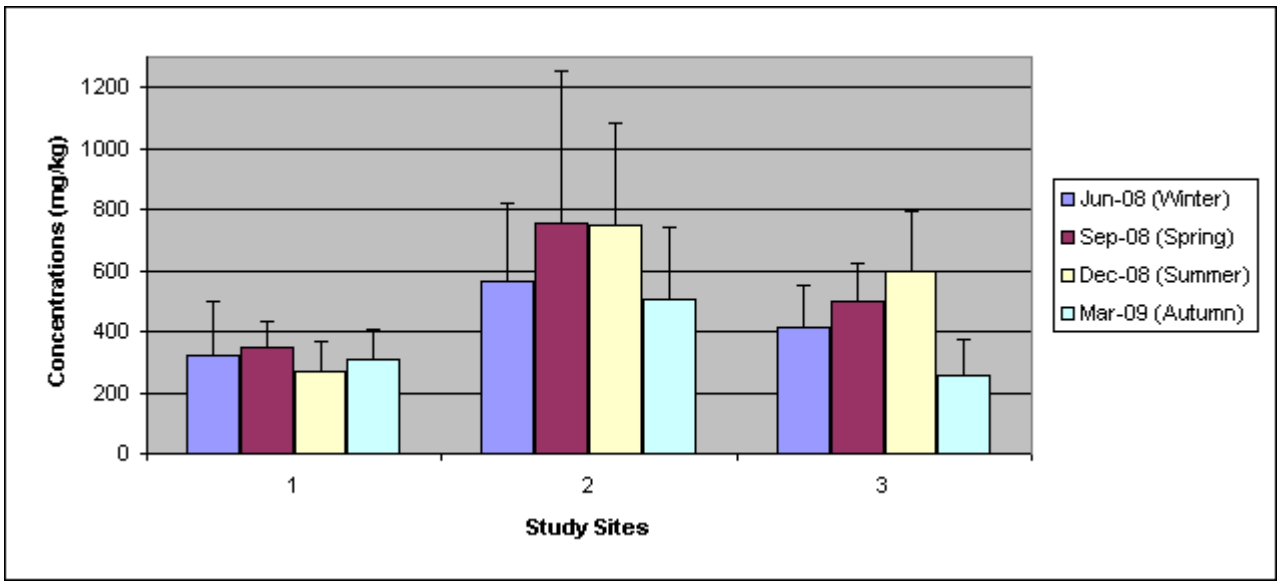

Figure 3.2.7 The mean (±SD) concentrations (mg/kg) of zinc in the tissues of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the zinc concentrations in the tissue of the oysters, decreased significantly ($p<0.001$) from summer (Dec 2008) to autumn (Mar 2009) at site 3 ($h=17.150$. d.f: 3, $p<0.001$). No other statistical differences were recorded.

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicate that the zinc concentrations in the oyster tissues in summer ($h=16.183$, d.f: 2, $p<0.001$) significantly differed between sampling sites 1 and 2 and also between sampling sites 1 and 3 ($p<0.001$), where sampling site 1 had the lowest mean in each instance. Also, pairwise multiple comparisons indicate that the oyster tissues zinc concentrations in spring ($h=9.177$, d.f: 2, $p=0.010$) at sampling sites 1 and 2, significantly differed ($p<0.05$) where site 2 had the highest mean. Sampling sites 2 and 3 indicated a significant difference ($p<0.05$) in the zinc concentrations of the oyster tissues in autumn, where site 2 had the highest mean. No other statistical differences were recorded.

3.2.2.4 Shells

The mean zinc concentrations measured in the shell samples for the duration of the study period are displayed in Figure 3.2.8 and Table 3.2.2.

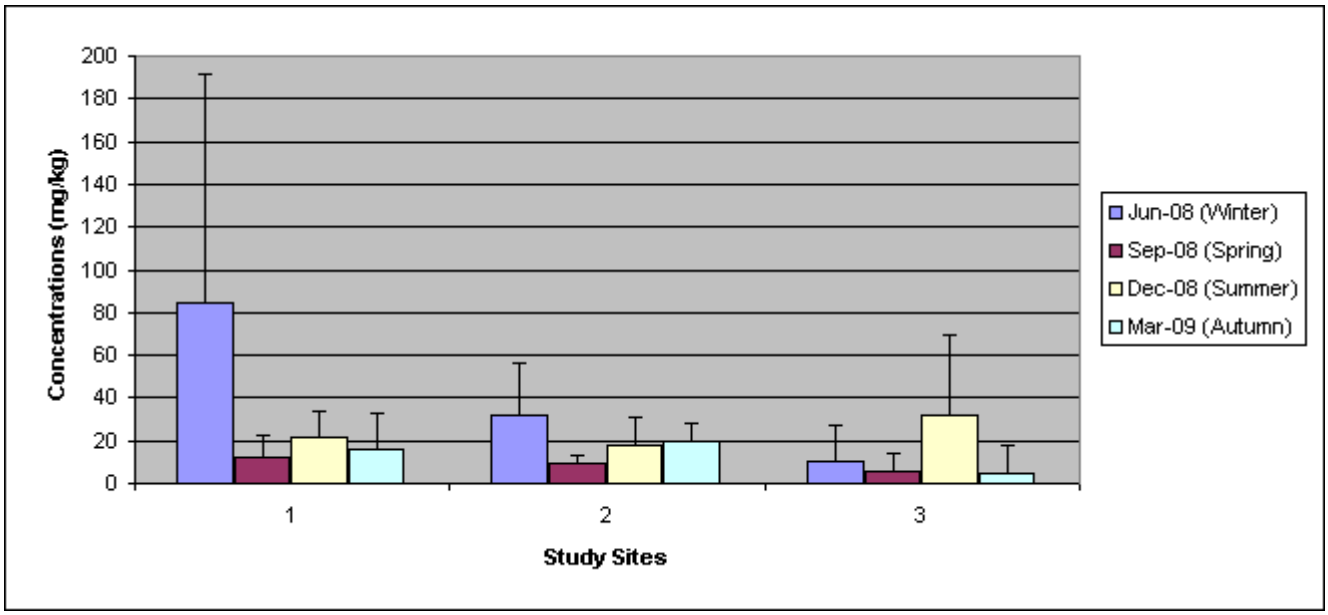

Figure 3.2.8 The mean (±SD) concentrations (mg/kg) of zinc measured in the shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the zinc concentrations in the shells of oysters at sampling site 2 decreased significantly (h=10.027, d.f: 3, p=0.018) from winter (Jun 2008) to spring (Sep 2009). No other statistical differences were recorded.

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicate that zinc concentrations measured in the oyster shells in autumn significantly differed (h=12.867, d.f: 2, p=0.002) between sampling sites 1 and 3 and also between sampling sites 2 and 3, where site 3 had the lowest mean on both occasions. No other statistical differences were recorded for zinc concentrations

Table 3.2.2: The mean (±SD) concentrations of zinc for water, sediment, oyster tissue and shells; collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

	Water (mg/L)		***Sediment (mg/kg)***		***Tissues (mg/kg)***		***Shells (mg/kg)***	
Sites and sampling occasion	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
June								
1	^{A} 0.00 ^{a}	0.00	^{A} 4.60 ^{a}	11.26	^{A} 324.77 ^{a}	173.11	^{A} 84.46 ^{a}	106.85
2	^{A} 0.06 ^{a}	0.16	^{A} 0.00 ^{a}	0.00	^{A} 562.80 ^{a}	254.81	^{A} 32.09 ^{a}	24.21
3	^{A} 0.18 ^{a}	0.43	^{A} 2.96 ^{a}	7.24	^{A} 415.67 ^{a}	135.58	^{A} 10.48 ^{a}	16.89
Sep								
1	^{A} 0.08 ^{a}	0.07	^{A} 3.16 ^{a}	1.05	^{A} 347.93 ^{a}	88.56	^{A} 11.89 ^{a}	10.25
2	^{A} 0.08 ^{a}	0.07	^{A} 2.65 ^{a}	0.81	^{A} 752.82 ^{b}	498.78	^{B} 9.01 ^{a}	4.50
3	^{A} 0.27 ^{a}	0.26	^{A} 2.55 ^{a}	0.81	^{A} 497.21 ^{a}	127.05	^{A} 5.55 ^{a}	8.75
Dec								
1	^{A} 0.10 ^{a}	0.24	^{A} 0.69 ^{a}	1.68	^{A} 269.12 ^{a}	99.67	^{A} 21.27 ^{a}	12.47
2	^{A} 0.01 ^{a}	0.03	^{A} 5.98 ^{a}	6.22	^{A} 749.68 ^{b}	333.35	^{B} 17.58 ^{a}	13.63
3	^{A} 0.14 ^{a}	0.18	^{A} 4.23 ^{a}	3.66	^{A} 598.01 ^{b}	199.69	^{A} 31.66 ^{a}	37.79
Mar								
1	^{B} 0.24 ^{a}	0.11	^{A} 17.37 ^{b}	19.62	^{A} 305.56 ^{a}	101.60	^{A} 16.13 ^{a}	16.55
2	^{B} 0.18 ^{a}	0.11	^{A} 3.61 ^{a}	1.67	^{A} 506.95 ^{b}	234.58	^{B} 19.56 ^{a}	8.43
3	^{A} 0.12 ^{a}	0.06	^{B} 5.28 ^{a}	3.09	^{B} 254.70 ^{a}	117.25	^{A} 4.91 ^{b}	13.35

*Statistical significant differences between sites per season are indicated by lower case letters (a,b) on the right hand side of mean values. Significant differences between seasons per site are indicated by capital letters (A, B) on the left hand side of mean values. Seasons were compared consecutively.

3.2.3 Copper

3.2.3.1 Water

The mean copper concentrations measured in the water samples for the duration of the study period are displayed in Figure 3.2.9 and Table 3.2.3.

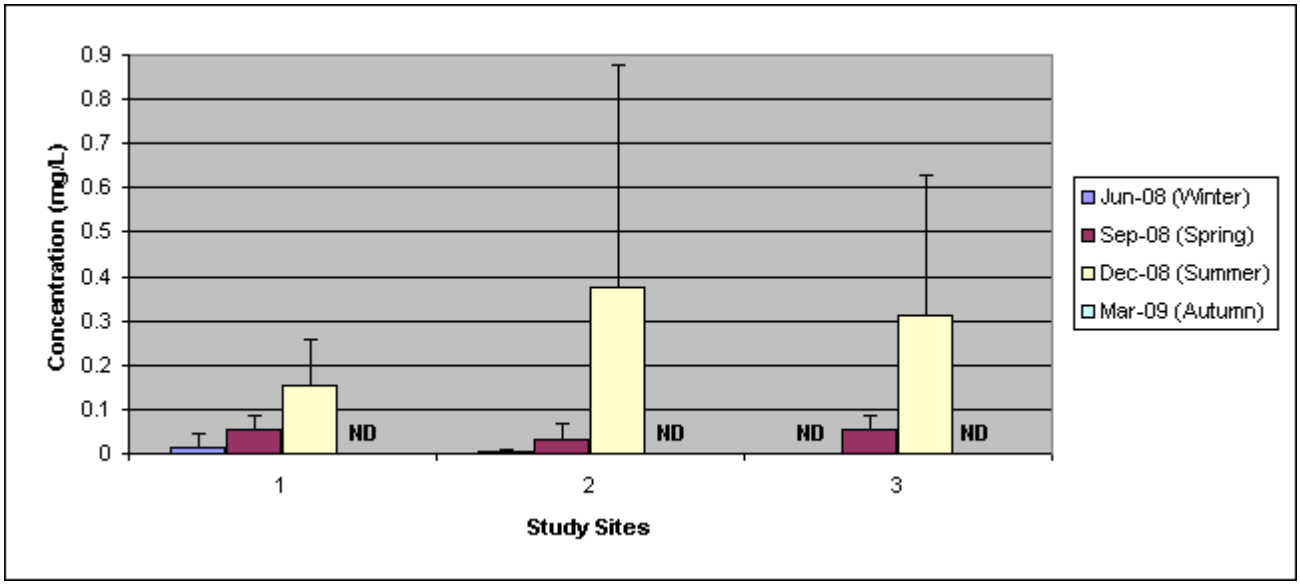

Figure 3.2.9 The mean (±SD) concentrations (mg/L) of copper in water from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72) with intervals for each season. ND = not detectable.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the copper concentrations in the water, decreased significantly ($p<0.001$) from summer (Dec 2008) to autumn (Mar 2009) at all the sampling sites (Site 1: $h=17.213$, d.f: 3, $p<0.001$; Site 2: $h=18.934$, d.f: 3, $p<0.001$; Site 3: $h=21.447$, d.f: 3, $p<0.001$) .

Comparisons of metal concentrations between sites

No significant differences ($p>0.05$) were recorded in the water copper concentrations between any of the sites during any sampling occasion.

3.2.3.2 Sediments

The mean copper concentrations measured in the sediment samples for the duration of the study period are displayed in Figure 3.2.10 and Table 3.2.3.

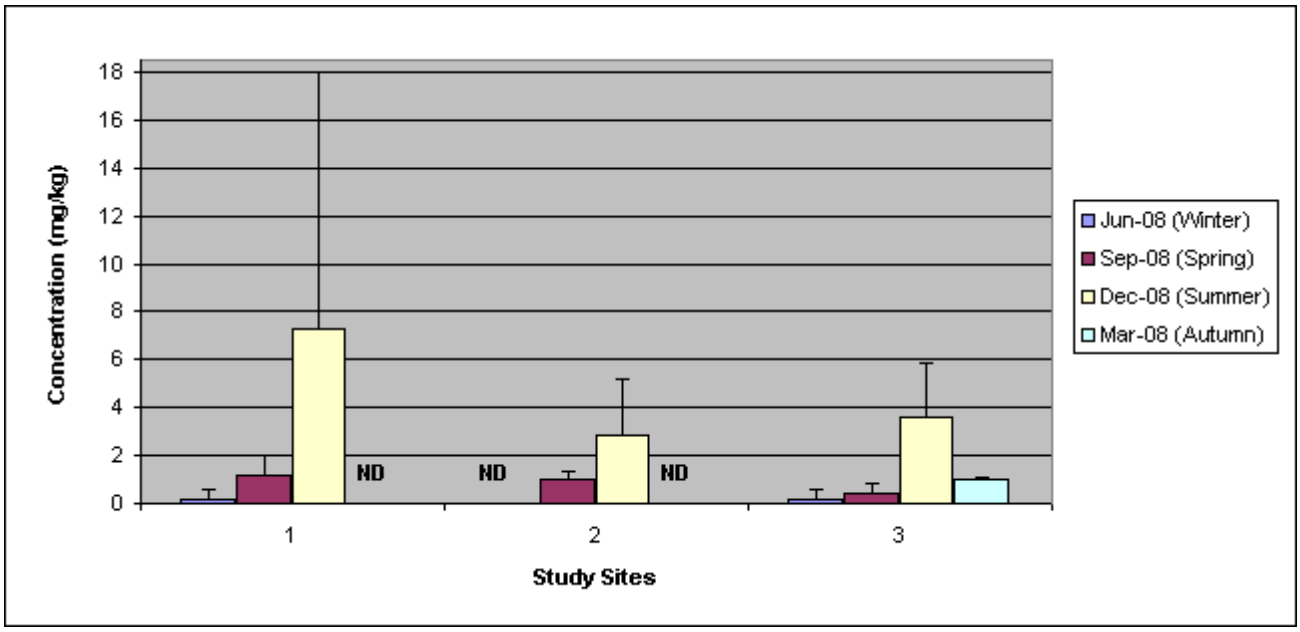

Figure 3.2.10 The mean (±SD) concentrations (mg/kg) of copper in the sediment from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72), with intervals for each season. ND= not detectable.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the copper concentrations in the sediment decreased significantly ($p<0.001$) at sampling site 1 ($h=16.856$, d.f: 3, $p<0.001$), from summer (Dec 2008) to autumn (Mar 2009). Also, comparisons indicated that from winter (Jun 2008) to spring (Sep 2008) the sediment copper concentration increased significantly ($p<0.001$) at sampling site 2 ($h=20.107$, d.f: 3, $p<0.001$), and then decreased significantly ($p<0.001$) from summer (Dec 2008) to autumn (Mar 2009). Sampling site 3 ($h=17.568$, d.f: 3, $p<0.001$) indicated a significant ($p<0.001$) increase in sediment copper concentrations from spring (Sep 2008) to summer (Dec 2008). No other statistical differences were recorded.

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicated that the copper concentrations measured in the surrounding sediments significantly differed ($p<0.001$) between sampling sites 1 and 3 and also between sampling sites 2 and 3 on the autumn ($h=16.129$, d.f: 2, $p<0.001$) sampling occasion, where site 3 had the highest mean. No other statistical differences were recorded.

3.2.3.3 Oyster tissue

The mean copper concentrations measured in the oyster tissue samples for the duration of the study period are displayed in Figure 3.2.11 and Table 3.2.3.

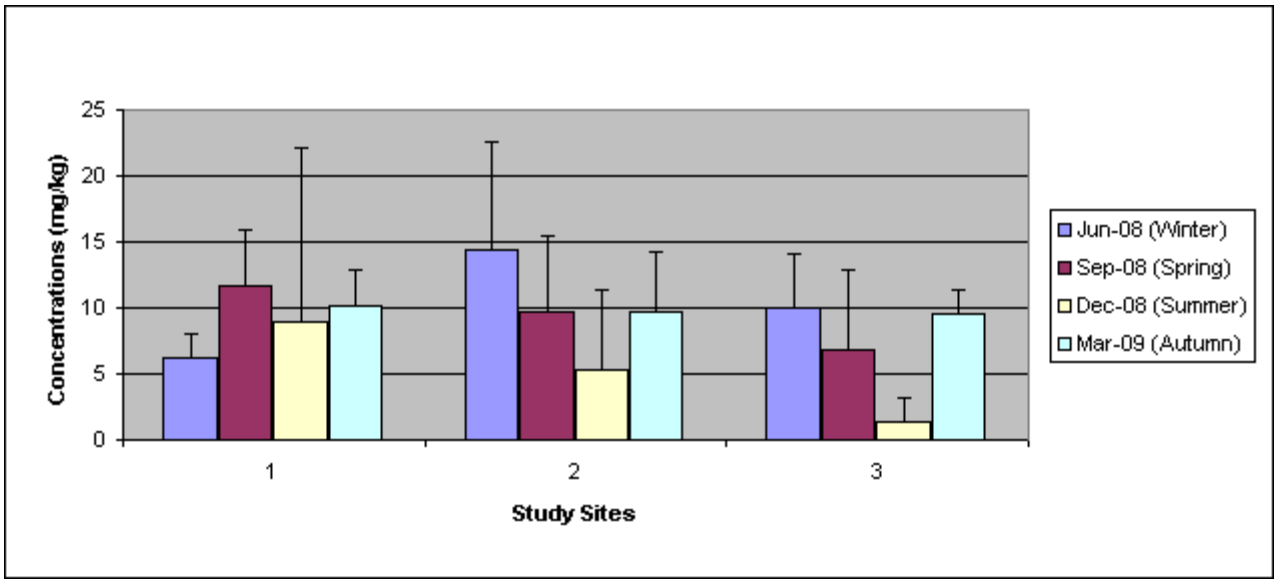

Figure 3.2.11 The mean (±SD) concentrations (mg/kg) of copper in the tissues of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that from winter (Jun 2008) to spring (Sep 2008) there was a significant increase ($p<0.001$) in copper concentrations in the tissues of the oysters at sampling site 1 ($h=15.597$, d.f: 3, $p=0.001$). Also, pairwise multiple comparisons indicated that the copper concentrations in the oyster tissues at sampling site 3 ($h=18.954$, d.f: 3, $p<0.001$), increased significantly from summer (Dec 2008) to the autumn (Mar 2009).

