The assessment of organic pollutant exposure and effects along the KwaZulu-Natal coastline

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It all starts here ™

NORTH-WEST UNIVERSITY
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The assessment of organic pollutant exposure and effects along the KwaZulu-Natal coastline

Marine pollution monitoring
PCB
PAH
OCP
Perna perna
Semi-permable membrane devices
Bioaccumulation

Marine pollution monitoring has been active in South Africa for the past 40 years, but with large periods of neglect where no research has been done. South Africa is ideally situated along the primary global shipping route, making its harbours, especially along the east coast, some of the largest and busiest ports in the world (Marshall et al. 2003). Several recent studies have been focussing on metal pollutants in Richards Bay Harbour (Greenfield et al. 2011, 2014; Degger et al. 2011b) and one study on the persistent organic pollutants polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) in Richards Bay Harbour (Degger et al. 2011a). The aim of this study was to successfully implement passive and active biomonitoring methods using semi-permeable membrane devices (SPMDs) and indicator organisms (mussels) for chemical and biochemical analyses in Durban Harbour and Richards Bay Harbour. Biomarker analyses were done to determine physiological effects to organic pollutant exposures and organochlorine pesticides (OCPs), PCBs and PAHs were analysed using QuEChERS extraction and GC-GC-TOFMS for chemical analyses of both SPMDs and mussel tissue. Significance was taken at $p < 0.05$. The results showed low levels of OCPs exposure in mussels, while the SPMDs were able to detect slightly higher levels in Durban Harbour. Both mussels and SPMDs were able to successfully accumulate PCBs and PAHs at all sites. Both harbours had higher levels of these pollutants than at Sheffield Beach, and Richards Bay Harbour had higher levels of PAHs during the dry season survey due to an oil spill a few weeks earlier. The biomarkers were able to confirm oxidative stress and exposure effects due to organic pollutant exposure, which were confirmed by chemical analyses. The biomarkers were also able to confirm oxidative stress due to other environmental factors (freshwater runoff from high rainfall, tidal influences, food scarcity) at Sheffield Beach, which are not associated with organic pollutant exposure. The data collected from this

study will contribute to baseline data on the state of Durban- and Richards Bay Harbours concerning persistent organic pollutants, and therefore open the door to further marine pollution monitoring studies along the east coast of South Africa.

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SUMMARY

Marine pollution monitoring has been active in South Africa for the past 40 years, but with large periods of neglect where no research has been done. The 'Mussel Watch' programme has been active since 1975, monitoring four major groups of pollutants: artificial radionuclides, chlorinated hydrocarbons, metals and petroleum hydrocarbons (Goldberg and Bertine 2000). South Africa is ideally situated along the primary global shipping route, making its harbours, especially along the east coast, some of the largest and busiest ports in the world (Marshall et al. 2003). Several recent studies have been focussing on metal pollutants in Richards Bay Harbour (Greenfield et al. 2011, 2014; Degger et al. 2011b) and one study on the persistent organic pollutants polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) in Richards Bay Harbour (Degger et al. 2011a). This project focusses on the organic contamination in Durban Harbour and Richards Bay Harbour using passive and active biomonitoring methods. The aim of this study was to successfully implement passive and active biomonitoring methods using semi-permeable membrane devices (SPMDs) and indicator organisms (mussels) for chemical and biochemical analyses. Brown mussels (*Perna perna*) were transplanted from Sheffield Beach to both harbour sites for two exposure periods of six weeks (March/April rainy season and June/July dry season) together with SPMDs containing PCB 8 spiked triolein lipid. Biomarker analyses were done to determine physiological effects to organic pollutant exposures and organochlorine pesticides (OCPs), PCBs and PAHs were analysed using QuEChERS extraction and GC-GC-TOFMS for chemical analyses of both SPMDs and mussel tissue. Significance was taken at $p < 0.05$. The results showed low levels of OCPs exposure in mussels, while the SPMDs were able to detect slightly higher levels in Durban Harbour. Both mussels and SPMDs were able to successfully accumulate PCBs and PAHs at all sites. Both harbours had higher levels of these pollutants than at Sheffield Beach, and Richards Bay Harbour had higher levels of PAHs during the dry season survey due to an oil spill a few weeks earlier. The biomarkers were able to confirm oxidative stress and exposure effects due to organic pollutant exposure, which were confirmed by chemical analyses. The biomarkers were also able to confirm oxidative stress due to other environmental factors (freshwater runoff from high rainfall, tidal influences, food scarcity) at Sheffield Beach, which are not associated with organic pollutant exposure. The data collected from this study will

contribute to baseline data on the state of Durban- and Richards Bay Harbours concerning persistent organic pollutants, and therefore open the door to further marine pollution monitoring studies along the east coast of South Africa.

LIST OF ABBREVIATIONS

µl – Microliter

µm – Micrometer

ABM - Active biomonitoring

AChE – Acetylcholinesterase

ANOVA - One-way analysis of variance

BHC - Benzene hexachloride

BSA - Bovine Serum Albumin

CAT – Catalase

CYP450 - Cytochrome P450

DCM – Dichloromethane

DDD - 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene

DDE - 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene

DDT - 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane

DEAT – Department of Environmental Affairs and Tourism

DH – Durban Harbour

DTPA - Diethylene triamine pentaacetic acid

g – Gram

GC – Gas chromatography

GHB – General homogenising buffer

H_2O_2 – Hydrogen peroxide

H_2SO_4 – Sulfuric acid

HCH – Hexachlorocyclohexane

HSB - Hendrikson Stabilising Buffer

IBR - Integrated Biomarker Response

K_2HPO_4 – Dipotassium phosphate

$KMnO_4$ – Potassium permanganate

LOD – Limit of detection

LOQ – Limit of quantification

m – Meter

M. galloprovincialis – *Mytilus galloprovincialis*

MDA – Malondialdehyde

ml – Millilitre

mm – Millimetre

N – Number of biomarkers

NaOH – Sodium hydroxide

ND – Not detected

ng – Nanogram

NIST – National Institute of Standards and Technology

NMDS - Non-metric multidimensional scaling

NMISA - National Metrology Institute of South Africa

OCP - Organochlorinated pesticides

P. perna – Perna perna

PAH - Polycyclic aromatic hydrocarbons

PC - Protein carbonyl

PCA – Principle component analysis

PCB - Polychlorinated biphenyls

PCDD - Polychlorinated dibenzo-*p*-dioxins

POP – Persistent Organic Pollutant

PPB -Potassium phosphate buffer

PRC - Permeability reference compound

RBH – Richards Bay Harbour

ROS - Reactive oxygen species

RPM – Revolutions per minute

SB - Sheffield Beach

SDS - Sodium dodecyl sulphate

SE - Standard error of the mean

SOD - Superoxide dismutase

SPE - Solid phase extraction

SPMD - Semi-permeable membrane device

TCA - Thiochloroacetic acid

TMP – Tetramethoxypropane

TOFMS – Time of Flight Mass Spectrometry

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CHAPTER 1

INTRODUCTION

1.1. MARINE POLLUTION IN SOUTH AFRICA

According to Lombard et al. (2004) there is a global increase in pollution along coastal regions, deteriorating the marine environment's productivity, health, integrity and diversity. These deteriorations have a negative influence on these systems' abilities to be sustainable resources, inadvertently deteriorating the livelihoods and life quality of people dependent on these resources (Taljaard et al. 2006). Pollution is now regarded as a very problematic environmental issue in South Africa (Lombard et al. 2003). Studies of coastal and estuarine regions of north-eastern KwaZulu-Natal support this statement (Vermeulen and Wepener 1999; Mzimela et al. 2002; Wepener and Vermeulen 2005; Wepener et al. 2008). There are many sources of marine pollution, which include effluents from mining, industrial and domestic activities due to increased development and 30% of South Africa's population residing along coastal areas (Mzimela et al. 2002; Greenfield et al. 2011; Wepener and Degger 2012).

Marine pollution monitoring has been active in South Africa for the past 40 years, but with large periods of neglect where no research has been done (Wepener and Degger 2012). The 'Mussel Watch' programme has been active since 1975, forming part of the Marine Coastal Management and concentrates on monitoring four major groups of pollutants: artificial radionuclides, chlorinated hydrocarbons, metals and petroleum hydrocarbons (Goldberg and Bertine 2000). International cooperation was made to develop and implement this program (Goldberg and Bertine 2000). A considerable amount of papers were published in the 1980s, but in the last 20 years there has been a distinct lack in publication and research efforts in South Africa. These local monitoring systems focused mainly on metal, organic and oil pollution (Wepener and Degger 2012). In the period 1960 – 1980 the majority of studies conducted were measurements of exposure (sediment, benthic, biota) along the coastline. It is only from the 1980s onwards that studies began to focus on effects rather than just exposure, thereby studying biological responses in marine organisms and the relationship to pollution levels in the environment.

A recent study by Agbohessi et al. (2015) focused on the exposure effects of pesticides on liver status, endocrine regulation and status of offspring in African catfish. Another recent study by Barnhoorn et al. (2015) focused on the effects of

pesticides on freshwater sharptooth catfish. Other studies concerning the levels and effects of POPs on organisms in both fresh- and saltwater environments of South-Africa (Bouwman et al. 2015; Daso et al. 2015; Kampire et al. 2015a; Maritz et al. 2015) indicate that there is an increase in monitoring of these contaminants in the aquatic and terrestrial environment. Even with this increase in POP monitoring studies in South-Africa, much more needs to be done if a complete, long-standing monitoring programme is to be implemented and a historic data basis constructed.