Comparisons of metal concentrations between sites

The pair wise multiple comparisons indicate that the copper concentrations in the oyster tissues significantly differed between sampling sites 1 and 2 on the winter sampling occasion (h=10.738, d.f: 2, p=0.005), where site 2 had the highest mean and site 1 the lowest. No other statistical differences were recorded.

3.1.3.4 Shells

The mean copper concentrations measured in the shell samples for the duration of the study period are displayed in Figure 3.2.12 and Table 3.2.3.

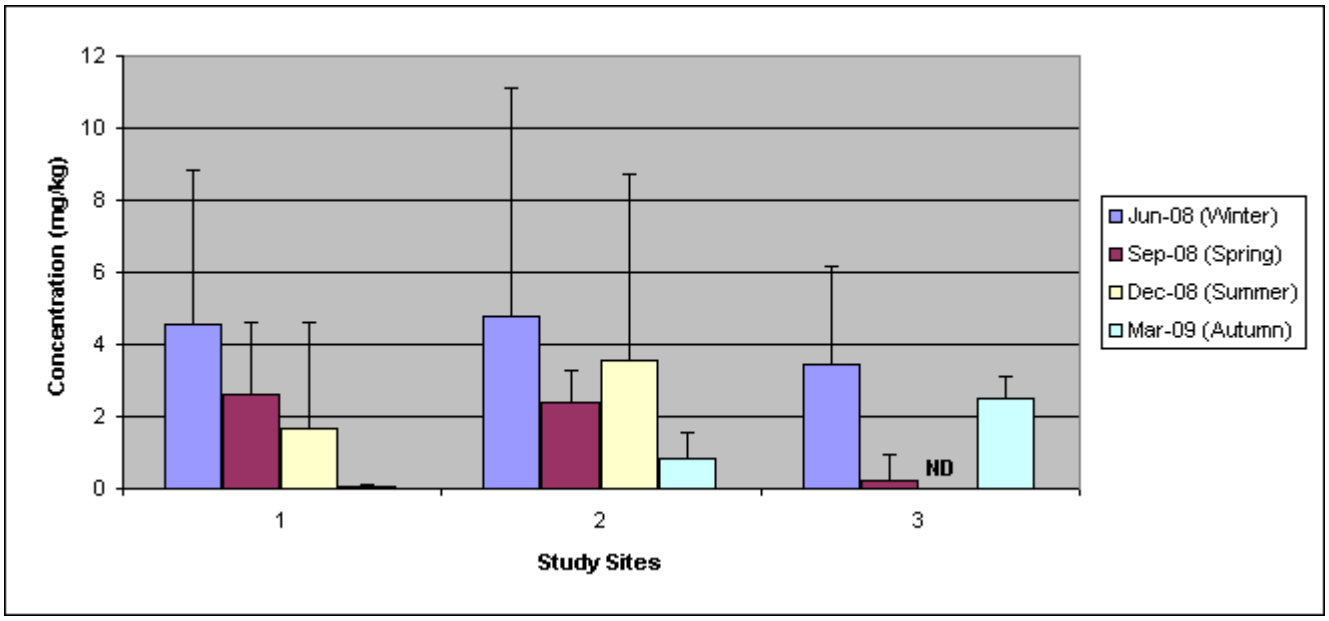

Figure 3.2.12 The mean (±SD) concentrations (mg/kg) of copper measured in the shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season. ND = not detectable.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the copper concentrations in the shells of oysters from sampling site 3 decreased significantly (h=30.441, d.f: 3, p<0.001) from winter (Jun 2008) to spring (Sep 2008), and then increased significantly (p<0.05) from summer (Dec 2008) to autumn (Mar 2009).

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicate that the copper concentrations measured in the shells of the oysters significantly differed ($p<0.001$) between sampling sites 1 and 3 and also between sampling sites 2 and 3 on both the spring ($h=15.805$, d.f: 2, $p<0.001$) and autumn ($h=22.528$, d.f: 2, $p<0.001$) sampling occasion. Site 1 had the highest mean for the spring sampling occasion and site 3 the lowest, where site 3 had the highest and site 1 had the lowest mean for the autumn sampling occasion.

No other statistical differences were recorded.

Table 3.2.3: The mean (±SD) concentrations of copper for water, sediment, oyster tissue and shells; collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

	Water (mg/L)		*Sediment (mg/kg)*		*Tissues (mg/kg)*		*Shells (mg/kg)*	
Sites and sampling occasion	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
June								
1	^{A} 0.013 ^{a}	0.032	^{A} 0.170 ^{a}	0.390	^{A} 6.180 ^{a}	1.780	^{A} 4.580 ^{a}	4.276
2	^{A} 0.003 ^{a}	0.007	^{A} 0.000 ^{a}	0.000	^{A} 14.420 ^{b}	8.115	^{A} 4.798 ^{a}	6.317
3	^{A} 0.000 ^{a}	0.000	^{A} 0.165 ^{a}	0.404	^{A} 9.972 ^{a}	4.173	^{A} 3.417 ^{a}	2.747
Sep								
1	^{A} 0.054 ^{a}	0.030	^{A} 1.154 ^{a}	0.829	^{B} 11.695 ^{a}	4.175	^{A} 2.629 ^{a}	1.990
2	^{A} 0.030 ^{a}	0.038	^{B} 1.039 ^{a}	0.298	^{A} 9.721 ^{a}	5.753	^{A} 2.374 ^{a}	0.881
3	^{A} 0.054 ^{a}	0.031	^{A} 0.425 ^{a}	0.407	^{A} 6.833 ^{a}	6.058	^{B} 0.225 ^{a}	0.713
Dec								
1	^{A} 0.155 ^{a}	0.102	^{A} 7.270 ^{a}	10.687	^{B} 8.952 ^{a}	13.181	^{A} 1.646 ^{a}	2.972
2	^{A} 0.375 ^{a}	0.503	^{B} 2.876 ^{a}	2.340	^{A} 4.712 ^{a}	6.009	^{A} 3.570 ^{a}	5.129
3	^{A} 0.313 ^{a}	0.314	^{B} 3.606 ^{a}	2.250	^{A} 1.308 ^{a}	1.885	^{B} 0.000 ^{a}	0.000
Mar								
1	^{B} 0.000 ^{a}	0.000	^{B} 0.000 ^{a}	0.000	^{B} 10.083 ^{a}	2.831	^{A} 0.040 ^{a}	0.091
2	^{B} 0.000 ^{a}	0.000	^{C} 0.000 ^{a}	0.000	^{A} 9.623 ^{a}	4.668	^{A} 0.819 ^{a}	0.761
3	^{B} 0.000 ^{a}	0.000	^{B} 0.985 ^{b}	0.093	^{B} 9.510 ^{a}	1.900	^{C} 2.502 ^{b}	0.584

* Statistical significant differences between sites per season are indicated by lower case letters (a,b) on the right hand side of mean values. Significant differences between seasons per site are indicated by capital letters (A, B, C) on the left hand side of mean values. Seasons were compared consecutively.

3.2.4. Iron

3.2.4.1 Water

The mean iron concentrations measured in the water samples for the duration of the study period are displayed in Figure 3.2.13 and Table 3.2.4.

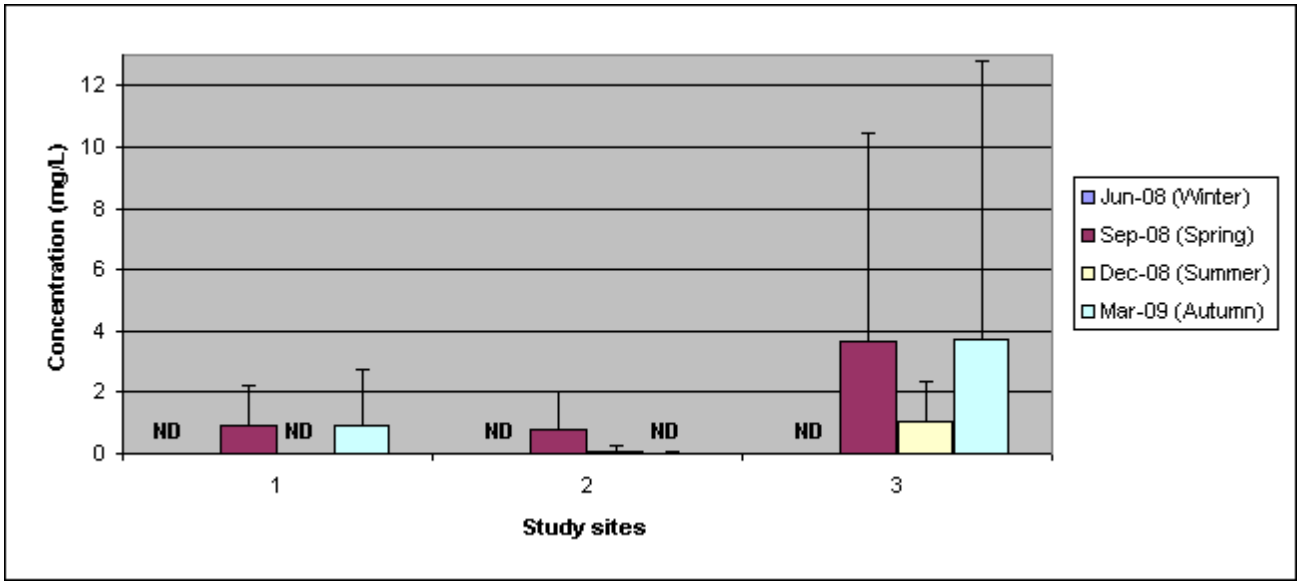

Figure 3.2.13 The mean (±SD) concentrations (mg/L) of iron in water from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72) with intervals for each season. ND = not detectable.

Comparisons of seasonal metal concentrations

No significant differences ($p>0.05$) were recorded in water iron concentrations between seasons, per site, in the course of the sampling period.

Comparisons of metal concentrations between sites

There were no significant differences ($p>0.05$) in water iron concentrations between any of the sites during any of the sampling occasions.

3.2.4.2 Sediment

The mean iron concentrations measured in the sediment samples for the duration of the study period are displayed in Figure 3.2.14 and Table 3.2.4.

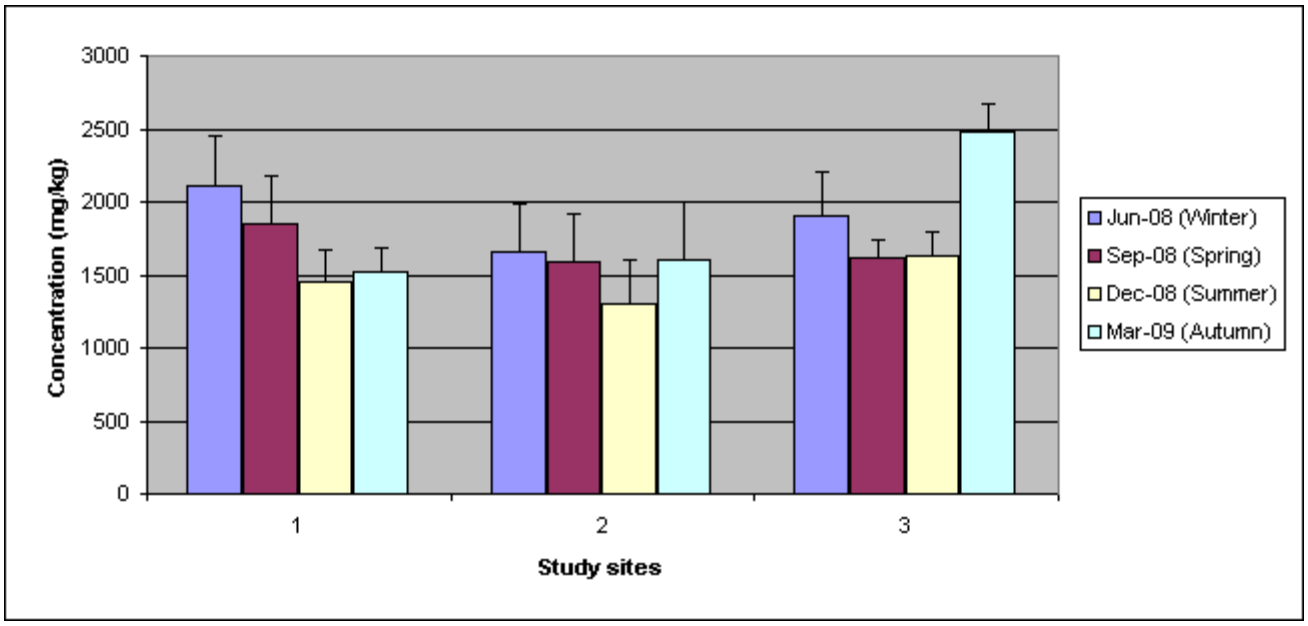

Figure 3.2.14 The mean (±SD) concentrations (mg/kg) of iron in the sediment from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=72), with intervals for each season.

Comparisons of seasonal metal concentrations

All pairwise multiple comparisons indicated that the iron concentrations in the sediment at sampling site 3, increased significantly (h=0.002, d.f: 3, p=0.002) from summer (Dec 2008) to autumn (Mar 2009).

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicated that the iron concentrations measured in the sediments significantly differed ($p<0.05$) between sampling sites 1 and 3 and also between sampling sites 2 and 3 on the autumn (h=11.415, d.f: 2, p=0.003) sampling occasion, where site 3 had the highest mean and site 1 the lowest. No other statistical differences were recorded.

3.2.4.3 Oyster tissue

The mean iron concentrations measured in the oyster tissue samples for the duration of the study period are displayed in Figure 3.2.15 and Table 3.2.4.

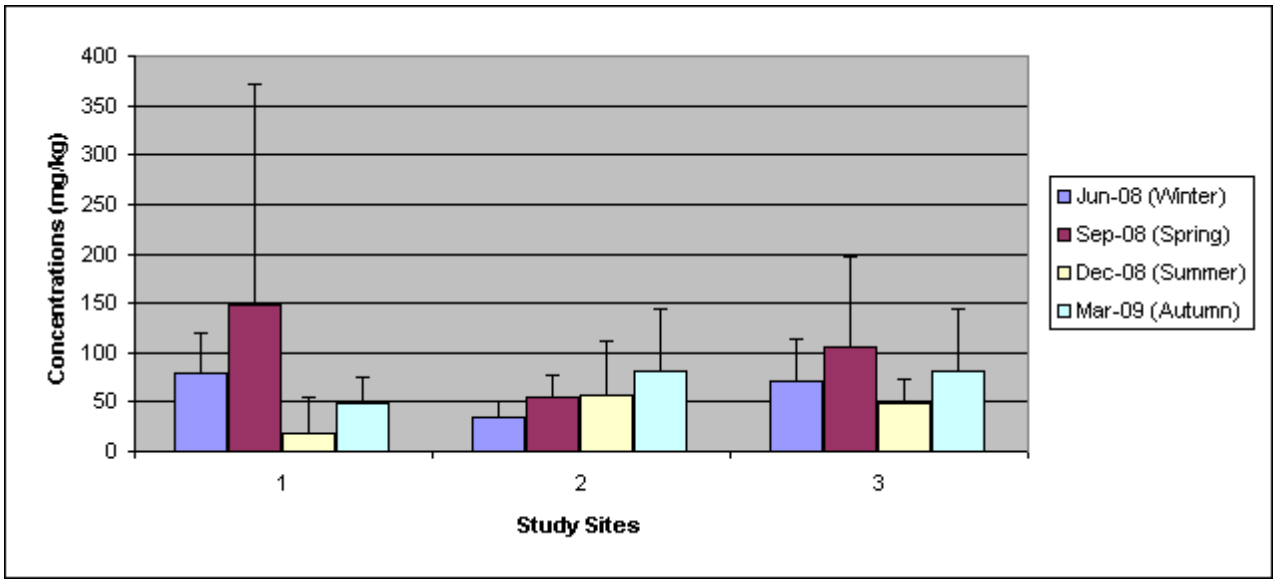

Figure 3.2.15 The mean (±SD) concentrations (mg/kg) of iron in the tissues of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season.

Comparisons of seasonal metal concentrations

No significant differences ($p>0.05$) were recorded in tissues of the oysters iron concentrations between seasons, per site, in the course of the sampling period.

Comparisons of metal concentrations between sites

The pair wise multiple comparisons indicate that the iron concentrations measured in the oyster tissues significantly differed ($p<0.05$) between sampling sites 1 and 2 on the winter ($h=10.196$, d.f: 2, $p=0.006$) sampling occasion, where site 1 had the highest mean and site 2 the lowest. No other statistical differences were recorded.

3.2.4.4 Shells

The mean iron concentrations measured in the shell samples for the duration of the study period are displayed in Figure 3.2.16 and Table 3.2.4.