1.2. CONTAMINATED HARBOURS ALONG THE EASTERN COAST OF SOUTH AFRICA

South Africa is ideally situated along the primary global shipping route, making its harbours (especially along the east coast) some of the largest and busiest ports in the world (Marshall et al. 2003). Richards Bay and Durban Harbours are important from both an economic and ecosystem perspective. These harbours are also estuarine habitats that give shelter and breeding areas for numerous aquatic and marine organisms. According to Cyrus and Forbes (1996), both Durban and Richards Bay Harbours are very productive marine ecosystems. These ecosystems supporting various fish species in their larval stages (acting as nurseries), habitat specific macro-benthic fauna, crustaceans and a large penaeid prawn fishery in Richards Bay. Both harbours are feeding grounds for migrant wading birds along the coast (Cyrus and Forbes 1996). Due to their ecological importance it is imperative to protect the functioning of these ecosystems. Not much research has been done in these systems regarding organic mico-pollution (Degger et al. 2011a), but a fair number addressed metal pollution, especially in Richard's Bay Harbour using sediment, water quality and biomonitoring (Vermeulen and Wepener 1999; Mzimela et al. 2002; Wepener and Vermeulen 2005; Greenfield et al. 2011). Therefore, it is important to implement organic contaminant monitoring programs in these systems as well.

Richards Bay Harbour, although a relatively new harbour constructed 37 years ago and located approximately 190km to the north of Durban, has become South Africa's premier port handling bulk cargo (Greenfield et al. 2011). It also has the largest single coal terminal in the world, contributing to its size and activity (Walmsley et al. 1998). This harbour also supports various other industries that benefit from its

location. These industries (phosphoric- and other acid plants, aluminium refinery, wood chip factory and fertilizer) greatly contribute to the contamination of this port (Vermeulen and Wepener 1999). Exports trade in steel, chrome ore and ferro-alloys, some of which are mined near Richards Bay (Vermeulen and Wepener 1999).

Durban Harbour was developed in the late 1800s, known as 'Port Natal', making it an old and well-developed harbour. It used to be permanently open to the ocean and quite shallow (approximately 3 m), but is now classified as an "estuarine bay" being tidal dominated (Harris and Cyrus 2000). Also considered one of South Africa's largest ports, there is still continuous development, recreational activities (e.g. angling) and increased pollution affecting this estuary. These activities have a large degrading effect on its functionality as a resource and estuarine habitat (Harris and Cyrus 2000).

1.3. PERSISTANT ORGANIC POLUTION

The global perspective of environmental pollution has changed over time, especially on persistent organic pollutants (POPs), now seen as a great contamination problem with toxic risks to both the environment and the human population (Fu et al. 2003). Rachel Carson's book "Silent Spring" has brought more focus on the effect of these contaminants on birds, bringing forth some of the first studies focusing on POPs and their toxic effects. As mentioned previously, estuaries are very important ecosystems that are greatly affected by various pollution sources, Durban and Richards Bay harbours being among some of these estuarine systems under threat. There has been a distinct lack in research for POPs monitoring in South Africa, the main reason being the expense of analyses, lack of necessary skills and shortage of available funding (DEAT 2008). The Department of Environmental Affairs and Tourism did fund a project for the "Development of National Inventory for Ten New POPs in South Africa" in 2013 (DEAT 2013), but not much research is done in terms of monitoring POPs in coastal areas.

There are many POP compounds that often come from a certain series of chemicals, differing only in chlorination of the molecule (Jones and de Voogt 1999). Persistent organic pollutants are persistent in environmental systems due to their long half-lives in biota, soil, sediments, air and their ability to travel great distances through ocean

currents, air and rivers (Fu et al. 2003; Braune et al. 2005). They mainly consist of polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs), polychlorinated dibenzo-*p*-furans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) all identified by the Stockholm Convention and polycyclic aromatic hydrocarbons (PAHs), which are not as yet identified as POPs by the Stockholm Convention. The abovementioned compounds are all lipophilic and giving them the property to partition strongly to organic matter (fat tissues of biota) and sediments (Jones and de Voogt 1999; Esteve-Turillas et al. 2008). Another great concern is POPs ability to be bioaccumulated in biota and biomagnified in food chains, having detrimental effects not only on sub-organism level, but on top-predators as well (Braune et al. 2003). There are many sources and transport mechanisms of POPs which include shipping and port operations, industrial and domestic effluents, metal refining and aerial deposition and combustion activities in various industries (Prest et al. 1995; Jones and de Voogt 1999). Therefore harbours have a high potential for POP contamination due to their port and shipping activities, not to mention other probable sources mentioned above.

1.3.1. Polychlorinated biphenyls

Polychlorinated biphenyls are synthetic, lipophilic chemicals that are produced by the catalysed chlorination of biphenyls. They consist of 209 isomers, of which all are classified as POPs (Cimenci et al. 2013; Kampire et al. 2015a). According to the ATSDR (2000), PCBs were introduced into the environment by accidental spills, leaks, illegal dumping exercises, improper waste disposal and fires of products containing these chemicals. The PCBs in the environment have a high persistence and do not readily break down, staying in the environment for long time periods and have the ability to be carried long distances away from the source pollution. Therefore PCBs have been identified worldwide since they are globally distributed and have harmful effects on both the environment and human life (Breivik et al. 2004; Cimenci et al. 2013; Gdaniec-Pietryka et al. 2013). Two groups of PCB mixtures have been classified: 1) dioxin-like and 2) non-dioxin like compounds, of which the non-dioxin like PCBs (no. 28, 52, 101, 138, 153 and 180) are monitored regularly in the environment because of their abundant releases in the past (Okay et al. 2009). These compounds with their persistent behaviour tend to settle onto

sediment particles, giving them the ability to be released further into the surrounding water (ATSDR 2000).

Since PCBs are very lipophilic hydrophobic in nature, they also tend to partition to organic matter and bioaccumulate, especially in fatty tissues of marine biota that include digestive glands of mussels, fish liver and tissues of marine mammals in general (Cimenci et al. 2013; Kampire et al. 2015b). Their tendency to attach to fat tissues of various marine biota gives rise to the ability to biomagnify in the food chain, i.e. increasing in concentrations the higher up they move in the food web (ATSDR 2000; Alava et al. 2012; Everaert et al. 2015). These biomagnified concentrations in marine biota are sometimes 40 times higher than the concentrations in the surrounding water (Echeveste et al. 2010). This biomagnification property of PCBs can easily be explained with Log K_{OW} (n-octonal/water partition coefficient) values. When persistent hydrophobic chemicals have Log K_{OW} values greater than three, they have the distinct capability to biomagnify and attach to fatty tissues. Polychlorinated biphenyls have Log K_{OW} values between 4.5 and 8.2 (Padmanabhan et al. 2006).

1.3.2. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons are chemicals formed during incomplete combustion activities. These activities may include burning processes involving oil, crude oil, gas, wood, coal, garbage, Tabaco or even meat (ATSDR 2000). They mainly consist of two or three aromatic rings of which each ring consists of either five or six carbons (Luna-Acosta et al. 2015). Unlike PCBs that have no natural source, PAHs can occur naturally in oil and coal but with all the current anthropogenic combustion activities involving fossil fuels and organic matter, the natural load is exceeded by a large extent (ATSDR 1995; Tierney et al. 2014). A group of these compounds have been identified for monitoring in the environment because of their dioxin-like toxic effects on biota and in turn humans due to their biomagnification properties (Wolska et al. 2012; Tierney et al. 2014).

As mentioned above, POPs can bioaccumulate and has the tendency to biomagnify with increasing trophic level. This makes it a potential risk for human consumption of all seafood, especially fish, as the concentrations tend to be 20 – 40 times higher

than in the surrounding environment (Echeveste et al. 2010; Wolska et al. 2012). Polycyclic aromatic hydrocarbons also have the ability to break down into longer-lasting products by reactions to light and other chemical compounds in the air. These light sensitive chemical reactions occur within a few days after the release of PAHs into the environment (ATSDR 1995).

The toxic and carcinogenic properties of PAHs are of great concern. An increase in certain PAHs may lead to not only carcinogenic effects, but also immunotoxic and neurotoxic effects, as established by several studies; (Casale et al. 2000; N'Diaye et al. 2006; Perera and Herbstman 2011). Oxidative damage is associated with PAH toxicity effects in on biota and photochemical induced toxicity, of which proteins (enzymes) and DNA are affected, that can be monitored using biomarker response analysis such as measuring Cytochrome P450 presence, oxidative stress metabolites and inhibition (Shimada and Fujii-Kurigama 2004; Gao et al. 2005; Obinaju et al. 2015).