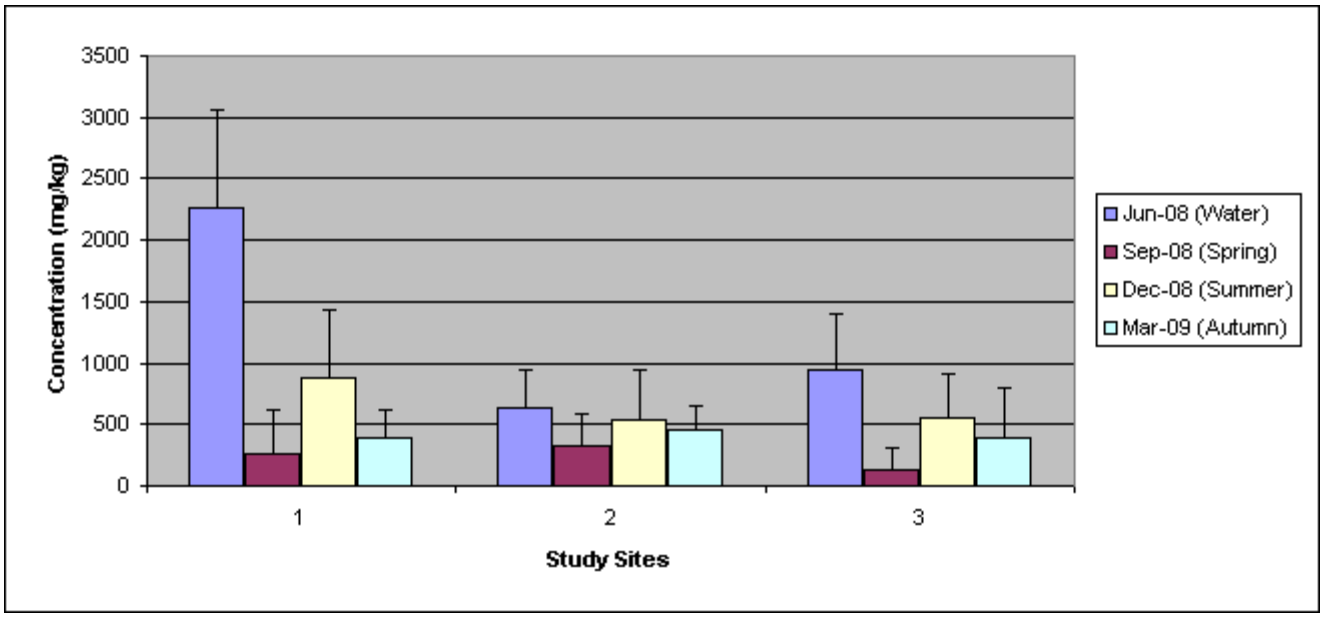

Figure 3.2.16 The mean (±SD) concentrations (mg/kg) of iron measured in the shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season.

Comparisons of seasonal metal concentrations

Pairwise multiple comparisons indicated that from spring (Sep 2008) to summer (Dec 2008) there was a significant decrease ($p<0.001$) in the iron concentrations in the shells of the oysters from sampling site 1($h=26.776$, d.f: 3, $p<0.001$). Also, pairwise multiple comparisons indicated that the iron concentrations in the oyster shells at sampling sites 1 (($h=26.776$, d.f: 3, $p<0.001$) and 3 ($h=19.117$, d.f: 3, $p<0.001$), decreased significantly ($p<0.001$) from winter (Jun 2008) to spring (Sep 2008). No other statistical differences were recorded.

Comparisons of metal concentrations between sites

The pairwise multiple comparisons indicate that the iron concentrations measured in the oyster shells significantly differed ($p<0.001$) between sampling sites 1 and 2 and also between sampling sites 1 and 3 on the winter ($h=17.631$, d.f: 2, $p<0.001$) sampling occasion, where site 1 had the highest mean and site 2 and 3 had the lowest. Also, pairwise multiple comparisons indicate that the oyster shells iron concentrations at sampling sites 2 and 3 on the spring ($h=7.556$, d.f: 2, $p=0.023$)

sampling occasion, significantly differed ($p<0.05$), where site 2 had the highest mean and site 3 the lowest. No other statistical differences were recorded.

Table 3.2.4: The mean (±SD) concentrations of iron for water, sediment, oyster tissue and shells; collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

	Water (mg/L)		***Sediment (mg/kg)***		***Tissues (mg/kg)***		***Shells (mg/kg)***	
Sites and sampling occasion	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
June								
1	^{A} 0.00 ^{a}	0.00	^{A} 2103.87 ^{a}	345.23	^{A} 79.89 ^{a}	39.52	^{A} 2268.44 ^{a}	789.64
2	^{A} 0.00 ^{a}	0.00	^{A} 1660.94 ^{a}	324.20	^{A} 34.19 ^{b}	16.92	^{A} 642.86 ^{a}	304.18
3	^{A} 0.00 ^{a}	0.00	^{A} 1909.29 ^{a}	290.61	^{A} 70.11 ^{a}	44.07	^{A} 936.18 ^{b}	462.22
Sep								
1	^{A} 0.91 ^{a}	1.33	^{A} 1855.23 ^{a}	328.12	^{A} 147.55 ^{a}	223.69	^{B} 254.70 ^{a}	363.14
2	^{A} 0.79 ^{a}	1.27	^{A} 1582.39 ^{a}	335.18	^{A} 55.38 ^{a}	20.88	^{A} 322.04 ^{a}	262.01
3	^{A} 3.63 ^{a}	6.82	^{A} 1614.06 ^{a}	131.05	^{A} 105.59 ^{a}	91.78	^{B} 124.19 ^{b}	183.41
Dec								
1	^{A} 0.00 ^{a}	0.00	^{A} 1451.23 ^{a}	226.11	^{A} 17.29 ^{a}	36.75	^{C} 885.80 ^{a}	544.82
2	^{A} 0.07 ^{a}	0.17	^{A} 1299.35 ^{a}	297.20	^{A} 56.51 ^{a}	55.65	^{A} 531.78 ^{a}	413.39
3	^{A} 1.04 ^{a}	1.31	^{A} 1635.51 ^{a}	163.62	^{A} 49.48 ^{a}	23.75	^{A} 548.55 ^{a}	362.16
Mar								
1	^{A} 0.93 ^{a}	1.81	^{A} 1517.79 ^{a}	164.22	^{A} 47.85 ^{a}	27.56	^{C} 388.41 ^{a}	222.36
2	^{A} 0.01 ^{a}	0.03	^{A} 1607.88 ^{a}	387.32	^{A} 81.80 ^{a}	62.34	^{A} 452.53 ^{a}	198.16
3	^{A} 3.76 ^{a}	9.05	^{B} 2478.16 ^{b}	194.51	^{A} 80.88 ^{a}	62.39	^{A} 388.59 ^{a}	408.57

* Statistical significant differences between sites per season are indicated by lower case letters (a,b) on the right hand side of mean values. Significant differences between seasons per site are indicated by capital letters (A, B, C) on the left hand side of mean values. Seasons were compared consecutively.

3.3 Comparison of metals between the oyster tissue and shells

The aluminium, zinc, copper and iron concentrations between the oyster tissues and shells measured for the duration of the study period, are displayed in the sections below.

*3.3.1 A*luminium

The mean aluminium concentrations measured in the tissue and shell samples for the duration of the study period are displayed in Figure 3.3.1.

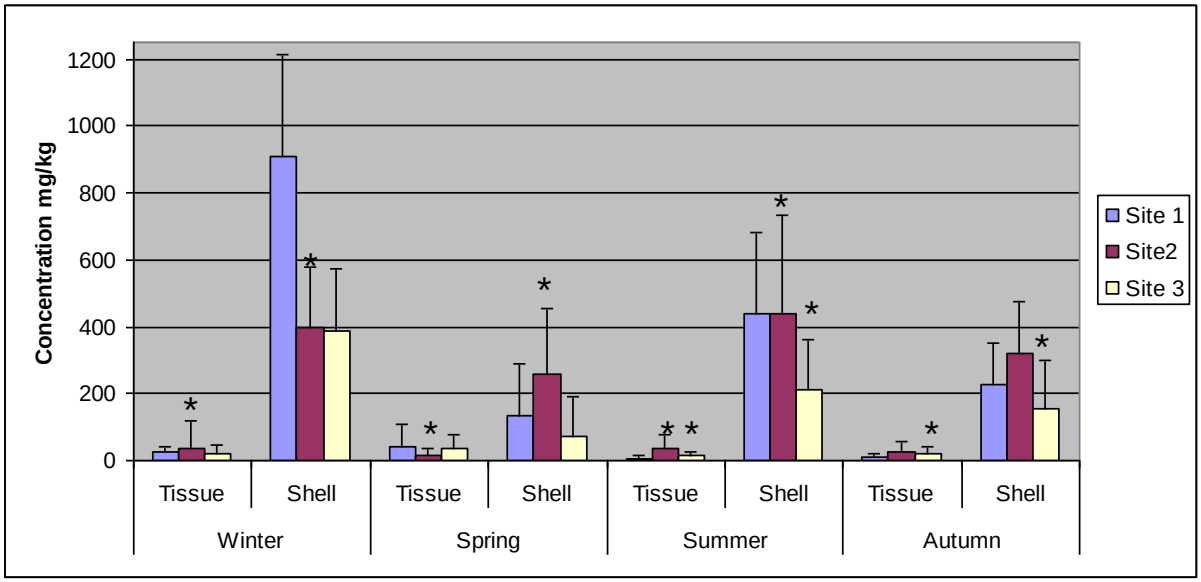

Figure 3.3.1 The mean (±SD) concentrations (mg/kg) of aluminium measured in the tissue and shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season. Asterisks indicate significant differences between tissues and shells per site, within respective sampling occasions/seasons.

The Mann–Whitney *U*-test indicated that the aluminium concentrations measured in the oyster tissues and shells differed significantly ($p<0.05$) at sampling site 2 for the winter, spring and summer sampling occasions. For each of these sampling occasions the aluminium concentrations were highest in the shells. Also, comparisons indicated that oyster tissue and shell aluminium concentrations at sampling site 3 differed significantly ($p<0.05$) for the summer and autumn sampling occasions. The aluminium

concentrations were highest in the shells for both occasions. No other statistical differences were recorded.

3.3.2 Zinc

The mean zinc concentrations measured in the tissue and shell samples for the duration of the study period are displayed in Figure 3.3.2.

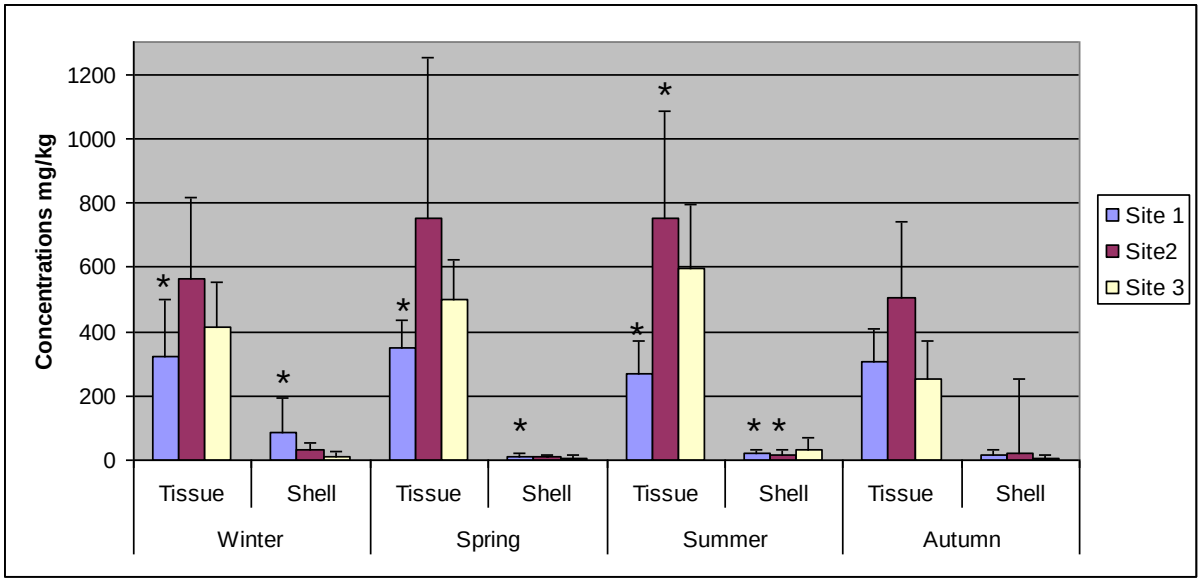

Figure 3.3.2 The mean (±SD) concentrations (mg/kg) of zinc measured in the tissue shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season. Asterisks indicate significant differences between tissues and shells per site, within respective sampling occasions/seasons.

The Mann–Whitney *U*-test indicated that the zinc concentrations measured in the oyster tissues and shells differed significantly ($p<0.05$) at sampling site 1 for the winter, spring and summer sampling occasions. For each of these sampling occasions the zinc concentrations were highest in the tissue concentrations. Also, comparisons indicated that oyster tissue and shell zinc concentrations at sampling sites 2 differed significantly ($p<0.05$) for the summer sampling occasion. The zinc concentrations were highest in the tissues. No other statistical differences were recorded.

3.3.3 Copper

The mean copper concentrations measured in the tissue and shell samples for the duration of the study period are displayed in Figure 3.3.3.

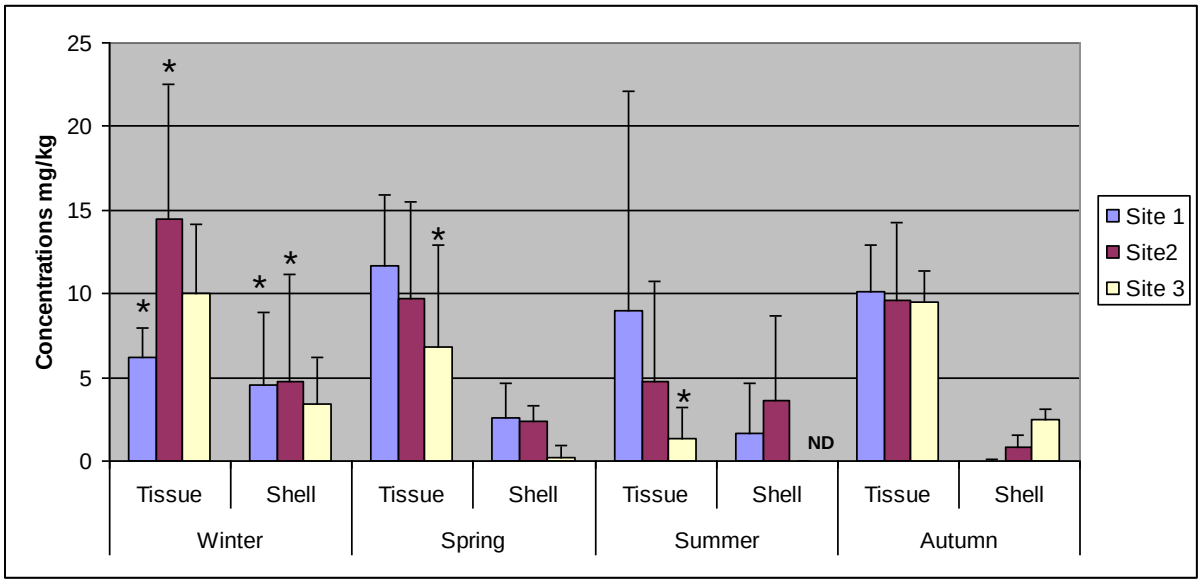

Figure 3.3.3 The mean (±SD) concentrations (mg/kg) of copper measured in the tissue and shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season. ND = not detectable. Asterisks indicate significant differences between tissues and shells per site, within respective sampling occasions/seasons.

The Mann–Whitney *U*-test indicated that the copper concentrations measured in the oyster tissues and shells differed significantly ($p<0.05$) at sampling site 1 and 2 for the winter sampling occasion. For each of these sampling occasions at both sites, the mean copper concentrations were highest in the tissues. Also, the comparisons showed that the copper concentrations in the tissues were significantly higher ($p<0.05$) at sampling site 2 for the spring sampling occasions. Furthermore, comparisons indicated that oyster tissue and shell copper concentrations at sampling sites 3 differed significantly ($p<0.05$) for the spring and summer sampling occasion. The copper concentrations were the highest in the tissues for both occasions. No other statistical differences were recorded.

3.3.4 Iron

The mean iron concentrations measured in the tissue and shell samples for the duration of the study period are displayed in Figure 3.3.4.

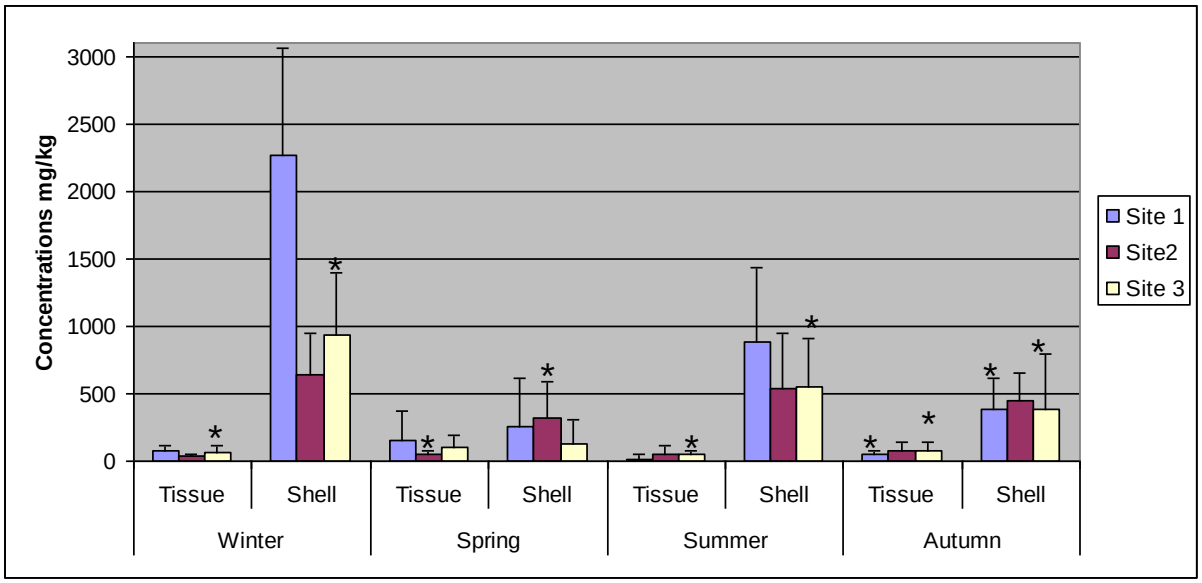

Figure 3.3.4 The mean (±SD) concentrations (mg/kg) of iron measured in the tissue and shells of oysters from site 1 (Wilderness), 2 (Witsand) and 3 (Goukamma, MPA) for the sampling period of one year (n=120), with intervals for each season. Asterisks indicate significant differences between tissues and shells per site, within respective sampling occasions/seasons.

The Mann–Whitney *U*-test indicated that the iron concentrations measured in the oyster tissues and shells differed significantly ($p<0.05$) at sampling site 1 for the autumn sampling occasion. The iron concentrations were highest in the shells for this sampling occasion. Also, comparisons indicated that oyster tissue and shell iron concentrations at sampling sites 2 differed significantly ($p<0.05$) for the spring sampling occasion with the shells containing the highest concentrations. Furthermore, comparisons indicated that the iron concentrations measured in the oyster tissues and shells significantly differed ($p<0.05$) at sampling site 3 for the winter, summer and autumn sampling occasions. The iron concentrations were highest in the shells on all occasions. No other statistical differences were recorded.

3.4 Bio-concentration factor (BCF)

The bio-concentration factors calculated for oyster tissues and shells for aluminium, zinc, copper and iron measured for the duration of the study period, are displayed in Tables 3.4.1, 3.4.2 and 3.4.3. Overall, BCF's were higher when water data was used as the environmental factor in the proposed BCF equation (refer to section 2.7.

Table 3.4.1: Bio–concentration factors for aluminium, iron, zinc and copper in oyster tissues and shells for site 1. CBC = could not be calculated

		Winter (Jun)	Spring (Sep)	Summer (Dec)	Autumn (March)
Tissues	**Water**				
	Aluminium	1.867	CBC	88.595	35.712
	Iron	CBC	161.997	CBC	51.651
	Copper	473.904	215.614	57.916	CBC
	Zinc	CBC	4544.725	2741.507	1283.858
	Sediment				
	Aluminium	0.052	0.157	0.025	0.052
	Iron	0.038	0.080	0.012	0.032
	Copper	36.318	10.137	1.231	CBC
	Zinc	70.643	110.038	392.428	17.589
Shells					
	Water				
	Aluminium	1288.629	CBC	5684.352	659.404
	Iron	CBC	279.648	CBC	419.222
	Copper	351.254	48.476	10.649	CBC
	Zinc	CBC	155.319	216.695	67.789
	Sediment				
	Aluminium	2.003	0.501	1.633	0.965
	Iron	1.078	0.137	0.610	0.256
	Copper	26.918	2.279	0.226	CBC
	Zinc	18.370	3.761	31.018	0.929

Table 3.4.2: Bio–concentration factors for aluminium, iron, zinc and copper in oyster tissues and shells for site 2. CBC = could not be calculated

		Winter (Jun)	Spring (Sep)	Summer (Dec)	Autumn (March)
Tissues	**Water**				
	Aluminium	CBC	CBC	135.085	749.687
	Iron	CBC	70.358	797.727	7668.694
	Copper	5224.741	328.309	12.570	CBC
	Zinc	8876.945	9731.366	69737.210	2895.769
	Sediment				
	Aluminium	0.116	0.066	0.163	0.106
	Iron	0.021	0.035	0.043	0.051
	Copper	CBC	9.361	1.638	CBC
	Zinc	CBC	284.496	125.272	140.524
Shells					
	Water				
	Aluminium	CBC	CBC	1535.466	8466.005
	Iron	CBC	409.171	7507.474	42424.938
	Copper	1738.309	80.165	9.523	CBC
	Zinc	506.093	116.443	1635.317	111.757
	Sediment				
	Aluminium	1.338	1.082	1.850	1.192
	Iron	0.387	0.204	0.409	0.281
	Copper	CBC	2.286	1.241	0.001
	Zinc	CBC	3.404	2.938	5.423

Table 3.4.3: Bio–concentration factors for aluminium, iron, zinc and copper in oyster tissues and shells for site 3. CBC = could not be calculated

		Winter (Jun)	Spring (Sep)	Summer (Dec)	Autumn (March)
Tissues	**Water**				
	Aluminium	CBC	87.213	24.152	21.079
	Iron	CBC	29.120	47.765	21.538
	Copper	CBC	126.413	4.182	CBC
	Zinc	2348.418	1815.790	4157.649	2130.775
	Sediment				
	Aluminium	0.051	0.155	0.051	0.043
	Iron	0.037	0.065	0.030	0.033
	Copper	60.418	16.067	0.363	1.000
	Zinc	140.635	195.021	141.298	48.242
Shells					
	Water				
	Aluminium	CBC	168.757	335.935	150.391
	Iron	CBC	34.249	529.489	103.478
	Copper	CBC	4.169	CBC	CBC
	Zinc	59.205	20.279	220.099	41.112
	Sediment				
	Aluminium	1.032	0.299	0.711	0.305
	Iron	0.490	0.077	0.335	0.157
	Copper	20.704	0.530	0.000	2.539
	Zinc	3.546	2.178	7.480	0.931

3.5 Neutral Red Retention Time Assay

The neutral red retention times in minutes, measured for the duration of the experiment, for oysters from all three sites, are indicated in Figure 3.5 with the statistical significant differences indicated in Table 3.5. The H statistic (h), degrees of freedom (d.f) and p values (p) have also been included for all Kriskal – Wallis One – Way ANOVA on Ranks tests for significant differences. Appendix A includes all H-statistics (h), degrees of freedom (d.f) and p values (p) for the NRRT assay in table A5.

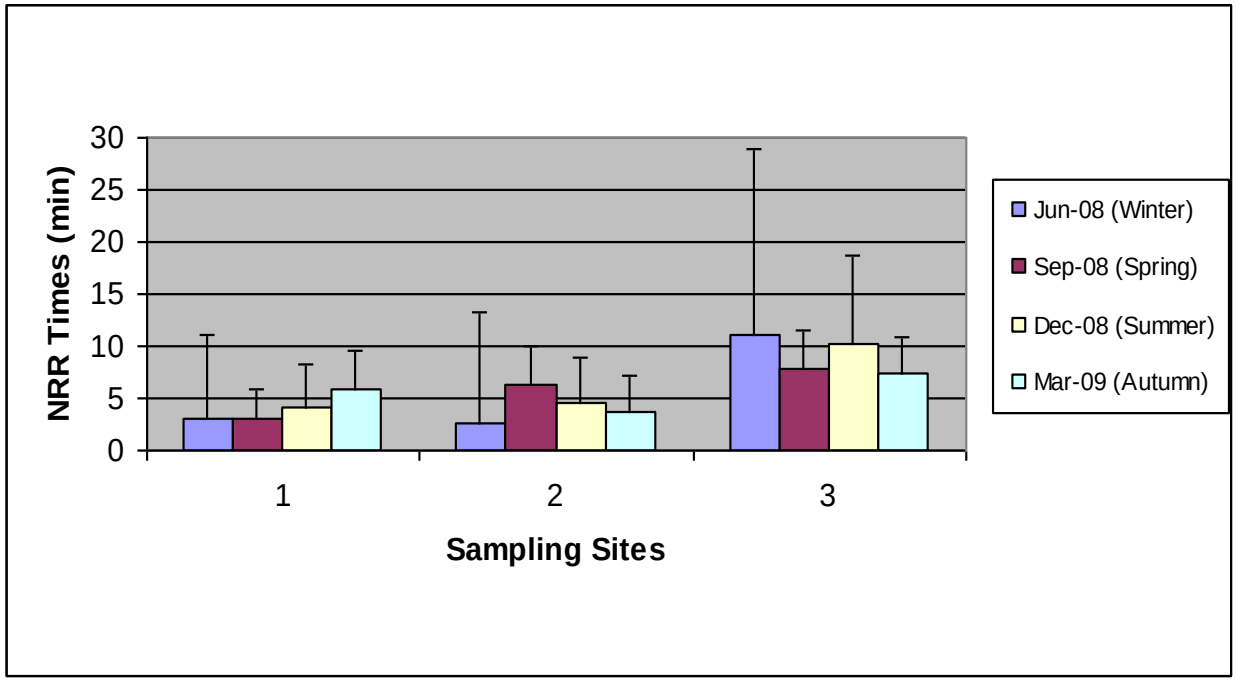

Figure 3.5 The mean neutral red retention times (NRRT) (minutes) recorded by counting haemocytes extracted from oysters (*Striostrea margaritacea)* collect from all three sampling sites, measured for the sampling period. N = 10 per site, per season.

Comparisons of seasonal neutral red retention times

No significant differences ($p>0.05$) in the neutral red retention times were recorded between seasons at any of the three sampling sites.

Comparisons of neutral red retention times between sites per season

The pairwise multiple comparisons indicated that the neutral red retention time assay were significantly different ($p<0.05$) between sampling sites 1 and 3 and also between

sampling sites 2 and 3 in winter (Jun 2008) (h=10.180, d.f: 2, p=0.006), where oysters from sampling site 3 had the longest retention time and site 2 the shortest (Table 3.1). Also, pairwise multiple comparisons indicated that the neutral red retention time for sampling sites 1 and 3 in spring (Sep 2008) were significantly different (h=8.440, d.f: 2, p=0.015), where sampling site 3 had the longest retention time and site 1 the shortest (Table 3.3). No other statistical differences were recorded.

Table 3.5: The mean neutral red retention times (minutes) recorded by counting haemocytes extracted from oysters (*Striostrea margaritacea)* at all three sites over the entire sampling period. N= 10 per sampling site, per occasion.

Sites	Mean	SD
Jun (Winter)		
1	$^{A}3^{a}$	8.13
2	$^{A}2.6^{a}$	10.57
3	$^{A}11^{b}$	17.85
Sep (Spring)		
1	$^{A}3^{a}$	2.83
2	$^{A}6.20^{a}$	3.79
3	$^{A}7.8^{b}$	3.69
Dec (Summer)		
1	$^{A}4.2^{a}$	4.13
2	$^{A}4.6^{a}$	4.41
3	$^{A}10.2^{a}$	8.44
Mar (Autumn)		
1	$^{A}5.8^{a}$	3.68
2	$^{A}3.8^{a}$	3.30
3	$^{A}7.4^{b}$	3.73

* Statistical significant differences between sites per season are indicated by lower case letters (a,b) on the right hand side of mean values. Significant differences between seasons per site are indicated by capital letters (A) on the left hand side of mean values. Seasons were compared consecutively.

CHAPTER 4

Discussion

4.1 Physico-chemical parameters

The ambient water and sediment in coastal and estuarine areas are subject to multiple anthropogenic or naturally occurring stress factors such as physical, chemical and microbiological agents (Urban *et al.,* 2010). Factors such as pH, salinity and temperature play a major role in the mobilization of metals, nutrients and other chemical compounds (Hutchings *et al.,* 2008).

The temperature range for the ambient water was 18ºC - 24.5ºC at site 1,17ºC - 24ºC at site 2 and 18ºC - 23.5ºC at site 3 (Table 3.1). According to Schumann & Beekman (1984) and Schumann *et al.* (1982, 1995), the south coast of South Africa is highly variable with regards to oceanic temperature as a result of wind-driven upwelling along the coast. The variability in these fluctuations has been recorded to be so dynamic and sudden that certain fish populations along the south coast have been affected in terms of mortality (Hanekom *et al.,* 1989). The temperatures recorded within the present study are common along the south coast and due to the variability, temperature ranges have been known to be even more extensive (Schumann *et al.,* 1995). Although the temperature range can be considered as being broad, it was uniformly so at all three sites, with all the sites having almost the same extent of range.

The salinity range measured at site 1 was 34.5‰ - 38.5‰, 27‰ - 34‰ at site 2 and 34‰ - 39‰ at site 3 (Table 3.1). The average range for sea water in South Africa is 34.5‰ - 35‰ (DWAF, 1995). Higher salinity events at shallow depths (<20m) are usually a result of evaporation with increasing temperatures, however, within the present study, temperatures were towards the bottom of the range recorded. The elevated levels of salinity at sites 1 and 3 could have been due to the lack of

freshwater input into the ocean as at both of these sites the river mouths were closed when high salinity was recorded.

Low salinities at shallow depths (<20 m) vary in both temporal frequency and duration in response to rainfall, associated runoff and wind stress (McLeod & Wing, 2008). This fact is supported by the data collected from site 2 (Witsand). As previously mentioned in section 2.2, the site is located at the mouth of one of South Africa's fastest flowing estuaries with a freshwater influx to the ocean all year round. The low salinity recorded at this site coincided with the season in which there was heavy rainfall and flooding events (own observation).

In chemistry, pH (potential Hydrogen) is a measure of the acidity or basicity of an aqueous solution. Pure water is considered to have a neutral pH of 7, with values >7 being basic or alkaline and values <7 are considered to be acidic (DWAF, 1996). The pH range measured in this study of 7.7 – 8 at site 1, 7.9 – 8 at site 2 and 7.7 – 8 at site 3 was within the range described by the South African Water Quality Guidelines (DWAF, 1995), which states that the pH of seawater usually ranges from 7.9 to 8.2, the majority of the recorded data fell within this range. pH and temperature are also directly linked as shown in a study conducted by Gieskes (1969), where pH was shown to increase with 0.0114 units as temperature increases in degrees Celsius. Both instances where the pH decreased within the present study, were in summer at site 1 and 3, respectively. The pH however did not increase with the increase in temperature but decreased by 0.2. Ocean acidification could be one of the causes for this and can be defined as the ongoing decrease in the pH of the oceans, caused by the uptake of anthropogenic carbon dioxide (CO_2) from the atmosphere (Caldeira & Wickett, 2003). With reference to metal availability, the pH of the ambient water determines the concentration and solubility of most natural occurring trace metals (DWAF, 1996).

To conclude, the south coast of South Africa is a highly variable and dynamic environment with physico-chemical parameters that fluctuate in wide ranges.

Temperature measurements recorded in this study, although highly variable, were within the same range at all the sites and can be considered as normal. The differentiation of the salinity range can be linked to the freshwater influx from rivers, rain or the subsequent runoff. Elevated levels can be due to the lack of these inputs. The pH range recorded was well within the range set by DWAF (1995) with the exception of two occasions within the summer months. Various other physical and chemical factors could have been responsible for this and additional measurements should be taken to confirm which of these factors could be responsible.

4.2 Aluminium *(Al)*

Aluminium is a silvery-white, ductile and malleable metal. It forms an average of 8% of the earth's crust and is one of the most reactive of common metals. Aluminium is released into the environment both by natural processes and from anthropogenic sources. Despite its prevalence in the environment, aluminium is not known to be used by any form of life, although plants and animals tolerate it well (UNEP, 1997).

4.2.1 Water

No significant differences in aluminium concentrations were found between the water samples collected for the entire sampling period. According to UNEP (1997), the tolerance range for aquatic invertebrates ranges from 0 – 59.6 mg/l based on a laboratory experiment. In the present study, the highest value of aluminium in water samples was recorded at 1 mg/l, which is well within the tolerance range of aquatic invertebrates. There are no water quality guidelines for marine ecosystems available for South African conditions to compare to the aluminium concentrations found in this study. According to the Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME, 2007), the limit for aluminium in freshwater is a maximum of 0.1 mg/l. However, no Canadian guidelines exist for aluminium for marine environments. In a study conducted by Okoro (2012) within the Cape Town harbour, aluminium concentrations ranged from 0.006 to 0.598 mg/l. Although the harbour is considered to be a contaminated site in terms of other substances, the aluminium concentrations recorded are below the LC_{50} threshold for invertebrates. When compared to the range

recorded by Okoro (2012), aluminium concentrations measured in the current study are much higher.

The overall low concentrations of aluminium measured in the water could be due to the chemistry and nature of the compound (UNEP, 1997). Aluminium occurs in the environment in the form of silicates, oxides and hydroxides, combined with other elements such as sodium (Na). It is not found as a free metal because of its reactivity. Also, aluminium-bearing solid phases in the environment are relatively insoluble, particularly at neutral pH values, resulting in low concentrations of dissolved aluminium in most natural water bodies (UNEP, 1997). Furthermore, the volume and tidal movements of the ocean could also be considered as a reason for the low concentrations of aluminium. All sites are located on the south coast, where the fast moving Agulhas current is prominent, but there are also counter currents inshore and upwelling taking place seasonally (Hancke, 2010).

Although there were no significant differences recorded between sites, it is of interest to note that the two highest concentrations were recorded at site 3, where they increased from summer to autumn almost two-fold (Goukamma, MPA). Factors such as pH and temperature influence the degree of solubility of aluminium in an aquatic environment, as they do for most other metals, where a decrease in pH results in an increase in mobility and availability for certain forms of aluminium (Vreysen & Maes, 2006). While temperature increased, pH decreased at site 3 within this sampling occasion. The increase of temperature and decrease of pH coincides with the increase of the aluminium concentration within the water from summer to autumn. As previously discussed, the pH of seawater usually ranges between 7.9 and 8.2 (DWAF, 1995). The pH values recorded during the study are generally within the range as prescribed by DWAF (1995) (Table 3.1), with the exception of 7.7 recorded on more than one occasion, including at site 3 within summer. This lower pH could be one of the factors contributing to the higher aluminium concentration within the water at site 3 for the summer occasion. As the pH increased in autumn, the increase of concentration within the autumn month could be due to the mobilization of aluminium

from other non-point sources. A variety of factors, such as the presence of other organic particles and total suspended solids (TSS) were not recorded within the water samples and could be responsible for some of these results.

In conclusion, the aluminium concentrations were generally low with the exception of two higher mean concentrations at Goukamma in the summer and autumn sampling occasions. Low concentrations can be attributed to physico-chemical parameters and the nature of the compound itself, whereas higher concentrations could possibly be due to TSS within the water column, which were not measured. The availability of the metal, as a result of these parameters, and the water movement could also contribute to both high and low water concentrations.

4.2.2 Sediment

Aluminium silicates (clay), a major compartment of sediments, contribute to the aluminium levels in sediments. This metal compound can be highly concentrated in sediments as a result of derived dust from activities such as agriculture and mining but natural processes far outweigh direct anthropogenic contributions to the environment (UNEP, 1997). Such natural processes may include weathering of rock formations and in this case, strong wave action.

Results indicated 240mg/kg and 500mg/kg as the lowest and highest concentrations, measured in the present study, respectively. There are no current and updated sediment quality guidelines for marine sediments available for South African conditions to compare to the aluminium concentrations found in this study. Other guidelines, such as the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life (CCEM, 2002) do not indicate the probable effects levels for aluminium in sediments. In a study by Vermeulen & Wepener (1999), in Richards Bay, KwaZulu Natal, aluminium had a range within the sediments between 4726 and 81368 mg/kg. The upper range was considered as being too high whereas the lower spectrum of the range was not viewed as problematic. As the results of the current study reflect

roughly 10% of the lowest value, it could be argued that the sediments of these sites are not heavily contaminated with aluminium.