1.3.3. Organochlorine pesticides

Organochlorine pesticides (OCPs) are a range of synthetic, commercial grade chemicals used as insecticides for agricultural purposes and pest control. Here, I will consider two types of OCPs: 1) Hexachlorocyclohexane (HCH), also known as benzene hexachloride (BHC) and 2) DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane). Although these pesticides are OCPs, their chemical structures differ greatly. There are eight isomers of HCH, of which four isomers (gamma-; beta-; alpha-; delta and epsilon) were used as a mixture, also known as technical HCH, for the purpose of agricultural pest control (ATSDR 2005; Vijgen et al. 2011). Technical-grade DDT consists of a mixture of three isomers (*o,p'*-DDT; *p,p'*-DDT and *o,o'*-DDT), but also contained DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) and DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene), which are by products of DDT (ATSDR 2005). Both these groups of OCPs have been banned in developed countries from production and usage for the last 20 to 30 years, although DDT is still used in some developing countries to control malaria for human health (ATSDR 2005; Sibali et al. 2008; Stockholm Convention 2014).

Generally OCPs are not easily degraded, giving them long lives and also the ability to travel long distances (Yang et al. 2010). With high Log K_{OW} values between 3.8 and 6.2 collectively, they have a tendency to attach to organic matter and fatty tissues (lipophilic), also giving them the ability to be bioaccumulated and biomagnified in the environment (Ahmed et al. 2015). Both DDT and HCH, with all their isomers and by products, are listed as some of the most persistent organic compounds, since they have high toxic and poisonous properties (Tang et al. 2013).

Water contamination of DDTs and HCHs occur from both point and diffuse sources of surface runoff and other means, which make them persistent in fresh water (rivers, ponds, lakes, and reservoirs), estuaries and lastly the ocean (Zhou et al. 2008; Hu et al. 2011). A study conducted on the South African Jukskei River catchment illustrated that there is definite OCP pollution, of which the compounds are all a risk to both biota and human health (Sibali et al. 2008).

1.4. IMPLEMENTATION OF POLLUTION MONITORING TECHNIQUES

1.4.1. Active biomonitoring

The concept of “active biomonitoring (ABM)” has been a ‘hot topic’ in pollution monitoring studies in both freshwater and marine systems due to its effectiveness to give accurate assessments of the environmental quality and toxic effects of pollution on organism populations (Nasci et al. 1999). The definition according to Wepener (2013) is: “…the translocation of organisms from one place to another and quantifying their biochemical, physiological and/or organismal responses for the purpose of water quality monitoring”. In this approach bio-indicator organisms are used, transplanted/relocated from an unstressed environment to a stressed/contaminated environment, making it possible to assess the ecotoxicological effect of this translocation over time and space in the ambient environment (Wepener 2013). The bio-indicators are deployed for a period of four to six weeks, giving the organisms enough time to recover from stress due to translocation and to respond to the environmental conditions monitored (Wepener 2013). In marine pollution ABM studies, bivalves such as mussels are often used due to their suspension feeding habits (Chandurvelan et al. 2015). They are able to bioaccumulate and concentrate toxic pollutants. Also being sedentary they provide

both spatial and temporal levels of contamination (Goldberg et al. 1978). According to Smolders et al. (2003), there are both advantages and disadvantages to the utilization of mussels. The advantages include the following: 1) mussels are not so sensitive to handling stress and crowding, they have minimal life space requirements, making the use of small cages possible; 2) mussels have relatively high resistance/tolerance to pollution, but they are not insensitive; 3) mussels are filter feeders, therefore exposed to various contaminant sources such as water, suspended matter and food; 4) mussels have high bioaccumulation potential for organic contaminants and low biotransformation potential and 5) marine mussels are easily collected due to their sedentary state. The disadvantages are the following: 1) reproduction has very high ecological relevance, difficult to measure and season dependent; 2) reference populations and proper history are not available, making comparisons between studies difficult and 3) with their low biotransformation of organic contaminants, certain biomarkers may not be used with limited biotransformation enzymes available. But as the advantages outweigh the disadvantages, mussels are able to act as good bio-indicators of marine pollution monitoring.

The brown mussel, *Perna perna*, is generally found in the intertidal and infralittoral zones of the rocky shores. They are reproductive at an early stage (less than one year) when still very small (Narváez et al. 2008). The brown mussel belongs to the family Mytilidae. The genus *Perna* is distinguished by a pattern of scars on the left hand side of the muscle attachment area (Vakily 1989). The three species belonging to the genus *Perna* are hardly distinguishable from each other except by geographical location (Vakily 1989). Although colour is not a characteristic to distinguish these species, adult *P. perna* have a typical brown to red-maroon colouring, with random flecks of a lighter brown and some green. Since *Perna* is easily adaptable to various environmental conditions, they can be found in brackish water, estuaries and therefore harbours as well, rocky shores and in less sheltered waters (Vakily 1989). Only a few studies have implemented the bivalve *Perna perna* as a bio-indicator for POPs monitoring, but these studies did demonstrate their usefulness as POPs accumulators (Francioni et al. 2005; Francioni et al. 2007; Degger et al. 2011a). A recent study of PCB pollution in Port Elizabeth Harbour did

demonstrate the success in using biomonitoring as a tool for POPs monitoring in the marine environment (Kampire et al. 2015a).

Essential assessment endpoints used in ABM studies are aimed at measuring effects at different levels of biological organisation (Wepener 2008). Lower levels of biological organisation, especially sub-organism level, have fairly rapid responses following deployment with lower ecological importance, represented in Figure 1.1. These assessment endpoints at sub-organism level are referred to as biomarkers (Wepener 2013). The ABM approach mainly concentrates on organism and sub-organism levels, as responses in higher levels of biological organization are very complex (Wepener 2013). The ABM approach, in which samples/bio-indicators are transplanted from an unstressed/reference environment to a contaminated site, has been investigated by several marine pollution monitoring studies, determining its success (Nasci et al. 1999; Damiens et al. 2007; Wepener et al. 2008; Bebiano and Marreira 2009; Brooks et al. 2009). To date ABM has only been applied to monitor metal and organic bioaccumulation in mussels from the South African coastline (Degger et al. 2011a; Degger 2011b; Greenfield 2014).

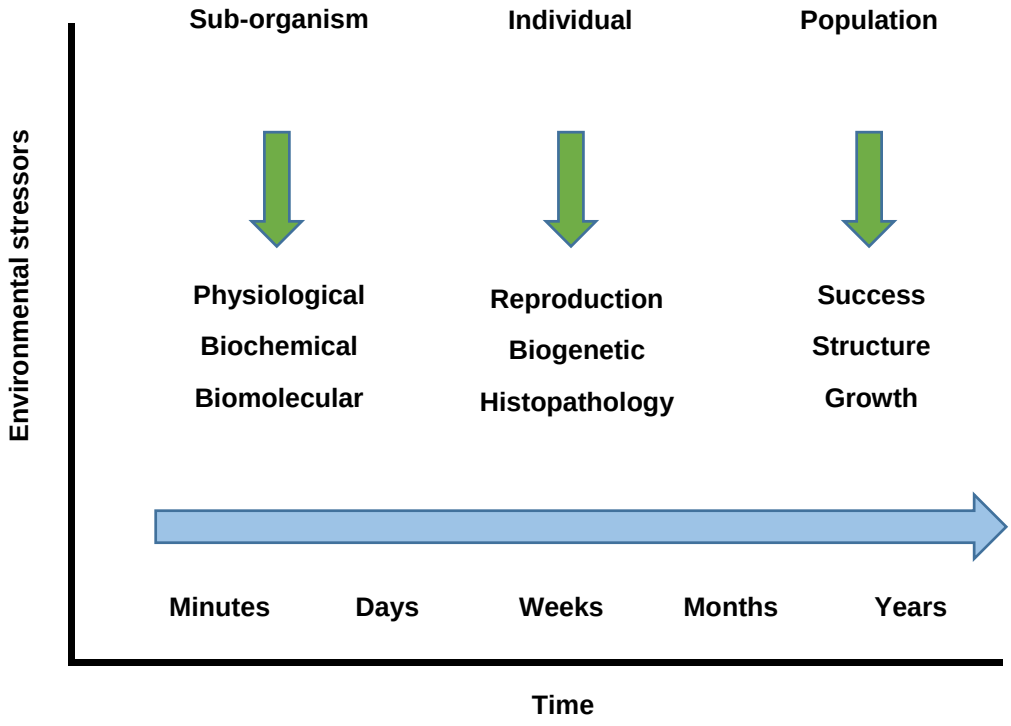

Figure 1.1. Ecological relevance against levels of biological organisation (adapted from Adams et al. 2000).

1.4.2. Biomarkers

A suite of biochemical biomarkers (assessment endpoints) is applied to these bivalves to detect exposure and effects of toxic contaminants in the environment. Biomarkers are biological responses at molecular or biochemical levels, involving enzymes and metabolites and are regarded as early warning signs for long-term pollution effects. They are ideal due to their ability to respond rapidly to stress and their high potential for toxicological relevance (Lau and Wong 2003). Biomarkers of exposure are induced when an organism is exposed to a pollutant e.g. acetylcholinesterase (AChE) and cytochrome P450 in the case of organophosphates, carbamates and organochlorines. Biomarkers of effect provide an indication of responses to pollutants e.g. through the induction of antioxidants in response to reactive oxygen species (ROS) formation. These antioxidant responses are determined by measuring enzymatic and non-enzymatic biomarkers such as catalase activity (CAT), malondialdehyde (MDA), superoxide dismutase (SOD) and protein carbonyl (PC) levels (Wepener et al. 2012).

The biotransformation of organic contaminants have an elevated effect on cytochrome P450 due to uncoupled electron transfer from cytochrome P450 to NADPH P450, in turn causing an increase in oxidative stress compounds like ROS and hydrogen peroxide (Zangar et al. 2004). Anti-oxygen defence systems will activate in the presence of increased ROS and hydrogen peroxide levels. The enzymes responsible for these anti-oxidant mechanisms are SOD, CAT and glutathione peroxidases (Almeida et al. 2015). With high levels of ROS, lipid peroxidation of cell membranes may also occur, where lipids are oxidized by ROS to create higher levels of MDA in organisms (Almeida et al. 2015; Almeida et al. 2005). Lipid peroxidation can result in the formation of PCs, where ROS are known for their ability to convert amino acid groups, changing their structure with lipid or sugar carbonyl moieties attached to the amino acids as side chains (Requena et al. 2003). Therefore an increase in protein damage occurs with an increase of oxidative stress (Almroth et al. 2005).

1.4.2.1. Acetylcholinesterase

The use of the enzyme AChE as a biomarker of exposure has been successful in a variety of environmental studies, utilizing different species of fish, mussels and other invertebrates in both fresh- and saltwater environments (Straus and Chambers 1995; Solé et al. 2010; Natalotto et al. 2015; Vidal-Liñán et al. 2015). Organic pollutants have a toxic effect on nervous tissue, initiating the inhibition of AChE activity and an increase of acytocholine at the nerve synapsis (Peakall 1992). The increase of acytocholine levels disrupts nerve function at the inhibition site (Peakall 1992). Acytocholine is the neurotransmitter that facilitates AChE activity, where it is responsible for the transmission of impulses in the nerve tissues making it an indicator of potential nerve damage/neurotoxicity (Peakall 1992; Natalotto et al. 2015). Corsi et al. (2007) demonstrated the importance of AChE in essential bivalve functions such as temperature resistance, valve opening, embryo development and ciliary activity.

1.4.2.2. Cytochrome P450

Enzyme responses to POP exposure, especially enzymes catalysing a phase I response, are good biomarkers of exposure. Two such enzymes are CYP450 (Khessiba et al. 2005) and ethoxyresorufin o-deethylase (EROD), triggered in the presence of PCB, OCP and PAH compounds (Kirby et al. 2004). The phase I response can also be called the oxidative metabolism, the first step in biotransformation, of which the enzyme CYP450 monooxygenases plays an important role (Stegeman and Lech 1991). Both fish and invertebrates possess these microsomal enzymes to transform organic compounds and activate carcinogens (Stegeman and Lech 1991). The general pathway of POPs in the oxidative metabolism is the formation of a complex with the aryl hydrocarbon receptor (AhR), which is transported into the nucleus and binds, through the AhR nuclear translocator, to dioxin responsive elements, activating the CYP450 response (Stegeman and Lech 1991). Benedetti et al. (2015) did a review of CYP450 emphasizing its uses and success in environmental monitoring and the different pathways of several organic compounds in the oxidative metabolism of different species.

1.4.2.3. Catalase activity

The main function of CAT is to convert hydrogen peroxide to water and oxygen, a process called hydrolyses (Limón-Pacheco and Gonsebatt 2009). These heme-containing enzymes are mostly found in peroxisomes, organelles within the cells of the organism, but endoplasmic reticulum and mitochondria hardly contain any CAT, making it difficult to transform intracellular hydrogen peroxide unless it diffuses into the peroxisomes (Limón-Pacheco and Gonsebatt 2009). The induction of CAT activity indicates an increase in oxidative stress and is an ideal biomarker for monitoring environmental stress, due to organic pollutant contamination, in mussel gills and digestive glands (Natalotto et al. 2015; Sellami et al. 2015).

1.4.2.4. Malondialdehyde (lipid peroxidation)

Lipid peroxidation have harmful effects on cells, degenerating cell membranes and organelle membranes and therefore changing their permeability (Storey 1996). This degenerative process is activated in the presence of harmful substances such as organic pollutants and metals with toxic accumulation and effects (Ameur et al. 2015). The MDA is a product of the lipid peroxidation process and is associated with both oxidative stress and protein damage (Traverso et al. 2004; Flohr et al. 2012). An increase in MDA levels have shown to be a biomarker of oxidative stress and has been successfully implemented in several environmental monitoring studies using bivalves and fish as indicator species (Flohr et al. 2012; Almeida et al. 2015; Ameur et al. 2015; Nnamdi et al. 2015).

1.4.2.5. Superoxide dismutase

The antioxidant enzyme SOD is the predecessor of the enzyme CAT in the antioxidant defence system and decomposes superoxides, which are produced by ROS, into hydrogen peroxide (Storey 1996). These enzymes contain metal, of which three isomers are identified and present in eukaryotic cells (Limón-Pacheco and Gonsebatt 2009; Nnamdi et al. 2015). Since CAT follows SOD responses, elevated levels of SOD will induce elevated levels of CAT during oxidative stress from pollutant toxicity.

1.4.2.6. Protein carbonyl

An increase in ROS production could overwhelm anti-oxidant defences, which results in lipid peroxidation and PC formation. As mentioned previously, amino groups are converted to other structures by ROS and their functionality altered. According to Requena et al. (2003) PCs form via adduction of oxidized sugars/lipids or via metal catalysed oxidation. But PC formation can also occur via secondary processes, of which reactions of free radicals with lipids, nucleic acids and carbohydrates, are one of them (Grune et al. 2000). The formation of PCs are not reversible and will affect catalytic activity and ultimately result in protein breakdown (Almroth et al. 2005).

1.4.3. Integrated biomarker response index

Even with the advantages in the application of biomarkers to monitor organic pollution in coastal and freshwater areas, there are a number of disadvantages. It is be difficult to both analyse and integrate responses to pollution for non-specialists, environmental managers and even decision makers, making it difficult to fully implement these monitoring tools successfully (Sanchez et al. 2012). According to Sanchez et al. (2013) several authors have tried to develop an integrative method to summarize a suite of biomarker responses in a single graph and value. Amongst these methods the most often used is the Integrated Biomarker Response (IBR) index (Beliaeff and Burgeot 2002) which makes use of starplots as graphical representation. However, the IBR index does have some drawbacks: 1) the result is dependent on the biomarker arrangement of the starplot and 2) up and down regulation are the sole considerations. Even with these shortcomings, the IBR index has been successfully applied to illustrate biomarker responses in a biomonitoring study using bivalve indicator species (Brooks et al. 2015).

1.4.4. Passive monitoring

The Mussel Watch Program (Goldberg 1975) has been an active global monitoring program for over 30 years. Many countries which include Japan, Canada and the United States and a few from Europe have successfully used and developed this program to monitor pollution levels in the marine environment (Farrington et al. 1983; Lauenstein 1995; Cantillo 1998; Monirith et al. 2003; Rodriquez y Baena and

Thébault 2007). However some shortcomings have been identified regarding the accuracy in monitoring bioaccumulation levels from toxic pollution in coastal regions. Not all bivalve mussel species bioaccumulate the same concentrations of toxic pollutants due to differences in species, size, age, gender, health, physical environmental factors (SANCOR 1985b) and the ability to metabolize toxic compounds (Richardson et al. 2003). Thus other techniques such as passive sampling devices are also used to obtain an integrated and accurate reflection of organic pollution levels (Prest et al. 1995). According to Prest et al. (1995), there are limitations in biomonitoring due to the different degrees of POPs bioaccumulation in species and individuals within a species group, motivating the need for developing passive monitoring methods such as semi-permeable membrane devices (SPMDs), in which these variations will be limited. Semi-permeable membrane devices have been successfully implemented for years in sampling both atmospheric and water systems as a monitoring tool for POPs (Prest et al. 1995; Söderström and Bergqvist 2004; Augulyte and Bergqvist 2007; Fernandez et al. 2009).

Semi-permeable membrane devices are able to 'mimic' the uptake of hydrophobic/lipophilic organic pollutants by bivalves and these devices are also effective in representing concentrations of pollutants in both water and sediment (Sabaliunas et al. 2000; Richardson et al. 2001). These passive samplers, originally designed by Huckins et al. (1990), consist of lipid (triolein) filled polyethylene tubes and function on the principle that lipophilic compounds (POPs) will partition from the water column through the polyethylene tubing into the lipid (Figure 1.2). This lipid has the ability to capture and concentrate non-ionic lipophilic pollutants when they diffuse from the environment through the polyethylene tubing into the lipid (Richardson et al. 2003). They are also able to give standardised values of organic pollution due to the ability to eliminate numerous variables (climate, physical environmental conditions, biological processes etc.), making comparisons between sites more accurate (Setkova et al. 2005). These SPMDs have shown to be a promising method to monitor POPs successfully as they have the ability to accumulate contaminants that are in some cases absent in mussel tissue concentration levels. Recent studies have validated this method and its usefulness in South African coastal regions (Degger et al. 2011a).