No significant differences were recorded between the sites within the study period. Site 3 (Goukamma, MPA) had the highest concentration and lowest concentration for the sampling period, although not significant. As previously discussed, physico-chemical parameters have direct impacts on the availability of metals within the environment. As discussed in section 4.2.1, the pH and temperature could be responsible for the lower and higher concentrations of aluminium, in conjunction with the dynamic water movement. Metal concentrations that are considered low within the sediments can also vary by orders of magnitude over relatively small spatial scales depending on the sediment mineralogy, granulometry and organic content amongst other factors (CSIR, 1997). Metals typically bind to fine grained sediments, such as mud and organic matter (Orr *et al.,* 2007). No data were collected in terms of grain size and sediment composition. This information could prove valuable and provide further information as to the movement of aluminium within the sediment. Also, sediment core samples were not taken, resulting in the reflection of surface sediment concentrations only.

Furthermore, results indicated a significant decrease of aluminium concentrations from winter to spring at site 3 (MPA) and then a significant increase from summer to autumn at the same site for sediments. The reason for the fluctuation of aluminium could be due to a decreased binding of this metal at lower pH, which enhances the availability of metals to surrounding waters and in turn re-deposits back into the sediments over time (Hutchings *et al.,* 2008). Many elements have low water solubility and are particle–reactive and as a direct result, concentrations of most elements in bottom sediments and at the sediment-water interface usually exceed those in the overlying water column by several orders of magnitude (Newman & Watling, 2007). This fact is again proven in the current study.

To conclude, the aluminium concentrations in the sediment are well below the proposed contamination concentration when compared to guidelines and other studies. Site 3 (Goukamma, MPA) had the highest and lowest concentration of aluminium which could be due to variability in the environment and also fluctuation in physico-chemical parameters. Grain size and sediment structure data are absent and should be incorporated for further studies.

4.2.3 Tissue

The mean range recorded within the oyster tissue was 6.85 – 41.88mg/kg. The concentrations of aluminium in the tissues cannot be compared to data for the same species, due to a lack of research using *Striostrea margaritacea* as a biomonitor. Studies that incorporate aluminium within the tissues of oysters in general are also not available. Studies that have been conducted do not reveal the actual range or measurement of aluminium within the tissues or the shell. Aluminium studies within other aquatic bivalve/mollusc species are also lacking. It is therefore difficult to conclude whether the concentrations in the tissues are high or low. As previously mentioned, aluminium is not a trace element that is used by plants and animals. They are however able to tolerate aluminium in low concentrations.

The bio–concentration factor (BCF) for the tissues, calculated using ambient water concentrations can be considered as mostly low and in some cases moderate at all three sites, as displayed in Tables 3.4.1, 3.4.2 and 3.4.3. The BCF does not necessarily indicate the source of available metals for uptake but does give an indication of the degree of accumulation within the animal when compared to environmental data. Tissues with BCF's greater than a 1000 are considered high, and under 250, low, with those in-between classified as moderate (Walker *et al.,* 2006). The BCF's using sediment data were exceptionally low which can theoretically indicate that the aluminium within the sediment might not have been available for uptake or remobilization into the water column, and the concentrations within the water column could have been from another non-point source of aluminium which was available for uptake. Metals enter the organism primarily through the digestive tract as

a result of the ingestion of enriched organic particles (Amaral *et al*; 2005). As oysters are filter feeders, water is ingested and organic compounds are absorbed from the water column (Roesijadi, 1996).

No significant differences were recorded between the sites. Site 1 had the highest concentration which was recorded within the spring sampling occasion. The lowest concentration was recorded at site 1 within the summer sampling occasion. The same trend is evident for the other two sites as well where concentrations fluctuated from season to season dramatically, but were not statistically reflected. This indicates high variability and could have been caused by a number of factors. As discussed in sections 4.2.1 and 4.2.2, the south coast is highly variable in terms of its hydrology and water dispersal. The lack of significant differences in aluminium within the oyster tissues between sites could also be directly linked to the bioavailability of aluminium. Bioavailability is influenced by many integrated processes, including physical, chemical and biological processes (Anderson & Hillwalker, 2008). Bioavailability, as well as inaccessibility, can determine whether or not certain elements, in this case aluminium, can cause adverse effects in organisms (Peinjnenburg & Jager, 2003). These processes include the mass transfer and uptake of substances into organisms, which are determined by the substance properties, compartment properties and the biology of the organism to name a few (Anderson & Hillwalker, 2008).

There were also no seasonal trends evident. As for the comparison between sites, bioavailability could be a major contributing factor and serve as an explanation for some of these results. Within the BCF results, there was a high variability between seasons using both water and sediment data. The aluminium concentrations found within the tissues can thus be from a source present within the animal, such as faecal matter or food particles, rather than an external factor. As aluminium is not a micronutrient, it stands to reason that the concentrations within the body are not necessarily due to bioaccumulation within the animal, but could be a reflection of the aliuminium present within the animal at a specific time. In a study conducted by Kennedy (1986), it was speculated that the concentration found within the animal

tissue is not always a true reflection of the actual concentrations within the animal. He suggested that the true concentration of an element within the tissue could be expressed as: total concentration minus (a) fluids lost during preparation minus (b) the extraneous material present in the visceral mass and intestine minus (c) the adhering sediment that has not been washed off and intestinal sediments minus (d) contamination picked up during preparation (Kennedy, 1986). Within the present study, some of these factors such as the contamination during preparation and the loss of fluids have been addressed. Sediments within the digestive tract were also taken into consideration and a purging period was introduced as previously mentioned in section 2.3. The possibility of sediment within the digestive tract can however not be overlooked and could be a possible source of aluminium within the organism.

It is inconclusive whether or not the range of aluminium within the tissues is high or not. Data sets are lacking and not available for comparison for oyster species and/or molluscs in general. The BCFs for water were low to moderate which indicated that the concentrations within the tissues were accumulated to a low/moderate degree when viewed as a fraction of the mean water concentration. The source of aluminium within the tissue could also be ingested food particles as well as the possible presence of sediment within the digestive tract.

4.2.4 Shell

No significant differences were recorded between sites or seasons for aluminium in the oysters' shells. The concentrations varied from 69.8 – 908.48 mg/kg. In a study conducted by Almeida *et al.* (1998), the concentrations in control oyster shells of the species *Crassostrea virginica* were in the range of 1631 – 3668 mg/kg, which is well above those concentrations found in the present study. Concentrations can by this association be considered as low. Further literature for concentrations of aluminium within the oyster shell is lacking, locally as well as internationally.

As displayed in Tables 3.4.1, 3.4.2 and 3.4.3, the bio-concentration factors are generally low for the oyster shells in terms of aluminium, except for a few incidences

where the BCF's using water data, well exceeded 1000, which is considered as high (Walker *et al.,* 2006). This indicates that the concentrations within the shell are orders of magnitude higher than that of the ambient water. According to Almeida *et al.* (1998) the incorporation of trace metals have been studied and observed in the calcium carbonate skeletons of many marine molluscs All shell components are secreted by the various mantle epithelium cells. The shell's internal layer, known as the nacreous layer or mantle, is never directly in contact with external factors. This indicates that all components incorporated into this layer are a result of the animal's metabolic activity which stems from the tissues or the animal itself (Almeida *et al.,* 1998). As excess debris was removed from the shell before analysis, it is unlikely that the aluminium concentrations recorded in the present study have been biased by other sources. A study done by Pierce & Mann (2006) supports this theory and states that aquatic organisms have the potential to record in their shells changes in the environmental conditions in which the organism lived.

4.2.5 Comparison between oyster tissue and shell

When the shell and tissue data sets were compared, significant differences were found. Figure 3.3.1 shows that during the winter, spring and summer sampling occasions the concentrations were significantly higher in the shell at site 2. Although there is not a significant difference at site 2 in the autumn sampling occasion, the concentrations in the shell are still much higher than in the tissues. With the theory of accumulation in mind, this site, although the concentrations within the water may be lower than at other sites, still has the most input of freshwater runoff and by association the most potential for anthropogenic input, as the mouth of the estuary is open all year around. Also, the size of the oysters should be taken into consideration as they are no longer juveniles and can be considered as adults, and accumulation within the tissues has been an ongoing process for not only the one year of sampling. The compartmentalization of aluminium within the oyster shell could be due to years of uptake via the oyster tissues and could account for the high concentrations. As previously mentioned in section 4.2.4, the shell's internal layer, known as the nacreous layer or mantle, is almost never in contact with external factors and this in

turn indicates that all components incorporated into this layer are a result of the animal's metabolic activity which stems from the tissues or the animal itself (Almeida *et al.,* 1998).

Significant differences were also recorded at site 3 for the summer and autumn sampling occasions. Once again the concentrations were much higher in the shells than in the tissues. It has been established that metals can substitute for the Ca^{2+} ion and so become incorporated in the calcium carbonate shell of bivalves, thus the shells of molluscs can act as a deposition centre for excess metals (Tynan *et al.*, 2005). As previously mentioned, the BCF's using water data were higher and could support the theory that uptake via the tissues was from the water column rather than the sediments. The water concentrations at site 3 for both the summer and autumn sampling occasions coincide with this as the concentrations were the two highest means recorded.

In conclusion, the aluminium concentrations within the shell were low when compared to other studies. The BCF's were generally low with a few high incidences reflected when using water data. The concentrations in the shells were much higher than that found in the tissues. Marine molluscs have the ability to metabolize and store metals within their shells (Almeida *et al.*, 1998). The concentrations at site 2 were higher in the shells throughout the year with the exception of the autumn sampling occasion. This could have been due to the high amount of freshwater and terrestrial runoff throughout the year and the potential aluminium contained within that runoff and in turn in the food sources of the oysters. The compartmentalization of elements that are not used within the tissue in routine metabolic activity could also explain the reservoir of aluminium within the shell. At site 3 in the summer and autumn sampling period, the concentrations were the highest within the range recorded.

4.3 Zinc (Zn)

Zinc is introduced to the environment by natural and anthropogenic processes (Meiller & Bradley, 2002). Zinc metal, in its free ion form, does not occur in the environment

naturally, but is present in the divalent state zinc (II), which can form complexes with a wide range of matter. Most rocks and many minerals contain zinc in varying amounts. This would explain why the largest natural emission of zinc to water results from erosion. The main anthropogenic sources of zinc are mining, iron and steel production, waste disposal and incineration, the use of zinc–containing fertilizers and pesticides, and runoff water (UNEP, 2001). Zinc is an essential micronutrient for all organisms, playing a critical role in a variety of biochemical processes including regulatory, structural, and enzymatic functions (Ju *et al.,* 2011). Although zinc is essential, it is toxic at high concentrations (Koh *et al.,* 1996).

4.3.1 Water

The range of zinc recorded within the water was 0.0 – 0.27mg/l. According to the South African Water Quality Guidelines for coastal marine waters, the maximum zinc concentration for unpolluted marine water is 0.005mg/l (DWAF, 1995; 1996) and the mean concentration commonly found in the surface water around South Africa is 0.00659 mg/l. Furthermore, at a concentration of 0.002mg/l, larval growth in oysters will cease and a LC_{50} test resulted in mortalities of larval oysters at a concentration of 0.0031mg/l (DWAF, 1995). In a recent study by Okoro (2012) zinc concentrations in water from Cape Town harbour were in the range of 0.02 – 1.8mg/l. The concentrations found in the present study were well above those reported by DWAF (1996) but lower than the range recorded by Okoro (2012). Higher concentrations can be attributed to a number of factors, including the chemical composition of the compound itself (UNEP, 2001). Concentrations in the present study were also above the Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME, 2007) which is 0.03 mg/l for freshwater. There are no Canadian guidelines for zinc available for marine waters.

Although zinc concentrations within the water were considered to be high at all three sites, site 3 had the highest concentrations for the winter, spring and summer sampling occasion and site 1 the highest in the autumn sampling occasion. When considering the geographical positioning of the sites and the hydrodynamics, site 3

(Goukamma, MPA) is the most isolated in terms of transport and circulation. Schumann *et al.* (1982) suggested that there are cases of down-welling in that area, which traps bodies of water and re-circulates them within the bay. If the water is re–circulated, the concentration of zinc will not be diluted as in most other cases and could become exceptionally high, as in this study.

The significant increase in zinc at sites 1 and 2 from summer to autumn could have been due to oceanic mixing during the summer months along the Agulhas bank (Schumann & Beekman, 1984). When compared to a study conducted by Watling & Watling (1982) this increase in the zinc concentration found in the present study from summer to autumn followed an opposite trend, as the concentrations decreased in their study from summer to autumn. The river mouths at both sites 1 and 2 were open in the summer months which could have been a contributing factor to the increase of zinc as rivers deposited run-off water in the area during this time.

As for all metals, the pH of the ambient water strongly influences the concentration of soluble zinc. Elevated zinc levels can be associated with a fluctuation in pH (DWAF, 1996). As shown in Table 3.1, the pH levels were always above the neutral value of 7. Although the solubility of trace metals is generally considered to increase with a decrease in pH, there are a few exceptions. Higher zinc concentrations at neutral and alkaline pH occur where zinc occurs largely as a colloidal suspension of zinc hydroxide (DWAF, 1996). As previously discussed, pH and temperature are also directly linked as shown in a study conducted by Gieskes (1969), where the pH will increase as temperature increases. As shown in Table 3.1, the water temperature in autumn at site 1 was 24.5 °C as opposed to 22°C that was recorded in summer. This temperature increase coincides with an increase in pH, strengthening the argument that physico-chemical parameters could be a reason for elevated zinc concentrations within the water column. Also, as previously mentioned, the upwelling that occurs along the south coast in summer (Hancke, 2010) can have a significant influence on the seasonality of elements.

To conclude, zinc concentrations within the water column were exceptionally high when compared to guidelines. Site 3 has the overall highest concentrations between sites. This could be attributed to the hydrodynamics of the area. Seasonal variability between sites coincided with fluctuations in the physico-chemical parameters and seasonal mixing due to upwelling.

4.3.2 Sediment

The mean range of zinc within the sediment was 0 – 6 mg/kg with one outlier value of 17 mg/kg. There are no current or updated sediment quality guidelines for marine sediments available for South African conditions, this no comparisons could be drawn. When the results are compared to the Canadian Sediment Quality Guidelines they can be considered as extremely low. The interim freshwater quality guideline is 124mg/kg with a probable effect level of 271mg/kg (CCME, 2007). In a study undertaken by Orren *et al.* (1981), they described the range of a “clean” beach to be 0.9 – 7.7 mg/kg in terms of zinc in the sediments. When the current results are compared to a study done by Mitileni *et al.* (2011) in the Limpopo Province, the range of zinc found around a mining site was 12.5 - 36.2mg/kg. Mitileni *et al.* (2011) did not conclude whether the concentrations were high. They did however look at the distribution of the metal within the sediment column (surface – 20cm depth) found along the mining sites. The zinc was found to be more concentrated towards the bottom of the sediment column. As previously mentioned, core samples were omitted in the present study and thus the results can only be seen as a “snapshot” of metal concentrations and should not be interpreted as the accumulative value for the sites. A recent study done by Sparks (2012) showed that the mean concentration of zinc from five sites around the Cape Peninsula was 14.26 mg/kg. The results of the present study are well below that.

When comparing sites, no significant differences were recorded. Also, there was high variability between sites and there was no trend visible where zinc at one site was consistently higher or lower than another. The variability between the sites could have been due to the dynamic nature of the hydrology in the surrounding areas. The

concentrations in which zinc were present could be directly attributed to availability of the metal in the immediate surroundings. Weathering of rocks is a direct result of wave action and when no point sources of input can be identified, the weathering of rocks is considered to have an input into the environment (Roesijadi, 1996). This could indicate that the source of input is not from anthropogenic activities but from a natural source and can be regarded as background concentrations for the sediments. As the water concentrations were exceptionally high, it can be argued that the zinc did not settle/deposit within the sediments, but were redistributed to other sources.

No trends were evident between the seasons. Zinc within the sediment significantly increased from summer (4.5mg/kg) to autumn (6 mg/kg) at site 3. The physico-chemical parameters all fluctuated from summer to autumn. As metals are affected by physico-chemical parameters making them either more soluble or less within the water column, the increase in the zinc concentration can be attributed to their variability in this case, as there is not a clear trend or pattern evident.

It is a well documented fact that the concentrations of various metals are generally higher in the sediments than in the water column (Orren *et al.*, 1981; CSIR, 1997; Newman & Watling, 2007). This finding is once again proven with the present study. The zinc concentrations in the sediments are considered to be exceptionally low and as previously discussed, zinc is known to be present in the natural environment with input sources such as, the weathering of rocks among other factors. An assumption can be made that the sediments are probably not contaminated through only anthropogenic sources. Concentrations that are present can be viewed as natural "background" concentrations.

4.3.3 Tissue

As previously mentioned, zinc is a micronutrient and it is naturally available in the environment and is used by the organism in routine metabolic processes (Roesijadi, 1996). The range of zinc recorded within the oyster tissues was 220mg/kg - 780mg/kg. In a study done by Roesijadi (1996), the range recorded for the Eastern flat

oyster, *Crassostrea virginica*, indicate that it had a tolerance of 300 – 13000 mg/kg for zinc within the tissues. The concentrations recorded for the current study fall well within this range, although it is a different species of oyster. Studies that incorporate the South African oyster, *Striostrea margaritacea*, are either lacking, outdated or difficult to compare with, as they used wet weights in analyses and calculations (Watling & Watling, 1976; Watling & Watling, 1982; Watling, 1983).

The BCFs can mostly be considered low when oyster tissue concentrations are compared to concentrations in the water and sediment (Tables 3.4.1; 3.4.2; 3.4.3). There were two occasions where the BCFs were well above the ceiling limit of 1000 as set by Walker *et al.* (2006). Water was the environmental medium used in both of these incidences and as discussed in 4.3.1, the concentrations in the water were well above the limits set by the South African Water Quality Guidelines (DWAF, 1996). These BCF results support the findings of authors such as Amiard *et al.* (1986), Viarengo (1989), Dallinger and Rainbow (1993), De Mora *et al.* (2004) and Damiens *et al.* (2006), who stated that oysters have the ability to accumulate and store high concentrations of metals within their tissues.

There was a trend visible between sites where animals at site 2 consistently had the highest Zn concentrations in their tissues throughout the sampling period. Site 2 (Witsand) has also been previously identified as the only site where the river has an input into the oceanographic system all year round. It could be argued that tissue zinc concentrations were higher at that site due to greater water volume entering the ocean and also probably due to a higher zinc bioavailability. In spring, zinc concentrations were higher at site 2 than site 1 and in summer, there were significant differences between site 1 and 2 and between site 1 and 3, where site 1 had the lowest mean concentration for zinc concentrations within the tissues. Site 1 (Wilderness) had very little freshwater input into the oceanic system as the mouth of the Touws River was closed for the majority of the study. Also, when drawing a comparison between site 1 and site 2's freshwater input, the Breede River (site 2) is open all year round, is one of the fastest flowing estuaries in South Africa and is much larger than the Touws River.

There were no seasonal trends for zinc concentrations within the oyster tissue. Only at site 3 did zinc decrease from summer (600mg/kg) to autumn (250mg/kg). It is well established that metal concentrations in oyster tissues are highly variable and influenced by natural and anthropogenically induced changes in the environmental conditions (Ju *et al.,* 2011). The fluctuation of zinc concentrations within the oyster tissue can also be as a result of the continuous processes that are defined physiologically as the outcome of the processes of influx (uptake rate) and efflux (release rate) (Roesijadi, 1996). Metals enter the organism primarily through the digestive tract as a result of the ingestion of enriched organic particles (Amaral *et al*., 2005). These ingested metals bind with metallothionein proteins and accumulate in lipofucines or amorphous granules (Viarengo, 1989) and because of the high similarity of metallothioneins and zinc, in terms of composition and structure, and in conjunction with low excretion rates, granules tend to keep metals inside the cell (in non–toxic forms) (Amaral *et al.*, 2005). Furthermore, Roesijadi (1996) suggested that the variability within the tissues in terms of metal concentrations are not always a result of the net movement of metals into or from organisms, but simply reflects changes in biomass associated with use and storage of nutrient reserves and the amount of gametes. These changes can alter metal concentrations by diluting or concentrating the pool of metals contained in the tissues. Oysters within the present study were spawning shortly before analysis and this could have had a direct effect on the concentrations of metals within the tissues.

In oysters, as in numerous other marine animals, the enhanced bioaccumulation that results in environments subject to anthropogenic contamination is superimposed on their natural ability to accumulate metals. Concentrations of metals in the tissues of marine organisms are subsequently four to five orders of magnitude higher than those in the surrounding seawater (Roesijadi, 1996). This is once again proven in the current assessment and by the bio-concentration factors for tissues and water, which ranged from 1283 – 69 737.

In conclusion, it is difficult to conclude whether or not zinc concentrations within the oysters are at a toxic level as this trace metal is highly variable within the oyster, although concentrations are relatively low when compared to other studies. The levels of variation could be caused by the oyster incorporating and using zinc within its own metabolic processes.

4.3.4 Shell

The range for zinc within the shells was 4.9 mg/kg – 32 mg/kg with one outlier of 84.5 mg/kg recorded at site 1 within the winter sampling occasion. Zinc concentrations recorded for "clean" shells of the oyster, *Crassostrea virginica,* in a study conducted by Almeida *et al.* (1998), were in the range 3.1 – 24.1 mg/kg. The range recorded for the current study falls generally within the range recorded for "clean" oysters with two concentrations being slightly elevated and one concentration that can be considered as exceptionally high. Carriker *et al.* (1991) found that the concentration of zinc varied as much as 300 times in different parts of the valves of oyster species. Only a sub-sample of 0.3 g was taken in the current study and provides a general overview of the zinc concentrations within the oyster shell, rather than a point specific concentration within the shell. As previously mentioned, studies that incorporated the South African oyster, *Striostrea margaritacea*, as a subject species, are either lacking or outdated.

As displayed in Tables 3.4.1, 3.4.2 and 3.4.3, the BCFs for the shell using ambient water and sediment data, were found to be relatively low, with two occasions where the BCFs were 506 and 1635 times higher in the shell than in the water. These factors are considered to be moderate and high respectively (Walker *et al.,* 1996), which indicate that the concentrations within the shell are orders of magnitude higher than that of the ambient water. This in turn supports the theory that the zinc that was available within the water column could have been bio-accumulated within the shell via the tissue of the animal. As previously discussed, the incorporation of trace metals have been studied and observed in the calcium carbonate skeletons of many marine molluscs (Almeida *et al.,* 1998) and as shell components are secreted by the shell's internal layer, known as the nacreous layer or mantle, it is never in contact with

external factors. This indicates that all components incorporated into this layer are a result of the animal's metabolic activity and not from external sources. Results indicated differences between site 1 and 3 and between site 2 and 3 in autumn, where site 3 had the lowest concentration, not only for this season, but for the entire sampling period. As previously discussed, marine bivalves have the ability to store metals within their shells and metals that are in the shells are contributed by the oyster tissue. The tissue zinc concentrations of oysters from site 3 were also the lowest within the sampling period, indicating the relationship between the oyster shell and tissues. Within the shells, zinc only displayed one seasonal difference at site 2, where the concentrations decreased from winter to spring. When compared to the tissue data, concentrations within the tissues were higher in the spring sampling period and lower within the shell when compared to winter. Seasonal variations and cycles in metal concentrations within both the oyster and its shell are governed by local environmental conditions that determine availability of metals, food supplies and biological processes, such as reproduction (Roesijadi, 1996). As zinc is used in metabolic activities, the shell of the oyster could possibly be used as a reservoir for surplus metals that are not needed within the tissue at any given point and are then remobilized into the tissues when there is a need for them, such as during reproduction (Carriker *et al.,* 1991). This could explain the decrease of concentrations within the shell and concomitant increase in the tissues.

4.3.5 Comparison between oyster tissue and shell

When the shell and tissue data sets are compared, significant differences were found. Figure 3.3.2 shows that the concentrations were higher within the tissues at site 1 within the winter, spring and summer sampling occasions and higher at site 2 within the summer sampling occasion. Although there were no other significant differences recorded, the concentrations in the shells were generally lower than in the tissues. As previously discussed, zinc is an essential element for all organisms, playing a critical role in a variety of biochemical processes including regulatory, structural, and enzymatic functions and will be retained within the tissues where it is readily available for these processes, rather than stored in the shell. When external concentrations of

zinc (from sediment and water) get too high, i.e. sources of input, the organism's homeostatic capacity will fail and toxic effects will occur (Ju *et al.,* 2011). In addition to this, Dineley *et al.* (2003) supplied evidence that shows high intracellular free zinc can promote neuronal death by inhibiting cellular energy production. Free zinc within the tissues that are not being incorporated within processes or stored within various organs seem to be shifted and stored in the shell as a detoxification or excretion mechanism (Carriker *et al.,* 1991). This phenomenon could explain why the concentrations of zinc are higher within the tissues than the shells of the animal.

In conclusion, zinc concentrations in oyster shells can be considered generally low to moderate when compared to other studies. When data is compared between shells and tissues, the movement of zinc is evident between them and provides some explanation for variation between sites and seasons. The use of zinc within the animal for metabolic activities can be considered the main reason why concentrations are lower within the shell than in the tissue.

4.4 Copper (Cu)

Copper is an essential trace element for plants, animals and humans (Fernandez *et al.*, 2007) and is essential for the normal functioning of cytochrome oxidase (Walker *et al.,* 2006). It is also responsible for the stabilization of lysosomal membranes (Roesijadi, 1996). Copper occurs in three oxidation states, namely metallic copper (0), cuprous copper (I) and cupric copper (II) (DWAF, 1996). Copper is introduced to the marine environment anthropogenically by sources such as anti – fouling paint used on aquatic vessels (DWAF, 1995).

4.4.1 Water

The range of copper for this study was recorded as 0 mg/l – 0.38 mg/l. According to Domestic Water Use Guidelines (DWAF, 1996) the characteristic concentration of copper in seawater is 0.0003mg/l while the range in the South African Water Quality Guidelines (DWAF, 1995) for coastal marine waters was reported to be 0.0328 mg/l. At the latter concentration, an LC_{50} test was conducted and concluded over a period of

12 days at a temperature of 25 degrees on larvae of *Crassostrea virginica* (DWAF, 1995) and it was found that half of the sample group died. The Canadian Water Quality Guidelines suggest a range of 0.002 – 0.004 mg/l for freshwater. There are no Canadian guidelines for copper available for marine waters.

Furthermore, in a study conducted by Orrin *et al.* (1981) it was found that the mean concentrations of copper in water samples taken from a "clean" site were within the range of 0.3 – 1.5 mg/l. A range of 0.007 – 0.07mg/l was recorded by Okoro (2012) at a polluted harbour-based site. The present study's results are well above the South African Domestic Water Use Guidelines, the South African Water Quality Guidelines, Canadian Water Quality Guidelines (CCME, 2007; DWAF, 1995; 1996) and the range given by Okoro (2012).

There were no significant differences between the sites. High variability was once again present. The concentrations of metals in the water column are subject to great temporal and spatial variability (Orr *et al.,* 2008) and this makes it difficult to obtain water samples that are representative of the contaminant status of the water body.

A seasonal trend is visible across all three sites where the concentration of copper within the water increased from winter to spring and then declined from spring to summer and again summer to autumn, with a mean concentration of 0.0 ± 0.0 mg/l in autumn. This could be explained by current movements and the upwelling phenomenon. Dynamic wave action caused by the prevailing winds in the winter months subside in summer and reduces the weathering of rocks, which are considered to be a major contributor of metals to the environment (Engel, 1988). The low concentrations and often high variability of metal concentrations in the water column can be a direct result of the difference in flow (e.g. currents) and variable anthropogenic inputs, which in turn causes researchers to obtain only a "snapshot" of contamination problems when working with water as a medium (CSIR, 1997). Anthropogenic activities are considered to be one of the major factors that influence the geochemical cycle, and as a consequence, the availability of metals (Roesijadi,

1996). The high copper concentrations found in the water samples could be due to anthropogenic inputs like boating and river run-off. The river mouths at all the sites were open when these higher concentrations were recorded.

pH is said to be the physico-chemical parameter that is very important for the availability of copper within the water column (DWAF, 1995). The environmental data in Table 3.1 show that the pH levels were slightly elevated at all three sites for the autumn sampling period. At neutral and alkaline pH, the concentration of copper in surface water is usually low due to the nature and solubility of the compound (DWAF, 1995). Temperature fluctuated over the sampling period between sites, but was uniformly high at all three sites in the autumn sampling occasion which could have caused the pH to lower and in turn the copper concentrations within the water column to rise.

To conclude, copper concentrations within the water column were high when compared to guidelines and other studies. Seasonal data followed a steady increase from winter to spring and in turn, summer, where after it decreased to 0 mg/l in the autumn months. This could be due to the availability of copper in the environment through inputs such as rivers and anthropogenic activities within the area.

4.4.2 Sediment

The range for copper within the sediment was 0mg/kg – 7mg/kg. With no South African Sediment Quality Guidelines available, a comparison was drawn with the Canadian Sediment Quality Guidelines. According to the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life, the limit for copper concentration within the sediment is 35.7 mg/kg and the probable effects limit is 197mg/kg (CCME, 2002). The results within the present study are well within the range proposed by the Canadian Sediment Quality Guidelines and could even be considered as low. In a study done by Orren *et al.* (1981), who used Witsand as a study site, the set range for copper in sediments of a “clean” beach was 0 – 0.005mg/kg. In comparison, the concentrations of the present study could be considered high. It should be noted that

the study conducted by Orren *et al.* (1981) was almost 30 years ago and with the rate of development and increased anthropogenic activity, the sources of contamination are presently almost certainly much more than 30 years ago. In a more recent study conducted by Sparks (2012), the mean concentration of copper from five sites in the Cape Peninsula was found to be 2.73 mg/kg. The present study's concentrations are above that when the range is compared, but is still well below the concentrations given in the Canadian Sediment Quality Guidelines.

Comparisons between the sites followed no trend throughout the study period and high variability was present. On one occasion there was a significant difference between sites where site 3 had the highest mean concentration and site 1 and 2 the lowest. In another instance there was a significant difference recorded where site 1 had the higher concentrations when compared to site 3. Metals do not degrade and with continued input and limited sediment distribution, metals can accumulate to high concentrations in certain areas (Newman & Watling, 2007). Although concentrations are still below the suggested range, the phenomena described by Newman & Watling (2007) could be responsible for the recording of elevated levels of copper within the sediments. As previously mentioned, sites that are more sheltered and incur less wave action will not have excessive sediment distribution and this could be a reason for the elevated metal concentrations.

There were a number of significant differences seasonally where the concentration increased at site 2 from winter to spring and then decreased again from spring to summer. At site 3, there was only a decrease from spring to summer and a decrease from summer to autumn at site 1. These results however do not deliver a visible trend in seasonal changes and once again indicates high variability of copper within the sediment. The fluctuations in copper concentrations between the seasons can be attributed to metals being remobilised and released from the sediments into the overlying water column through natural and anthropogenic disturbances such as bioturbation, storms and dredging (Eggleton & Thomas, 2004; Newman & Watling, 2007). As discussed in 4.4.1, the copper concentrations in water are high for the

present study and would support this statement for increasing concentrations. When looking at the decreases between seasons, it can be attributed to the fact that most of the naturally occurring copper in marine and estuarine water is considered to be unavailable as a result of binding to dissolved and particulate organic matter (Roesijadi, 1996). The particulate organic matter contributed to the sediment composition. Consequently, concentrations of most trace metals / contaminants in the bottom sediment and at the sediment – water interface usually exceed those in the overlying water column by several orders of magnitude (Newman & Watling, 2007).

As with the zinc concentrations, copper concentrations in the sediments are considered to be low and as copper is also known to be available in the natural environment, the assumption can be made that the sediments are probably not contaminated with copper. Concentrations that are present can be viewed as natural "background" concentrations.

4.4.3 Tissue

As for zinc, copper is also a micronutrient and is naturally available in the environment and is used by the organism in routine metabolic processes (Roesijadi, 1996). The range of copper recorded within the oyster tissues were 1.30 mg/kg – 14.420 mg/kg. In a study done by Roesijadi (1996), the range recorded for the European flat oyster, *Crassostrea virginica*, indicated that it had a tolerance of 14.7 – 1603 mg/kg for copper within the tissues. The concentrations that are presented in the current study fall well within this range, although it is a different species of oyster. Studies that incorporated the South African oyster, *Striostrea margaritacea*, are either lacking, outdated or difficult to use for comparison, as wet weights were used in analyses, as previously mentioned. Concentrations exceeding 3000 mg/kg have been recorded for the Eastern flat oyster, *Ostrea edulis*, without causing apparent toxicity to the oyster (Roesijadi, 1996). Cellular mechanisms for metal detoxification can be expected to be responsible for this tolerance.

BCF's for oyster tissues can mostly be considered low when water and sediment data are used, with one exception where the BCF was 5 times the ceiling limit of 1000. Water was the environmental medium responsible for this high value and as discussed in 4.4.1, the concentrations in the water were well above the limits set by the South African Water Quality Guidelines (DWAF, 1996). The ability of marine molluscs to accumulate and store metals has been discussed in 4.3.3 and is once again evident within these results as well as the high degree of bioaccumulation by the oysters, as shown by the BCF data.

Comparisons between sites did not deliver any obvious trends where one site was consistently higher or lower with regards to copper concentrations. One significant difference was recorded where site 2 had a higher concentration than site 1. The dynamics of site 2 have been previously discussed and can be incorporated within these results once again. Also as previously mentioned, the interaction of metals with one another and biological processes within the organism can be a contributing factor to variability. Processes that control bioaccumulation and intracellular distribution of metals are responsible for the observed concentrations of metals in tissues (Roesijadi, 1996) and could result in the compartmentalization of some of these metals. The differences in bioavailability of copper between sites could also be a pertinent factor with regards to variability within the tissues.

There were no seasonal trends visible for the copper concentrations within the tissues although two significant differences were recorded. An increase occurred from winter to spring at site 1 and another increase from summer to autumn at site 3 was recorded. As discussed in 4.3.3, the fluctuation in oyster metal content can also be as a result of the continuous processes that are defined physiologically as the outcome of the processes of influx (uptake rate) and efflux (release rate) (Roesijadi, 1996). Copper is also taken into the tissues by means of the digestive tract, although in some cases copper can be incorporated by the Na–uptake processes in the gills (Ju *et al.,* 2011). The environmental copper bioavailability can once again be a pertinent factor explaining the variability between seasons.

In conclusion, bioaccumulation from the environment has been proven by BCF data sets but copper within the tissues cannot necessarily be considered as a product of environmental contamination or to be toxic to oysters as it is relatively low when compared to other studies. Levels of variation could be caused by the oyster incorporating and using copper within its own metabolic activity. Further contamination/toxicity studies using *Striostrea margaritacea* as a subject species could assist in concluding whether or not these concentrations of copper within the tissue are high or low.

4.4.4 Shell

Copper concentrations in the shells were in the range of 0 mg/kg – 4.5 mg/kg. In the study conducted by Almeida *et al.* (1998), the range for copper in the oyster shells of *Crassostrea virginica,* was found to be 3 – 4.4 mg/kg for control oysters. The copper content in the oyster shells of the present study, are well within this range, except for one outlier value. Studies using *Striostrea margaritacea* as a test species are either lacking or outdated. As previously mentioned, concentrations within the shell tend to vary in terms of distribution within the shell (Carriker *et al.,* 1997).

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As displayed in Tables 3.4.1, 3.4.2 and 3.4.3, the BCF's for the shell when using ambient water and sediment data were found to be low, with one occasion where the BCF was 1738 times higher in the shell than in the water. As previously discussed, factors that exceed 1000 are considered to be high (Walker *et al.,* 1996). As previously discussed, the incorporation of trace metals has been studied and observed in the calcium carbonate skeletons of many marine molluscs (Almeida *et al.,* 1998). As in the case of aluminium and zinc, copper is also accumulated within the oyster tissues and shell. All shell components are secreted by the various mantle epithelium cells. As previously discussed, the oyster shell's internal layer is never in contact with external factors. This indicates that all components incorporated into this layer are a result of the animal's metabolic activity and come from the tissues of the oysters and not from external sources.

Results indicated differences between sites 1 and 3 and between sites 2 and 3 in autumn, where site 3 had the highest concentrations. When the tissue data were compared to the shell data in this instance from site 3, the tissue concentrations were not the highest within the sampling period. As previously discussed, copper, as for zinc, is also used in the metabolic activity of the oyster. The reason for the differences between sites is not necessarily site dependant but could simply be due to the availability of copper within the oyster tissues.