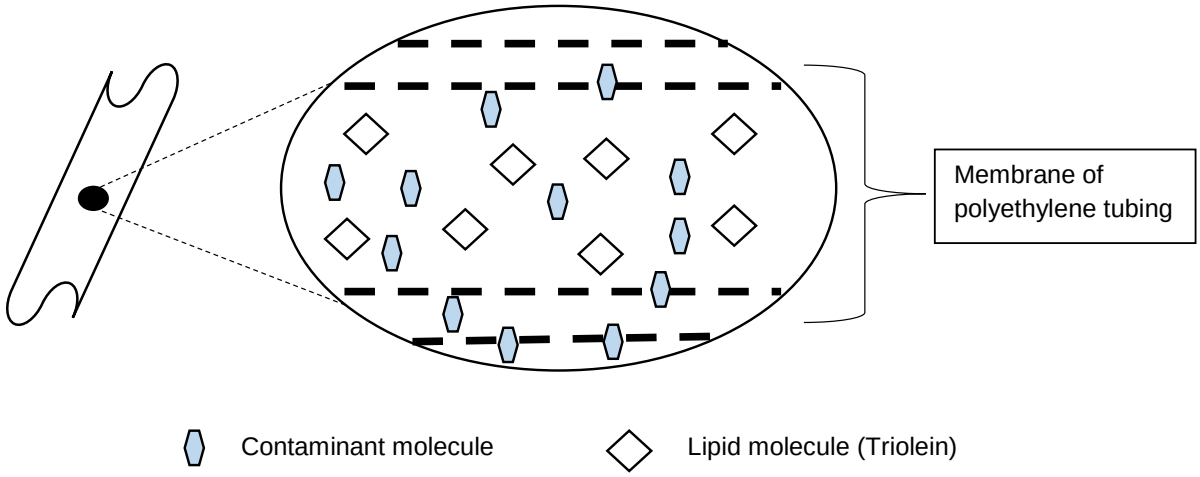

Figure 1.2: Detailed scheme of contaminant partitioning through semi-permeable membrane device (adapted from Setkova et al. 2005).

1.5. HYPOTHESIS, AIMS AND OBJECTIVES

The aims of the present study were to:

- Develop and test the most appropriate extraction and analytical techniques to undertake analyses of trace POPs (selected PAH, PCB and OCP contaminants) in environmental and biological samples.
- Use SPMDs and bio-indicator organisms (ABM - transplanted and resident brown mussels - *P. perna*) to determine the extent of POPs exposure on a spatial (two harbours along the KwaZulu-Natal coastline) and temporal (during high rainfall and low rain fall periods) scale.
- Apply a suite of biomarkers in the ABM and resident mussels to determine whether POPs exposure result in biological effects in these marine organisms.

The specific objectives were to:

- Refine and validate extraction and analytical techniques for environmental matrices in collaboration with the National Metrology Institute of South Africa (NMISA) as part of their reference material development activities.

- Conduct field studies comparing SPMDs and mussels for monitoring exposure to POPs in the marine environment at Durban and Richard's Bay Harbours during a high and low rainfall period.

- Determine the levels of the POPs in the mussels from Sheffield Beach, which were used as reference mussels for the ABM studies.

- Determine the extent to which POPs exposure results in biological effects through relating exposure to biomarker responses.

- Provide recommendations to promote the inclusion of SPMDs and biomarkers in a Marine Pollution Monitoring Program for South Africa.

This research tests the following hypotheses:

- The SPMDs and bio-indicator organisms (transplanted and resident mussels) will show similar bioaccumulation patterns for organic pollutants;

- provides an indication of oxidative stress indicative of organic pollutant exposure;

- And exposure to POPs and the ensuing biomarker responses are influenced by increased run-off during the high rainfall periods.

- Both transplanted (ABM) and passive monitoring methods are efficient in monitoring organic pollutants along the KwaZulu-Natal coastline.

CHAPTER 2

MATERIALS AND METHODS

2.1. SITE SELECTION

Richards Bay Harbour and Durban harbour are two of South Africa's shipping ports, located along a primary international shipping route (Marshall and Rajkumar 2003). These ports were developed in pristine estuarine environments but, due to increased port-, industrial activities and mining activities/development, the degradation of these systems have come of great concern (Marshall and Rajkumar 2003; Greenfield et al. 2011). Due to their sheltered nature, ecological and economic importance, and exposure to contaminants from industrial and shipping activities, they are ideal for organic pollution monitoring. Thus, SPMDs were deployed at these harbours, along with the brown mussel *P. perna*, collected from the reference site at Sheffield Beach (Figure 2.1) for spatial bioaccumulation analysis.

Richards Bay Harbour (28° 48' 00" S; 32° 05' 00" E) is situated along the South African east coast, approximately 190 km north of Durban (Figure 2.2). Developed 36 years ago, Richards Bay harbour has become one of South Africa's largest and busiest ports with various industrial and mining activities in the surrounding area (see Chapter 1).

Durban Harbour (29.8732° S; 31.0245° E) is situated in Durban, also along the South African east coast (Figure 2.3). Durban harbour was constructed in the late 1800s, making it an old and well developed port with continuous development, recreational activities, increased contaminant exposure and industrial effluents (see Chapter 1).

Sheffield Beach (29°29' 00" S; 31°16' 00 E) is situated approximately 60 km to the north of Durban and 125 km to the south of Richards Bay, along the South African east coast (Figure 2.4). Also known as the Dolphin Coast for its many dolphin sightings, it is regarded as an unstressed environment with minimal industrial influences and development. Due to the rocky shores, brown mussel *P. perna* was also easily collected during low tide conditions. Greenfield et al. (2014) also demonstrated the use of this mussel population in ABM of South African harbours.

Brown mussels, between 40 – 50 mm in size, were collected from Sheffield Beach and translocated to the selected monitored sites for an exposure period of six weeks.

Two exposure surveys were conducted, March/April 2014 referred to as the rainy season and June/July 2014 referred to as the non-rainy season.

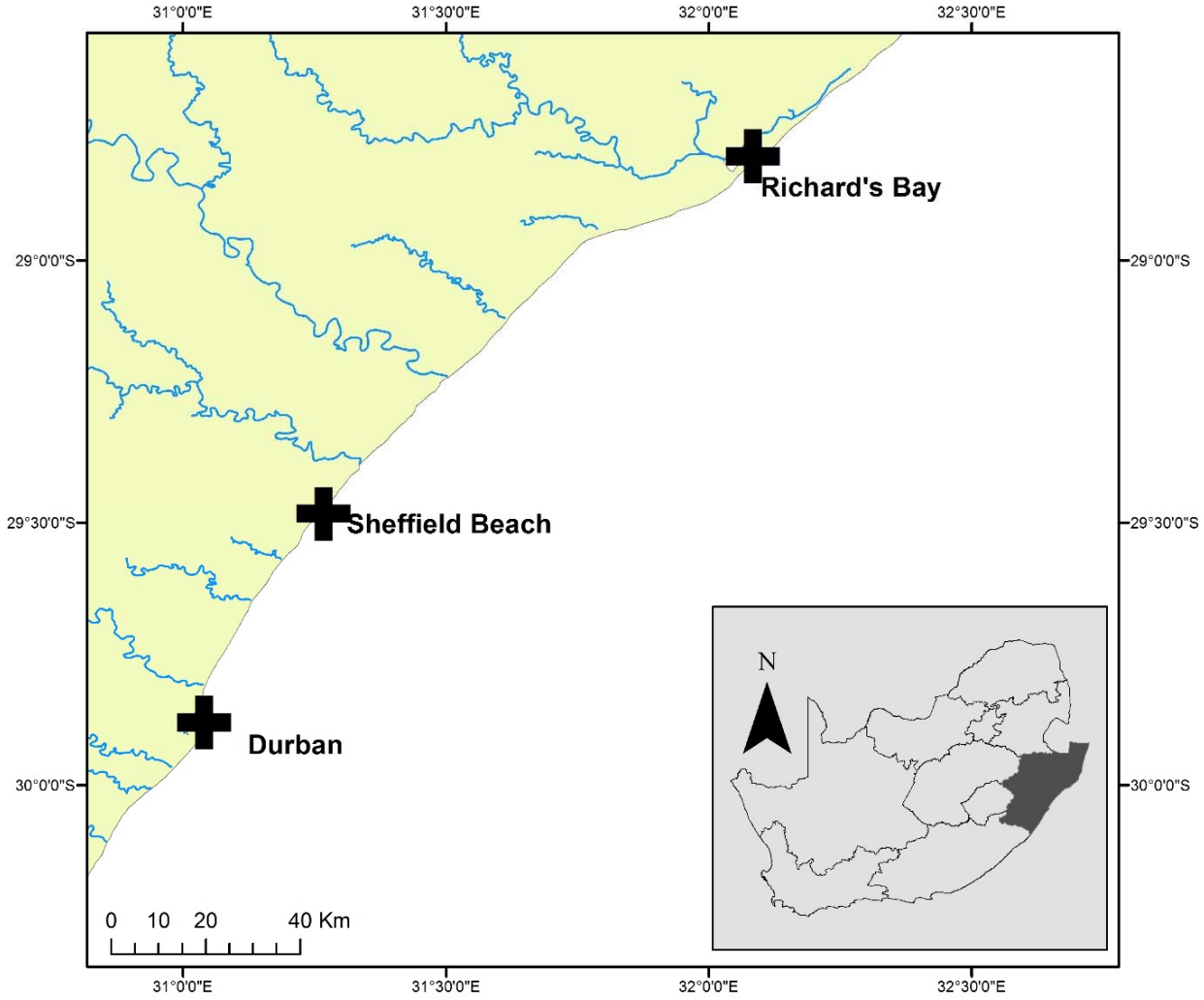

Figure 2.1. Map of study sites selected for monitoring persistent organic pollutants (POPs) along the east coast of South Africa.

Figure 2.2. Richards Bay Harbour (Google earth images).

Figure 2.3. Durban Harbour (Google earth images).

Figure 2.4. Sheffield beach reference site.

2.2. SPMD PREPARATION, DEPLOYMENT AND RETRIEVAL

The SPMDs (Figure 2.5) consisting of polyethylene lay-flat tubing, 5 cm wide and 0.05 cm thick containing PCB 8 spiked triolein lipid as a permeability reference compound (PRC), were deployed in stainless steel canisters. Replicate SPMD canisters, each containing duplicate SPMDs, were deployed at the two monitoring sites (Figure 2.1). The SPMDs were woven through the prongs of the canisters to maximise the surface area exposed to the environment (Lohmann et al. 2001). The devices were prepared in the field, therefore field blanks were exposed to the air for background concentration correction. The canisters were attached to a navigational buoy in Durban harbour (Figure 2.3) and a free-standing jetty in Richards Bay harbour (Figure 2.2) with chains, at a depth of approximately 2 m. The SPMDs were left for an exposure period of six weeks, after which they were retrieved. Upon retrieval, field blanks were exposed to the air for background concentration correction, the SPMD canisters wrapped in aluminium foil, after which the SPMDs were removed from the canisters, cleaned of biofouling with Milli-Q water, placed separately in aluminium foil and frozen at - 20°C, pending analysis. The frozen samples were transported to NMISA (National Metrology Institute of South Africa) for analysis.

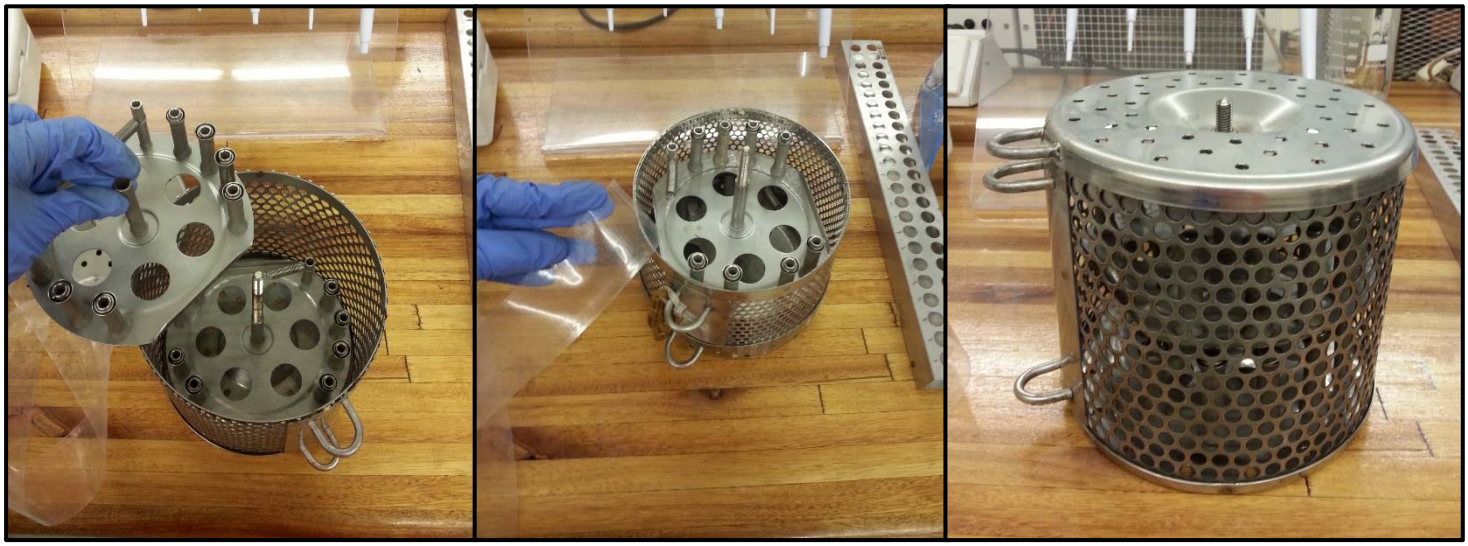

Figure 2.5. Semi-permeable membrane devices (SPMDs) and canisters.

2.3. MUSSEL PREPARATION, DEPLOYMENT AND RETRIEVAL

Brown mussels, *P. perna*, 40 – 50 mm in size were collected from Sheffield Beach and transplanted to the two monitoring sites (Figure 2.1). Approximately 50 mussels were deployed at each site in stainless steel containers (Figure 2.6), attached with chains to a navigational buoy in Durban harbour (Figure 2.3) and a free-standing jetty in Richards Bay harbour (Figure 2.2). Mussels were exposed for a six week period (exposures described in Section 2.2). Upon retrieval, 30 transplanted mussels and 30 resident mussels from the reference site and monitored sites were immediately dissected. Five mussels were pooled to form six resident samples per site and six transplanted samples per monitored site. Half of each mussel's digestive gland was removed for ABM purposes and the rest of the tissue used for chemical analysis. The dissected digestive glands were placed in 2 ml microtubes with Hendrikson Stabilising Buffer (HSB), placed in liquid nitrogen and transported to the laboratory at North-West University. The samples were removed from the liquid nitrogen and placed in a - 80°C freezer, pending analysis. The dissected mussels were placed in 50 ml polypropylene Falcon tubes, frozen in liquid nitrogen and transported back to the laboratory at North-West University. The samples were removed from the liquid nitrogen and freeze-dried for four days. Freeze-dried samples were transported to the National Metrology Institute of South Africa (NMISA) for chemical analysis.

Figure 2.6. Transplanted mussels in stainless steel canister ready for deployment.

2.4. CHEMICAL ANALYSIS

All extracts, standards and calibration curves were prepared in toluene, which were also used as a rinse solvent and solvent blanks during analytical runs. The native standards as well as carbon labelled (Cambridge Isotope Laboratories) and deuterated isotopes (Table 2.1) were obtained commercially with purities between 97-99 %. All solvents used were high purity HPLC grade, Supelclean dual layer LC-Florosil®/ LC silica solid phase extraction (SPE) cartridges (Sigma-Aldrich) were used during extract purification. The QuEChERS used during mussel tissue analysis were Q-sep™ Q110 packets compliant with European EN15662 method (Restek) and Q-sep™ QuEChERS dSPE 15 ml centrifuge tubes with AO/AC 20070.1 method (Restek).

Table 2.1. Standards used during analysis.

Compound group	**Native Standard**	**Labelled Isotope**
PCBs	Restek PCB congener standard 2	EN-1948 Marker PCBs
OCPs	Restek individual standards	CIL individual labelled standards
PAHs	SRM 1947s (NIST)	PAH cocktail for Carb method

2.4.1. Semi-permeable membrane analysis

The analysis method was developed and validated by NMISA (2015a). The samples were removed from the freezer and allowed to return to room temperature. During thawing, 50 ml centrifuge tubes were labelled and spiked with an internal standard. The SPMDs were removed from the alumina foil and further manually cleaned when needed, using Milli-Q water. A new set of nitrile gloves were used in between each sample to prevent cross contamination. After cleaning the SPMDs were cut into smaller segments and rinsed three times with hexane to a total volume of 50 ml. The SPMD segments were cut into smaller strips and placed in a 50 ml centrifuge tube with 30 ml hexane. Both sets of extracts were spiked on top of the solvent with the labelled isotope mixture of all three organic compound groups (PCBs, OCPs and PAHs). The extracts were left overnight to equilibrate.

The sample extracts were placed in an ultrasonic bath at ambient temperature (20˚C) for two hours to extract. Thereafter the extracts were centrifuged at 5 ˚C, 3000 RPM (rotations per minute) for 20 minutes and stored overnight at -20 ˚C to separate the solvent and triolein components. The solvent phase was removed and evaporated under a stream of nitrogen to 10 ml. After evaporation, the extracts were purified using dual layer LC-Florosil®/ LC silica SPE open column cartridges. The

SPE cartridges were conditioned with 10 ml hexane, the sample loaded and the cartridge eluted with 9 ml 1:1 DCM:hexane. The extracts were evaporated under a under a stream of nitrogen till dry and reconstituted in 100 µl toluene for analysis.

A LECO Pegasus IV chromatographic system and a comprehensive two-dimensional gas chromatography coupled to time of flight mass spectrometry (GC-GC-TOFMS) was used for analysis. The columns used for analysis were a non-polar Rxi®-XLB (30 m, 0.25 mm ID, 0.25 µm df) as the primary column and a mid-polar Rxi®-17SilMS (2 m, 025 mm ID, 0.25 µm df) as the secondary column. The autosampler, gas chromatography and mass spectrometry were optimised in the presence of triolein to ensure optimal separation between the target analytes and matrix interferences.