Differences recorded in the shells in terms of copper do not reflect an obvious trend. There were differences between site 3 in winter and in spring where the concentration decreased, and then increased from summer to autumn again, which could be due to the mobilization of this metal. As reflected in Table 2.3, in the winter sampling occasion at site 3, 30% of animals were spawning in comparison to 15 % spawning in the spring sampling occasion. The reason for the difference between seasons can be attributed to copper being used for metabolism and reproduction within the oyster tissues. As previously discussed, seasonal variations and cycles in metal concentrations within both the oyster and its shell are governed by local environmental conditions that determine availability of metals, food supplies and biological processes, such as reproduction (Roesijadi, 1996). The increase in concentrations in the shell from summer to autumn coincided with an increase in copper in the tissues at the same site. The concentrations in the shell were still much lower than that of the tissues. Twenty percent of the animals were spawning in the summer sampling occasion compared to 60% spawning in the autumn sampling occasion. There was a clear increase of spawning activity between these seasons. This coincides with the increase in copper concentrations in the tissues and the shells. Copper is thus available in higher concentrations within the oyster during these months and could be attributed to the necessity of this metal for reproductive activity (Roesijadi, 1996).

4.4.5 Comparison between oyster tissue and shell

When the shell and tissue data sets for copper concentrations were compared, significant differences were found (Figure 3.3.3). Concentrations were higher within

the tissues at sites 1 and 2 in the winter sampling occasion when compared to site 3. Site 3 had the highest copper concentrations within the tissues in spring and summer sampling occasions when compared to the other sites. Although there were no other significant differences recorded, the concentrations in the shell were generally lower than in the tissues. Copper is an essential element for all organisms, playing a critical role in a variety of biochemical processes including regulatory, structural, and enzymatic functions and will be retained within the tissues where it is readily available for these processes, rather than stored in the shell (Carriker *et al.,* 1991).

In conclusion, copper concentrations in *Striostrea margaritacea* can be considered generally low to moderate when compared to other studies. When data are compared between shells and tissues, the movement of copper is evident between them and can provide some explanation for variation between sites and seasons. The use of copper within the animal for metabolic activities can be considered a major reason for why concentrations are lower within the shell than in the tissue.

4.5 Iron (Fe)

Pure iron is silvery in colour but usually appears as greyish black or brown deposits as a result of oxidation (DWAF, 1996). Iron is found in three oxidation states, namely, 0, II and III of which the III oxidation state is the most common. In water, iron can be present as dissolved ferric iron, iron(III), as ferrous iron, iron(II) or as suspended iron hydroxides. Biologically, iron is an essential micronutrient required by all living organisms (DWAF, 1996).

4.5.1 Water

The range of iron concentrations within the water samples for this study was recorded as 0 mg/l – 3,76 mg/l. According to Domestic Water Use Guidelines (DWAF, 1996) the characteristic concentration of iron in seawater is 0.002mg/l. The Canadian Water Quality guidelines (CCME, 2007) indicated that the concentration of iron for freshwater is set at 0.3 mg/l. There are no Canadian guidelines available for iron for marine waters. A study done by Okoro (2012) at a polluted harbour-based site, recorded a

range of 0.06 – 1.1 mg/l iron in the water. The present study's results are well above the South African Domestic Water Use Guidelines (DWAF, 1996) and the Canadian Water Quality Guidelines (CCME, 2007) and could be considered as high when compared to other studies. In some instances, iron concentrations within the water were not detectable.

When drawing a comparison between sites, no statistical trends were evident,. High variability was once again present. As previously discussed, the concentrations of metals in the water column are subject to great temporal and spatial variability (Orr *et al.,* 2008) and this makes it difficult to obtain water samples that are representative of the contaminant status of the water body. Site 3 did however have the highest concentration for the duration of the study with the exception of the winter sampling period where concentrations at all sites were recorded to be 0mg/l. As previously discussed in 4.3.1 and 4.4.1, the hydrodynamics of site 3 seem to have had an impact on concentrations due to its "isolation" and down–welling events. This theory can once again be reinforced by the fact that the concentrations at site 3 were not only the highest but were three fold the concentrations found at the other sites.

No seasonal trends were recorded across all the sites. In a study conducted by Vermeulen & Wepener (1999) in Richards Bay, they found that there was no seasonal trend for iron in water samples and the range varied from 0.8 – 1.2 mg/l. This is significantly lower than the range found in the present study. Once again, this could be due to the oceanic structure around the various sites. Small retention eddies can often form an isolated pocket (Hancke, 2010) within the ocean and can be the cause for high concentrations of metals or contaminants. Furthermore, the high concentrations can be attributed not only to anthropogenic input but to natural occurrences such as the weathering of organic compounds and re–release of metals from sediments. Not only does the bottom sediment act as a sink for metal accumulation, but it could be a significant source as well (CSIR, 1997).

High concentrations of iron are also a visual concern since ferrous salts are unstable under the pH conditions prevailing in water as insoluble ferric hydroxide, which settles out as a rust-coloured silt (DWAF, 1996). No silt was present at any of the sites upon sampling occasions and from this observation the conclusion can be drawn that the pH could possibly not have contributed significantly to the elevated concentrations of iron in the water column.

To conclude, iron concentrations within the water column are high when compared to a variety of guidelines. Site 3 has the overall highest concentrations between sites. As mentioned, this could be attributed to the hydrodynamics of the area. No seasonal trends were visible.

4.5.2 Sediment

Iron concentrations in the sediments were recorded within the range of 1300 - 2478 mg/kg. Neither South African Sediment Quality Guidelines nor Canadian Sediment Quality Guidelines are available for comparisons. A study done by Orren *et al.* (1981) found iron concentrations for a “clean” beach ranging from 620 – 4110 mg/kg. A study conducted on the East coast of South Africa almost 20 years later by Vermeulen & Wepener (1999), indicated iron concentrations in sediment to be within the range of 3673 – 72 437 mg/kg. The concentrations recorded in the present study are well below the range as described by Vermeulen & Wepener (1999) and fall within the range of “clean” beaches as described by Orren *et al.* (1981).

Differences between the sites followed no trend throughout the study period and high variability was once again present. On one occasion there was a significant difference between sites where site 3 had the highest concentration and sites 1 and 2 the lower respectively. Although concentrations are below the suggested range, the phenomena described by Newman & Watling (2007), with reference to metal degradation and limited sediment distribution, could be responsible for the recording of elevated concentrations of iron within the sediments. As previously mentioned, sites that are

more sheltered and incur less wave action will not have excessive sediment distribution.

There was also a seasonal variation where iron increased from summer to autumn at site 3. These results however do not deliver a visible trend in seasonal changes and once again indicate high variability within the sediment for iron. In Table 2.1, there is a clear indication that from December 2008 to May 2009, the mouth of the Goukamma River had been closed at site 3. The source of the iron input can thus be correlated with the high concentrations in the ambient water of the ocean and cannot be due to runoff from the river. The highly dynamic nature of the marine environment allows for very rapid assimilation of materials by processes such as dilution, dispersal, oxidation, degradation or sequestration into sediments (Fatoki & Mathabatha, 2001).

As previously discussed, the fluctuations in sediment iron concentrations between the seasons can be attributed to metals being remobilised and released from the sediments into the overlying water column through natural and anthropogenic disturbances such as bioturbation, storms and dredging (Eggleton & Thomas, 2004; Newman & Watling, 2007). As discussed in 4.5.1, the water iron concentrations were high in the present study and would support this statement for increasing concentrations.

As with the zinc and copper concentrations, iron concentrations in the sediments are considered to be low and as iron is also known to be available in the natural environment, the assumption can be made that the sediments are not greatly contaminated with iron. Concentrations that are present can be viewed as natural "background" concentrations.

4.5.3 Tissue

The range recorded within the oyster tissue was 34.19 – 147.55 mg/kg. The concentrations of iron in the tissues cannot be compared to data of the same species, due to a lack of research using *Striostrea margaritacea* as a biomonitor. Studies that

investigate iron within the tissues of oysters are not well cited within the literature. South African studies that have been conducted use oyster wet weights for metal analysis and cannot easily be compared to dry weight samples used in the current study (Watling, 1983). A study conducted by Soto–Mimenez *et al.* (2001) in the southeast Gulf of California showed the concentrations of iron in the tissues of *Crassostrea iridescens,* to be within a range of 1237 – 4148 mg/kg. These samples were taken at a sewage outfall and are considered to be high. The range within the present study is well below that stated by Soto–Mimenez *et al.* (2001) and can be considered as low.

The BCFs for the tissues, when using ambient water data can be considered as low with the exception of one high factor as displayed in Tables 3.4.1, 3.4.2 and 3.4.3. The BCFs using the sediment data are exceptionally low, enforcing the theory that the iron within the sediment might not have been available for uptake whereas the iron within the water column was concentrated within the tissues. As previously discussed, metals enter the organism primarily through the digestive tract as a result of the ingestion of enriched organic particles (Amaral *et al.*, 2005). As oysters are filter feeders, water is ingested and organic compounds are absorbed from the water column. There were occasions were the iron concentrations within the water column were recorded to be 0mg/l or not detectable. In these instances, it could be argued that iron concentrations were absorbed from other sources, such as food particles.

The results indicate only one significant difference between sites where site 1 had a higher concentration when compared to site 2 in the winter sampling occasion. Although the concentrations can be considered as low, the higher concentration at site 1 could be due to the presence of the metal within the surrounding sediments, as well as certain water quality parameters. No significant differences were recorded between seasons. High variability between sites could be caused by a number of factors. As discussed in 4.2.1, 4.3.1, and 4.4.1, the south coast is highly variable in terms of its hydrology and water dispersal. The lack of significant differences of iron in the oyster tissues could also be directly linked to the incorporation of iron within the animal.

Within oysters, iron has been detected within the haemocytes (Roesijadi, 1996). Variability is not always the result of net movement of metals into and from organisms, but can also reflect changes in biomass associated with use and storage of nutrient reserves and amount of gametes. The reproductive cycle of the oyster, which is characterized by seasonal changes in nutrient reserves and release of gametes, also contributes to the variability in metal content in oysters (Engel, 1988). As displayed in Table 2.3, spawning occurred throughout the sampling period with high variability between sites and seasons, which could have been a contributing factor to the fluctuating concentrations within the tissues. Physico-chemical parameters have been previously discussed and could also be responsible for changes in concentration within the animal.

Iron concentrations within the tissues can be considered low when compared to international studies. High variability was present between sites and seasons and could have been due to a number of physico-chemical parameters as well as processes within the animal itself.

4.5.4 Shell

The concentrations of iron within the shell varied from 124 – 2268 mg/kg. Studies conducted using *Striostrea margaritacea* as test subject are either outdated or lacking. In a study conducted by Almeida *et al.* (1998), the concentrations in control oyster shells of the species *Crassostrea virginica* were in the range of 87.7 - 467mg/kg, which is well below those concentrations found in the present study. The iron concentrations in shells in the present study can by this association be considered as possibly high, but further studies using *Striostrea margaritacea* as a subject species should be considered for more reliable conclusions.

As displayed in Tables 3.4.1, 3.4.2 and 3.4.3, the BCFs are generally low to moderate for the oyster shells in terms of iron when compared to the ambient water and sediment concentrations, with one occasion where the BCF was 42424 times higher in the shell than in the water.

Results between sites indicated some significant differences but no trend was visible. The concentrations were highest at site 1 in relation to sites 2 and 3 in the winter sampling occasion. With reference to the tissue data, site 1 also had the highest concentration when compared to the other sites for this sampling occasion. This once again proves that metals are accumulated and stored from within the tissues to the shell and that the shell can be a reservoir of metals. Furthermore, in the spring sampling occasion, sites 2 and 3 significantly differed from one another where site 2 had the higher concentration. Within this sampling occasion the tissue concentrations were not the highest between sites. This high concentration in the shell could be due to the continuous mineralization of the iron within the shell. The study undertaken by Carriker *et al.* (1997) suggested that metals such as iron might represent normal constituents of the oyster's shell in minimal quantities or could be present due to the incorporation of the metal within the shell during mineralization. The fact that the concentrations in the shell tended to follow that of the ambient water and sediment, suggest the latter.

The shell data presented a seasonal variation where the concentration of iron decreased at site 1 from winter to spring and increased from spring to summer. Iron in shells from site 3 also decreased from winter to spring. The seasonal trend can be noted here with both sites 1 and 3 significantly decreasing, and site 2 also decreasing ($p>0.05$) from winter to spring. Once again the values recorded in this study are high when compared to other studies (Almeida *et al.*, 1998). Seasonal cycles in metal concentrations in oysters are governed by local environmental conditions that determine availability of metals and food supplies and by biological processes such as reproduction, which influence endogenous cycles (Roesijadi, 1996). The oysters used in this study were spawning shortly before metal analysis. As previously mentioned, the reproductive cycle of the oyster, which is characterized by seasonal changes in nutrient reserves and release of gametes, also contributes to the variability in metal content in oysters (Engel, 1988).

4.5.5 Comparison between oyster tissue and shell

When the shell and tissue data sets were compared, significant differences were found. Figure 3.3.4 shows that within the winter, summer and autumn sampling occasions the concentrations were significantly higher within the shell at site 3. Sites 1 and 2 also had higher concentrations within the shell for the autumn and spring sampling occasion respectively. Although no other significant differences were present, the concentrations were higher within the shell than in the tissues for all occasions. Marine molluscs have the ability to metabolize and store metals within their shells. As previously discussed in 4.1, the physico-chemical parameters have effects on the mobility of the metals within the water column, as well as within the organism itself. The fluctuations between sites and seasons can be attributed to these factors as well as the high variability between spawning occurrences and physiological processes involving iron in general.

In conclusion, the iron concentrations within the shell are high when compared to other studies. As for aluminium, the shell concentrations are much higher than that found within the tissues and mineralization seems to have occurred.

4.6 Neutral Red Retention Time Assay (NRRT)

Lysosomal alterations in marine molluscs induced by metals have been reported in several laboratory exposure and field monitoring programs (Harrison *et al.*, 1983; Viarengo *et al.,* 1987; 1991, Moore *et al.,* 1988; Regoli, 1992; Krishnakumar *et al.,* 1994; Lowe *et al.*, 1995).

Results indicated that there were no significant differences ($p>0.05$) between seasons over the sampling period of a year at any of the sites. The neutral red retention times indicated in Table 3.1 are short for all the sites compared to studies undertaken on different subject species, such as mussels (Lowe *et al.,* 1995). Caution should be exercised when comparing two different sets of data which incorporate different subject species. It must be remembered that the experimental conditions and the choice of experimental animal were different in the two studies (Hauton *et al.,* 1998).

Oysters can be considered as stressed when compared to these data sets and are so throughout the entire sampling period. As previously discussed, spawning fluctuated throughout the sampling period and could have been a contributing factor to the stress within the animal. The handling of the oysters should also be considered as a stress factor.

Depledge & Kure (1994) pointed out that while biomarkers which signify exposure to pollutants are useful, those which signify that organisms are experiencing toxic/stressed effects are more ecologically relevant. Taking into account the short neutral red retention times, it is a fair assumption to make that although there are no significant differences between seasons at any particular site, the oysters appear to be in a stressed state throughout the year. This phenomenon could be a result of immunological parameters such as detoxification, reproduction and routine metabolic activities (Roesijadi, 1996). Alterations in blood cell function, including lysosomal destabilization, as a biomarker of contaminant impact and effect have tended to address changes in immunological parameters (Lowe *et al.,* 1995). A study undertaken by Coles *et al*. (1994) has also addressed alterations in molluscan blood cells that give insight into mechanisms of the immune response. In their role as components of the immune response, blood cell lysosomes release acid hydrolase which is in turn able to degrade circulating pathogens. However, unscheduled release of acid hydrolase may have disastrous consequences. Once the functional integrity of the lysosomal membrane has been compromised and the acid hydrolases gain free access to the cytoplasm, further damage and disruption to the cell is inevitable (Lowe *et al.,* 1995). Also, Viarengo & Moore (1987) indicated that the destabilization of lysosomal membranes is associated with enhanced protein catabolism and failure of membranes to recover in the short to medium period (Lowe *et al.,* 1995), whereas Lowe *et al*. (1992) speculated that release of the neutral red dye into the cytosol following membrane damage may be due to impairment of the lysosomal membrane proton pump. The internal acid environment of lysosomes is maintained by a Mg^{2+}ATPase dependent H^{+}ion proton pump (Lowe *et al.,* 1995). Dysfunction of the pump would lead to a marked increase of the intra-lysosomal pH, and in the absence

of any gradient, free passage of the lysosomal contents including neutral red into the cytosol (Lowe *et al.,* 1995).

Important issues regarding the use of cellular biomarkers include understanding the effects of natural stressors. Ringwood *et al*. (1998) suggested that lysosomal destabilization may not be affected by environmentally relevant temperature. Oysters from variable salinity regimes indicated that lysosomal destabilization in either short term or long term responses show no significant differences, according to another study by Ringwood *et al*. (1998). The environmental conditions for the present study fluctuated throughout the sampling period, although not to an extreme degree (Table 3.1). Taking into account the previous statement, the present results continued to support lysosomal integrity indicators as potentially valuable biomonitoring tools for estuarine species.

The possibility that seasonal changes in physiological conditions may contribute to seasonal differences in lysosomal destabilization should not be eliminated. Ringwood *et al.* (1998) suggested that tissue metal concentrations may be higher during the winter due to higher bioavailability of metals or other pollutants during cooler periods. The availability for aluminium, zinc, copper and iron varied throughout the seasons with fluctuating concentrations at all sites. As a result, an explanation for the significant differences in NRR times that were found between sites 3, 2 and 1 for the winter sampling occasion may not be a singular one. As previously mentioned, spawning was also recorded throughout the seasons and between sites with no evident trend visible. A study done by Cho & Jeong (2005) found that significant decreases were present in both haemocyte density and NRR time in spawning oysters, *Crassostrea gigas*.

Study sites were chosen for their unique positioning in terms of their river inputs. The Goukamma River mouth at site 3 is closed for the duration of the winter months and would eliminate the potential for input of contaminants into the marine environment via river run-off. Also, it is a Marine Protected Area (MPA) and by regulations and law,

shipping traffic is prohibited within the boundary of the MPA which extends 1 nautical mile seawards, which translates to 1.8 km. By this association, the input from anthropogenic sources should be less than other sites and contribute to the fact that this site (site 3), when compared with the other two sites, significantly differed from both, having the longest NRRT in both instances. When compared to the metal data sets, site 3 had the highest concentrations on more than one occasion for the various metals. As previously discussed, zinc, copper and iron are trace elements that are incorporated within the animal for biological processes. The concentrations recorded for these metals were generally low when compared to guidelines and other studies. The presence of these metals could thus have a positive effect on cellular metabolism and help the animal with coping with external stress factors.