For quantification and quality control purposes, a ten-point matrix matched calibration curve for each of the target analytes was constructed using triolein extracts. The matrix matched calibration for PCBs ranged from 0 – 100 ng/SPMD, for OCPs 0 – 150 ng/SPMD and for PAHs 0 – 2000 ng/SPMD, collectively. The quality of analytical runs was tested running a blank sample, solvent blank and non-matrix matched standards. Recovery was assessed by gravimetrically spiking blank triolein samples.

2.4.2. Mussel tissue analysis

The analysis method was developed and validated by NMISA (2015b). The mussel samples were freeze-dried and manually ground in the 50 ml tubes until a powder-like consistency was obtained. Approximately 2 g of sample was placed into a new 50 ml centrifuge tube and spiked with labelled isotope, after which 10 ml of water was added to each sample and thoroughly mixed. The mixed samples were left for an hour prior to extraction to equilibrate.

After the hour, the samples were vortexed for two minutes and a 10 ml volume of 1:1 DCM:acetone was added to each sample. The samples were again vortexed for two minutes and placed in an ultrasonic bath at ambient temperature (20˚C) for 20 minutes. When the consistency of the rehydrated mussel samples changed to a gel-like appearance, the samples were placed in an orbital shaker for 15 minutes to ensure the samples are properly equilibrated prior to the QuEChERS extraction

procedure. The QuEChERS dispersive salt (magnesium sulphate, sodium chloride, trisodium citrate dehydrate and disodium hydrogen citrate sesquihydrate) was added and the samples were vigorously shaken for two minutes, vortexed and placed in the orbital shaker for 10 minutes. After shaking, the samples were centrifuged for 15 minutes at 3000 RPM and the organic phase transferred to a 15 ml test tube. The remaining solids were washed three times with 5 ml hexane and after each wash centrifuged for 15 minutes at 3000 RPM. The organic phase was removed in between washes and added to the 15 ml test tube with the previously retained organic phase.

The organic phase was evaporated under a stream of nitrogen to approximately 5 ml and added to the QuEChERS dispersive salt (magnesium sulphate, primary and secondary amine and C18 modified silica) in a 15 ml centrifuge tube. The extracts were vigorously shaken for two minutes, vortexed and placed in the orbital shaker for 15 minutes. The extract was centrifuged and the organic phase removed after which it was evaporated to approximately 5 ml. Thereafter 3 ml of hexane was added prior to SPE using a dual layer LC-Florosil®/ LC silica SPE open column cartridge. The eluent was evaporated to dryness and reconstituted in 100 µl toluene prior to GC-GC-TOFMS analysis.

The same chromatographic analysis system mentioned in section 2.4.1 was also used in the mussel tissue analysis. For quantification and quality control purposes, commercial frozen, cleaned and prepared blue mussels (*Mythilus edulis*) were homogenised and tested to ensure the samples did not contain traces of the target analytes. The same homogenised blue mussels were used to construct a matrix matched ten-point calibration curve with the ranges mentioned in section 2.4.1. The quality of analytical runs was tested running a blank sample, solvent blank and non-matrix matched standards. Recovery was assessed through the analysis of certified reference material (NIST SRM 1974c; Organics in mussel tissue, *M. edulis* and NIST SRM 2974a Organics in freeze-dried mussel tissue *M. edulis*). When in some cases a target analyte was not certified in these CRMs, gravimetrically spiked blank mussel samples (*M. edulis*) were used to calculate recovery.

2.5. BIOMARKER ANALYSES

In preparation of biomarker analyses, the samples were defrosted, weighed and homogenised, 0.1 g tissue in general homogenising buffer (GHB) or 0.05 g tissue in tris/sucrose homogenising buffer, depending on the biomarker response measured. The samples were placed in a -80°C freezer after homogenisation, pending analysis.

2.5.1. Acetylcholine esterase (AChE) activity

Biomarker activity was measured using the protocol of Ellman et al. (1961). The following solutions were made on the day of analysis: 1) 0.09 M potassium phosphate buffer (PPB) consisting of 1.566 g K_2HPO_4 (base, Rochelle) in 100 ml deionised water, 0.612 g K_2HPO_4 (acid, Rochelle) in 50 ml deionised water mixed together to pH 7.4; and could be stored up to three months; 2) 30 mM s-Acetylthiocholine iodide (Sigma-Aldrich) 0.214 g in 25 ml deionised water and 3) 10 mM Ellman's reagent (Sigma-Aldrich) 0.099 g in 25 ml methanol (Rochelle). Solutions 1 and 2 were kept cold at 4°C and solution 3 kept in the dark at room temperature.

Mussel tissue (0.05 g) was homogenised in 250 µl Tris/Sucrose Homogenising Buffer (pH 7.4) and centrifuged at 9 500 g (4°C) for 10 minutes. The supernatant was used for analysis. Only seven samples in triplicate were analysed per micro plate, whilst working on ice. Absorbance was measured with a Biotek Elx 800 micro plate reader. In each micro plate well, 210 µl PPB, 10 µl s-Acetylthiocholine iodide and 10 µl Ellman's reagent were added, mixed carefully and incubated for five minutes at 37°C. The sample/blank was added to each well, mixed and read immediately at 405 nm in one minute intervals over a six minute period (seven readings in total).

Protein content was determined using the protocol of Bradford (1976). In each micro plate well, 5 µl of blank, standards and samples were added in triplicate, together with 245 µl Bradford reagent (Sigma-Aldrich) and left for five minutes. The absorbance was read at 595 nm. Standards for protein content analysis were prepared using a concentration gradient of Bovine Serum Albumin (BSA) supplied by Merck/VWR. A stock solution of 0.01 g BSA in 4 ml deionised water was prepared

and the standards made up to the concentrations, with deionised water, represented in Table 2.2.

Table 2.2. Standard curve preparation for protein content analysis.

Standard	BSA (µg/ml)	BSA stock (µl)	Water (µl)
1	0	0	100
2	250	10	90
3	500	20	80
4	1000	40	60
5	1500	60	40
6	2000	80	20
7	2500	100	0

Acetylcholine esterase activity was determined by absorbance/min/mg protein, the absorbance being the average of the triplicate readings, and absorbance/min the gradient of the seven average absorbance readings over time.

2.5.2. Cytochrome P450 (CYP 450) activity

The P450 Demethylating Fluorescent Activity kit, Arbor Assays was used in this analysis, with all its provided standards, buffers, micro plates and protocol. Samples were analysed in duplicate and the fluorescence read at 450 nm with excitation at 510 nm with a Berthold Tristar LB 941, Germany micro plate reader.

2.5.3. Catalase Activity (CAT)

This biomarker of exposure was measured using the protocol of Cohen et al. (1970). The following solutions were made on the day of analysis: 1) 6 mM H_2O_2 (Rochelle) 60 µl in 100 ml PPB; 2) 5 ml H_2SO_4 (98 %, Rochelle) in 50 ml deionised water and 3) 0.0316 g $KMnO_4$ (Rochelle) in 100 ml deionised water. Solution 1 was kept cold at 4°C, solution 2 at room temperature and solution 3 kept in the dark.

Mussel tissue (0.1 g) was homogenised in 1 ml GHB (pH 7.4) and centrifuged at 10 000 g (4°C) for 10 minutes. The supernatant was used for analysis. Only 10 samples in triplicate were analysed per micro plate, whilst working on ice. Absorbance was measured with Biotek Elx 800 micro plate reader. In each micro plate well 10 µl blank/sample and 93 µl H_2O_2 was added and mixed. After three minutes, 19 µl H_2SO_4 was added to stop the reaction sequentially at the same time fixed intervals after which 130 µl $KMnO_4$ was added immediately and read at 490 nm within 30 – 60 seconds. For the standard, the 10 µl sample and 93 µl H_2O_2 were replaced with 102 µl PPB. Protein content was determined as described in section 2.5.1. Final CAT activity was expressed in µmol H_2O_2/min/mg protein.

2.5.4. Malondialdehyde content (MDA)

This biomarker of effect was measured using the protocol of Ohkawa et al. (1979) as modified by Üner et al. (2005). The following solutions were made on the day of analysis: 1) 8.1 % Sodium dodecyl sulphate (SDS, Sigma-Aldrich): 1.01 g dissolved in 12.5 ml deionised water; 2) 20 % Acetic acid (pH 3.5, Rochelle): 20 ml made up to 100 ml deionised water, pH adjusted with NaOH (Rochelle); 3) 0.8 % Thiobarbituric acid (Sigma-Aldrich): 0.8 g in 100 ml deionised water and 4) n-Butanol (Sigma-Aldrich) and Pyridine (Sigma-Aldrich) in ratio 15:1.

The homogenate and centrifuge procedure described in section 2.5.1. was used to obtain a supernatant and used for analysis, the samples and standards analysed in triplicate. Absorbance was measured with a micro plate reader Berthold Tristar LB 941, Germany. In 2 ml microtubes, 12.5 µl sample/standard/blank, 25 µl SDS, 187.5 µl acetic acid, 187.5 µl thiobarbituric acid and 87.5 µl deionised water were added together and placed in a boiling water batch at 95°C for 30 minutes. It was allowed to cool down at and to room temperature, after which 125 µl deionised water and 625 µl

butanol:pyridine solution was added, vortexed and centrifuged at 2 700 g for 10 minutes at room temperature. In each micro plate well 245 µl of supernatant was added and absorbance read at 540 nm.