Another contributor to lysosomal destabilization could be the metabolic rates of the organism that tend to be lower during the cooler months, hence the bioavailability of contaminants could actually be greater since acid volatile sulfides (AVS) and organic carbon levels tend to be lower during winter months (Ringwood *et al.,* 2002). Likewise, since metabolic rates during the warmer months are higher, it may be expected that bioaccumulation would be higher, but higher AVS levels and organic loads may reduce bioavailability. Fundamental biological processes such as reproduction, feeding activities, energy reserves accumulation and storage are mainly concentrated in the short summer season, when anthropogenic activities (and consequently, their impacts) are at their maximum (Regoli, 1992). Within the results of the present study, bioaccumulation did not always follow seasonal trends.

In the spring sampling occasion it was once again noted that oysters from site 3 had a significantly longer mean NRRT when compared to site 1. Factors such as temperature, pH, and salinity, have previously been discussed and can once again be incorporated here. It is conceivable that the retention of dye within the lysosomal compartment of the haemocytes varied as a function of the sex of the individual oysters as well (Hauton *et al.,* 1998). The sex of the oysters was not determined as oysters were selected randomly. When comparing the activities at site 1 and 3, site 1

has a considerable higher amount of anthropogenic activity all year around. As previously mentioned, site 1 has a lake and river that is open to recreational activities such as fishing and power boats. Low NNR times can in this instance perhaps be attributed to other contaminants beside heavy metals.

Metals could have an indirect effect mediated by the formation of oxyradicals. These reactive species could enhance lysosomal damage by promoting the peroxidation of membranes and in the meantime would further reduce the antioxidant cellular defences. In this respect, it could be speculated that lysosomal damage is at least in part dependant on the efficiency of antioxidant mechanisms. In fact, as more of these defences are depleted, the more severe the indirect effects of metals on lysosomal membranes become (Regoli *et al.,* 1998).

The significant damage to the lysosomal vascular system, for an individual or a population, can be inferred from studies by Regoli (1992). Using depressed lysosomal latency in mussel digestive tissues as a biomarker of contaminant induced damage, Regoli (1992) demonstrated that 4 month depuration following metal contamination did not result in any increase in the latency periods and could indicate that lysosomal stability had improved..

In conclusion, lysosomal destabilization in oysters was correlated within the present study to tissue metal concentrations. These data also support a second hypothesis that seasonal differences in physico-chemical factors (such as reduced levels in nutrients) may increase the bioavailability of metals during the winter so that adverse effects are more pronounced (Ringwood *et al.,* 1994). One cannot eliminate the possibility that seasonal changes in physiological conditions may contribute to seasonal differences in lysosomal destabilization. Ringwood *et al*. (1994) suggested that tissue metal concentrations may be higher during the winter due to higher bioavailability of metals or other pollutants during cooler periods. Some environmental measurements were not measured within the present study and could possibly be responsible for the short NRR times. It should also be noted that metals interact with

various other substances as well as other metals. This could also have contributed to the results of the present study. A controlled laboratory experiment could yield more concrete results.

CHAPTER 5

Conclusion

The south coast of South Africa is a highly variable and dynamic environment with physico-chemical parameters that fluctuate in wide ranges. Temperature measurements recorded in this study, although highly variable, are within the same range at all the sites and can be considered as normal. The differentiation of the salinity range can be linked to the freshwater influx from rivers, rain or the subsequent runoff. Elevated levels can be due to the lack of these inputs. The pH range recorded was well within the range set by DWAF (1995). Various other physical and chemical factors could have been responsible for this and additional measurements should be taken to confirm which of these factors could be responsible.

Aluminium concentrations found in the water column at all sites can generally be considered as low and the sites uncontaminated when compared to guidelines and other studies. Iron, zinc and copper concentrations within the water column can on the other hand be considered to be high when comparisons are drawn with other studies and data sets. This does not necessarily indicate contamination. Site 3 had the overall highest concentrations between sites. Seasonal trends are not present with the exception of copper where there was a steady increase in concentrations from winter to spring and in turn, summer, where after it decreased to 0 mg/l in the autumn months.

The sediment concentrations for all the metals within the present study are considered to be low when compared to other studies and guidelines. There were not many significant differences recorded between sites and no seasonal patterns were present. Grain size and sediment structure data are absent for the present study and should be incorporated for further studies to verify results.

Within the tissues of the oysters, the metal ranges are considered to be low when compared to other studies. Data sets are lacking that incorporate *Striostrea margaritacea* as target species and this makes it difficult to draw a concrete conclusion. Although concentrations within the oysters are low, this does not mean that they are not toxic to the animal. Also, most of the metals that were tested in the current study are considered to be micronutrients and their presence within the oysters is necessary for survival. A field study in conjunction with a laboratory experiment should yield more concrete results. There were also no seasonal trends present and very few differences between sites. A more comprehensive data set could reveal more concrete results for seasonal trends.

Concentrations for aluminium, zinc and copper in the shells can be considered low when comparisons are drawn, with the exception of iron that was observed to be high. There were also no seasonal trends present and a prolonged sampling period is suggested to further investigate these findings. Also, very few differences were recorded between sites and a more comprehensive data set could prove useful to determine further results.

When a comparison was drawn between the tissue and shell data a clear pattern was evident. Aluminium and iron had higher concentrations within the shell and zinc and copper concentrations were highest within the tissues of the animal. The theory of mineralization is supported by these findings where bivalves will use their shell as a reservoir for micronutrients and other substances. They also seem to have the ability to remobilize nutrients and use them within the animal when biological processes demand them.

The NNRT assay revealed that lysosomal membrane destabilization had occurred and that the animals appeared to be stressed for the duration of the sampling period. The retention times that were recorded were short when compared to other studies. In this study, the whole animal was sacrificed, thereby removing the opportunity of follow up studies to investigate recovery and interactive effects. Perhaps further investigation

into haemocyte harvesting of *Striostrea margaritacea* could be undertaken as to deliver a more comprehensive data set. Clearly this assay has potential as a basic monitoring tool from which more thorough investigations can be initiated more cost effectively. Other contaminants should also be measured when doing an *in situ* study to yield more reliable results. It should also be considered to use a "suite of biomarkers", in future studies, in conjunction with the NRRT to delivery more and broader results.

More vigorous and lengthy studies should be undertaken to contribute to current knowledge of our indigenous species, *Striostrea margaritacea* and to aid in the development of better management of this resource as well as an ongoing monitoring programme.

Final concluding remarks per research objective:

1. To determine the degree of metal contamination in the water and sediments at sites selected in Witsand, Wilderness and Goukamma.

There does not seem to be an immediate threat in terms of metal contamination on the south coast at the investigated sites. Concentrations of all metals were mostly below regulatory guidelines on a local and international scale, with the exception of water, which included instances of high zinc, copper and iron concentrations

2. To determine the degree of metal bioaccumulation in the wild oyster, *Striostrea margaritacea*.

As there were increases in the concentrations of various metals between sites and from one season to another, *Striostrea margaritacea* has proven to be a species that can bioaccumulate metals. The current BCF data sets support this statement. A laboratory experiment may prove useful to determine the degree or rate of bioaccumulation within this species.

3. To determine the main site for metal storage in the wild oyster, *Striostrea margaritacea*, in terms of shell and tissue comparisons.

Metal concentrations for aluminium, zinc, copper and iron were present in both the shells and the tissues of the oyster. There were however significant differences that show that zinc and copper concentrations were higher in the tissues whereas aluminium and iron were more concentrated within the shell.

4. To determine if there is seasonal variability in the metal concentrations at the three study sites and in metal bioaccumulation by the oyster tissues.

No seasonal trends were consistently visible within the study period. A lengthier sampling period over 2 years may reveal possible seasonal trends.

5. To determine the toxicity of selected metals to the oysters, by using a biomarker (lysosome membrane destabilization) as tool. The biomarker will be tested using the neutral red retention time assay.

No final conclusions can be drawn as the NNR times were exceptionally short in the current field study. A controlled laboratory experiment, which will eliminate some environmental stressors, may yield more reliable results.

6. To establish if *Striostrea margaritacea* qualifies as a successful biomonitor.

Striostrea margaritacea has shown promise to be used as a biomonitor in conjunction with environmental data sets. A laboratory study should be undertaken to further assess its worth as a potential biomonitor species.

7. To determine if the Goukamma Marine Protected Area (MPA) can be used in field studies as control sites by doing an *in situ* study

The Goukamma MPA served as a possible control site within this study. The concentrations recorded at the site were at times the highest. This does not necessarily mean that those concentrations were toxic. This study revealed that the hydrology of the surrounding water bodies plays a vital role when sites are selected and hydrology charts should be incorporated before choosing possible field study control sites.

8. To establish if there is a need for a monitoring programme for metal contamination at the selected study sites

Further research will have to be conducted, but there is no immediate threat in terms of metal contamination on the South African south coast with relevance to the study sites. There is however the possibility of the presence of other chemicals e.g. organic pollutants within the study sites that were not tested for. Further monitoring may also be needed at these sites along the coast as development increases.

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Annexure A

Table A1: The H- statistic (h), degrees of freedom (d.f) and p-values (p) are represented in the table below for aluminium concentration for water, sediment, oyster tissue and shells, collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

Aluminium							
Water concentrations	h	d.f	p	*Tissue concentrations*	h	d.f	p
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	4.235	2	0.120	winter (Jun 2008)	5.267	2	0.072
spring (Sep 2008)	2	2	0.368	spring (Sep 2008)	1.082	2	0.582
summer (Dec 2008)	1.28	2	0.527	summer (Dec 2008)	6.598	2	0.037
autumn (Mar 2009)	2.539	2	0.281	autumn (Mar 2009)	3.161	2	0.206
Differences between seasons per site				Differences between seasons per site			
Site 1	6.803	3	0.078	Site 1	6.824	3	0.078
Site 2	13.802	3	0.003	Site 2	4.688	3	0.196
Site 3	10.407	3	0.015	Site 3	0.648	3	0.885
Sediment concentrations				*Shell concentrations*			
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	4.924	2	0.085	winter (Jun 2008)	13.985	2	<0.001
spring (Sep 2008)	2.842	2	0.241	spring (Sep 2008)	11.279	2	0.004
summer (Dec 2008)	2.772	2	0.250	summer (Dec 2008)	7.463	2	0.024
autumn (Mar 2009)	11.661	2	0.003	autumn (Mar 2009)	5.840	2	0.054
Differences between seasons per site				Differences between seasons per site			
Site 1	13.207	3	0.004	Site 1	25.021	3	<0.001
Site 2	1.940	3	0.585	Site 2	4.782	3	0.188
Site 3	18.373	3	<0.001	Site 3	17.666	3	<0.001

Table A2: The H- statistic (h), degrees of freedom (d.f) and p-values (p) are represented in the table below for zinc concentration for water, sediment, oyster tissue and shells, collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

Zinc							
Water concentrations	h	d.f	p	*Tissue concentrations*	h	d.f	p
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	1.069	2	0.586	winter (Jun 2008)	5.917	2	0.052
spring (Sep 2008)	3.871	2	0.144	spring (Sep 2008)	9.177	2	0.010
summer (Dec 2008)	2.098	2	0.350	summer (Dec 2008)	16.183	2	<0.001
autumn (Mar 2009)	4.257	2	0.119	autumn (Mar 2009)	8.519	2	0.014
Differences between seasons per site				Differences between seasons per site			
Site 1	15.525	3	0.001	Site 1	3.385	3	0.336
Site 2	10.490	3	0.015	Site 2	3.549	3	0.314
Site 3	4.973	3	0.174	Site 3	17.150	3	<0.001
Sediment concentrations				*Shell concentrations*			
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	1.069	2	0.586	winter (Jun 2008)	5.434	2	0.066
spring (Sep 2008)	1.310	2	0.519	spring (Sep 2008)	4.221	2	0.121
summer (Dec 2008)	4.218	2	0.121	summer (Dec 2008)	0.546	2	0.761
autumn (Mar 2009)	3.591	2	0.166	autumn (Mar 2009)	12.867	2	0.002
Differences between seasons per site				Differences between seasons per site			
Site 1	13.091	3	0.004	Site 1	4.262	3	0.235
Site 2	10.960	3	0.012	Site 2	10.027	3	0.018
Site 3	5.924	3	0.115	Site 3	4.157	3	0.245

Table A3: The H- statistic (h), degrees of freedom (d.f) and p-values (p) are represented in the table below for copper concentration for water, sediment, oyster tissue and shells, collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

Copper							
Water concentrations	h	d.f	P	*Tissue concentrations*	h	d.f	p
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	1.069	2	0.586	winter (Jun 2008)	10.738	2	0.005
spring (Sep 2008)	4.110	2	0.128	spring (Sep 2008)	4.932	2	0.085
summer (Dec 2008)	1.310	2	0.519	summer (Dec 2008)	5.205	2	0.074
autumn (Mar 2009)	0.000	2	1	autumn (Mar 2009)	0.126	2	0.939
Differences between seasons per site				Differences between seasons per site			
Site 1	17.213	3	<0.001	Site 1	15.597	3	0.001
Site 2	18.934	3	<0.001	Site 2	10.059	3	0.018
Site 3	21.447	3	<0.001	Site 3	18.954	3	<0.001
Sediment concentrations				*Shell concentrations*			
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	2.023	2	0.364	winter (Jun 2008)	0.302	2	0.860
spring (Sep 2008)	6.041	2	0.049	spring (Sep 2008)	15.805	2	<0.001
summer (Dec 2008)	0.222	2	0.895	summer (Dec 2008)	6.133	2	0.047
autumn (Mar 2009)	16.129	2	<0.001	autumn (Mar 2009)	22.528	2	<0.001
Differences between seasons per site				Differences between seasons per site			
Site 1	16.856	3	<0.001	Site 1	19.713	3	<0.001
Site 2	20.107	3	<0.001	Site 2	10.267	3	0.016
Site 3	17.568	3	<0.001	Site 3	30.441	3	<0.001

Table A4: The H- statistic (h), degrees of freedom (d.f) and p-values (p) are represented in the table below for aluminium concentration for water, sediment, oyster tissue and shells, collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

Iron							
Water concentrations	h	d.f	p	*Tissue concentrations*	h	d.f	p
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	0.000	2	1.000	winter (Jun 2008)	10.196	2	0.006
spring (Sep 2008)	0.943	2	0.624	spring (Sep 2008)	0.472	2	0.790
summer (Dec 2008)	4.812	2	0.090	summer (Dec 2008)	5.310	2	0.070
autumn (Mar 2009)	1.879	2	0.391	autumn (Mar 2009)	4.552	2	0.103
Differences between seasons per site				Differences between seasons per site			
Site 1	7.468	3	0.058	Site 1	13.663	3	0.003
Site 2	5.565	3	0.135	Site 2	7.155	3	0.067
Site 3	7.062	3	0.070	Site 3	1.819	3	0.611
Sediment concentrations				*Shell concentrations*			
Differences between sites per season				Differences between sites per season			
winter (Jun 2008)	4.924	2	0.085	winter (Jun 2008)	17.631	2	<0.001
spring (Sep 2008)	4.667	2	0.097	spring (Sep 2008)	7.556	2	0.023
summer (Dec 2008)	3.520	2	0.172	summer (Dec 2008)	2.795	2	0.247
autumn (Mar 2009)	11.415	2	0.003	autumn (Mar 2009)	1.680	2	0.432
Differences between seasons per site				Differences between seasons per site			
Site 1	11.987	3	0.007	Site 1	26.776	3	<0.001
Site 2	3.560	3	0.313	Site 2	7.020	3	0.071
Site 3	14.487	3	0.002	Site 3	19.117	3	<0.001

Table A5: The H- statistic (h), degrees of freedom (d.f) and p-values (p) are represented in the table below for aluminium concentration for water, sediment, oyster tissue and shells, collected in the study period from June 2008 – March 2009, at three different study sites. Site 1 = Wilderness, Site 2 = Witsand and Site 3 = Goukamma (MPA).

Neutral Red Retention Time (NRRT)			
-	**h**	**d.f**	**p**
Differences between sites per season			
winter (Jun 2008)	10.18	2	0.006
spring (Sep 2008)	8.44	2	0.015
summer (Dec 2008)	3.615	2	0.164
autumn (Mar 2009)	4.736	2	0.094
Differences between seasons per site			
Site 1	4.115	3	0.249
Site 2	4.953	3	0.175
Site 3	0.663	3	0.882

Annexure B

Summary of all significant differences for the period of one year where Y = Significant differences between sites per season; X = Significant differences between seasonal variation; aluminium = aluminium; zinc = zinc; copper = copper; iron = iron;
↑ = Increase of concentration; ↓ = Decrease in concentration; ↑↓ = Higher concentration between sites per season

	Winter			Spring			Summer			Autumn		
	site 1	site 2	site 3	site 1	site 2	site 3	site 1	site 2	site 3	site 1	site 2	site 3
Tissues	Y Fe ↑	Y Fe										
	Y Cu	Y Cu ↑										
	X Cu↑			X Cu↑					X Cu↑			X Cu↑
				Y Zn	Y Zn ↑		Y Zn	Y Zn ↑	Y Zn ↑		Y Zn ↑	Y Zn
									X Zn↓			X Zn↓
Shells	Y Fe ↑	Y Fe	Y Fe		Y Fe ↑	Y Fe						
	X Fe↓		X Fe↓	X Fe↓↑		X Fe↓	X Fe↑					
				Y Cu ↑	Y Cu ↑	Y Cu				Y Cu	Y Cu	Y Cu ↑
			X Cu↓			X Cu↓			X Cu↑			X Cu↑
										Y Zn ↑	Y Zn ↑	Y Zn
		X Zn↓			X Zn↓							
	Y Al ↑	Y Al	Y Al				Y Al ↑	Y Al ↑	Y Al			
	X Al↓		X Al↓	X Al↓		X Al↓			X Al↓			X Al↓
Sediment										Y Fe	Y Fe	Y Fe ↑
									X Fe ↑			X Fe ↑
										Y Cu	Y Cu	Y Cu ↑
		X Cu↑			X Cu↓↑	X Cu↑	X Cu↓	X Cu↓	X Cu↑	X Cu↓		
									X Zn↑			X Zn↑
										Y Al	Y Al	Y Al ↑
			X Al↓			X Al↓			X Al↑			X Al↑
Water							X Cu↓	X Cu↓	X Cu↓	X Cu↓	X Cu↓	X Cu↓
							X Zn↑	X Zn↑		X Zn↑	X Zn↑	