Standards for MDA analysis were prepared using a concentration gradient of 1,1,3,3-tetramethoxypropane (TMP, Sigma-Aldrich). A stock solution of 6 µl TMP in 1.94 ml deionised water was prepared and the standards made up with deionised water to the concentrations represented in Table 2.3. All standards followed the same procedure as samples and blanks. Protein content was determined as described in section 2.5.1. Final MDA content was expressed in nmol/mg protein.

Table 2.3. Standard curve preparation for malondialdehyde content analysis.

Standard	TMP (nmol)	TMP stock (µl)	Water (ml)
1	0	0	2.00
2	0.5	1	1.99
3	1	2	1.98
4	1.5	3	1.97
5	2	4	1.96
6	2.5	5	1.95
7	3	6	1.94

2.5.5. Superoxide dismutase (SOD)

This biomarker of effect was measured using the adapted protocol of Greenwald (1989). The following solutions were made on the day of analysis: 1) Tris/DTPA

buffer, 1.214 g Tris (Sigma-Aldrich) in 200 ml deionised water pH adjusted to 8.2 with HCl (Rochelle) and 0.039335 g diethylene triamine pentaacetic acid (DTPA, Sigma-Aldrich) in 50 ml pH adjusted Tris and 2) 0.003 g pyrogallol (Sigma-Aldrich) in 49.95084 ml deionised water and 0.04916 ml HCl. Solution 2 was kept in the dark as pyrogallol is light sensitive.

The mussel tissue homogenised in section 2.5.3 was used in this assay. After centrifugation at 10 000 g for 10 minutes at 4°C, the supernatant was used in triplicate for this analysis. Absorbance was measured with micro plate reader Berthold Tristar LB 941, Germany. Only seven samples were analysed per micro plate in triplicate. The blank consisted of 4 µl deionised water, 242 µl tris/DTPA buffer and 4 µl pyrogallol in each well. The samples consisted of 4 µl of sample, 242 µl tris/DTPA buffer and 4 µl pyrogallol in each well. Absorbance was read kinetically for 5.5 minutes, with one reading every 30 seconds that gave a total of 11 readings. Protein content was determined as described in section 2.5.1. Final SOD activity was expressed in ng SOD/mg protein.

2.5.6. Protein carbonyls (PC)

This biomarker of effect was measured using the protocol described by Parvez and Raisuddin (2005). The following solutions were made on the day of analysis: 1) 0.099 g 2,4-dinitrophenylhydrazine (DNPH, Sigma-Aldrich) in 50 ml 2 M HCl; 2) 6 % thiochloroacetic acid (TCA, Merck) 3 g in 50 ml deionised water and 3) 6 M guanidine hydrochloride (Sigma-Aldrich) 28.66 g in 50 ml 50 % formic acid (Rochelle). Solution 2 was kept cold at 4°C.

The mussel tissue homogenised in section 2.5.3 was used in this assay after the first centrifugation. An aliquot of 600 µl of the supernatant was taken and centrifuged further at 10 500 g for 25 minutes at 4°C. The newly derived supernatant was used for analysis and samples and standards analysed in triplicate. Absorbance was measured with the Biotek Elx 800 micro plate reader.

In 2 ml microtubes, 500 µl of supernatant and 500 µl of DNPH was added and incubated for 1 hour at room temperature (37°C), vortexed every 10 – 15 minutes. An equal volume (500 µl) of 6 % TCA was added to the microtubes and centrifuged at 10 000 g for three minutes. The supernatant (TCA) was discarded and the

pelleted protein washed thrice by resuspension in 1 ml ethanol, let stand for 10 minutes, centrifuged and the supernatant (ethanol wash) discarded. The pelleted protein was resuspended in 400 µl guanidine hydrochloride and let stand for 15 minutes at room temperature (37°C), after which it was centrifuged at 16 000 g for five minutes. In each micro plate well 100 µl of supernatant was added and read at 390/405 nm.

Standards for PC analysis were prepared using a concentration gradient of Bovine serum albumin (BSA). A stock solution of 5.28 mg BSA in 2 ml GHB was prepared and the standards made up with GHB to the concentrations represented in Table 2.4. All standards followed the same procedure as samples and blanks. Protein content was determined as described in section 2.5.1. Final PC content was expressed in nmol carbonyl/mg protein.

Table 2.4. Standard curve preparation for protein carbonyl content analysis.

Standard	BSA (nmol)	BSA stock (µl)	GHB (µl)
1	0	0	500
2	5	62.5	437.5
3	10	125	375
4	20	250	250
5	30	375	125
6	40	500	0

2.6. STATISTICAL ANALYSIS

The graphical presentations were performed using Excel 2013, GraphPad Prism 5 and Prism 6 software and data are reported as mean + SE (standard error of the mean) for all organic pollutant and biomarker data. The data of chemical analysis of SPMDs and mussels are reported as total compound fraction of total percentages. The variations in each exposure were tested by one-way analysis of variance (ANOVA) for parametric data and Kruskal-Wallis for non-parametric data, considering the sites as variables for biomarker data and both sites and compounds as variables for chemical analysis data. Data were tested for normality and homogeneity using Kolmonogorov-Smirnoff and Levene's tests, respectively. When significant differences were revealed by one-way ANOVA or Kruskal-Wallis, post-hoc multiple comparisons between sites were made using Turkey test for parametric data and Dunn's test for non-parametric data, to determine which values differed significantly. Significance was determined at $p<0.05$ (Wepener et al. 2011).

The relationships between OCP bioaccumulation and biomarkers were assessed using the approach followed by Wepener et al. (2008). In this study Principle Component Analysis (PCA) (Canoco for Windows Version 4.53) was used to assess the spatial patterns associated with water and sediment quality, bioaccumulation in fish tissue, biomarker responses and fish community structures (Ter Braak and Smilauer, 2004). The PCA is based on a linear response model relating species and environmental variables (van den Brink et al., 2003). Results of the ordination are a map of the samples being analysed on a two dimensional basis, where the placements of the samples reflect the dissimilarities or similarities between the samples; in this case the sampling sites. To determine which factors were responsible for the structure or groupings obtained in the PCA a redundancy analysis (RDA) assessment was carried out. An RDA is a derivative of a PCA with one additional feature which allows for the selection of the driving variables which are intended to be overlaid onto the PCA.

The integrated biomarker response index (IBR) was calculated in the same manner as in Brooks et al. 2015. The following formulas were used in that order:

$xi' = (xi - x)/s$

$yi = xi' + |xmin'|$

$Ai = [(yi*yi-1)/2]$

$IBR/N = \Sigma Ai/N$

xi´: standardised value of the biomarker

xi: mean value of each biomarker from each sample

x: general mean value of xi from the data set

s: standard deviation

yi: absolute standardised value

yi + 1: absolute standardised value of the following biomarker

Ai: starplot triangular areas

N: number of biomarkers

The calculations and the starplot triangular areas were performed and plotted using Excel, and the IBR/N values plotted in Graphpad Prism 6.

CHAPTER 3

RESULTS

3.1. CHEMICAL ANALYSES

Chemical analyses results of mean ± standard error of OCPs, PCBs and PAHs for SPMDs and mussels are presented in Table 3.1 for the rainy season exposure period and Table 3.2 for the dry season exposure period. The analytical limit of detection (LOD) and limit of quantification (LOQ) values for the OCPs, PCBs and PAHs reported in Table 3.3. All the chemical analysis data for both transplanted and resident mussels reported are representative of a samples size of 30 mussels per site per exposure. All SPMD chemical analysis data reported are representative of four SPMDs for each exposure site. Those compounds that were not detected (ND) are also reported in Table 3.1 and 3.2. The SPMDs did have a level of biofouling that in some cases caused some small tears.

3.1.1. Semi-permeable membrane (SPMD) analysis rainy season survey 2014

For spatial comparison of OCPs, PCBs and PAHs the results and significant differences (significance taken at $p < 0.05$) are presented in Table 3.1. The fraction of total value for each chemical compound is illustrated in Figure 3.1A-B. The OCPs were mostly not detected in the SPMDs, with the exception of *p,p'*-DDE and Alpha-HCH. There were no significant OCP differences between sites. All the analysed PCBs (28, 52, 101, 118, 153, 138 and 180) were present in the SPMDs from DH, but CB 180 was not present in the SPMDs from the RBH site. The concentrations of CB 28, 52 and 101 were significantly higher ($p < 0.05$) in the SPMDs from DH than in the SPMDs from RBH site. It was only the CB 118 concentrations that were higher in RBH compared to DH ($p > 0.05$), whereas the concentrations of CBs 13, 138 and 180 were all higher in DH than in RBH site ($p > 0.05$). Most of the PAHs analysed were present in both DH and RBH exposure sites, with the exception of benzo[a]pyrene. The concentrations of phenanthrene and fluoranthene in RBH were higher than the concentrations in DH, but did not differ significantly.

Table 3.1. Organochlorine (OCP), polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbon (PAH) concentrations in resident and transplanted Brown mussels (ng/g lipid) and semi-permeable membrane devices (SPMD) (ng/SPMD triolein) from Richards Bay Harbour (RBH) and Durban Harbour (DH) during the rainy season (March 2014). Values represent mean ± standard error of the mean and not detected (ND) compounds. Rows with common numerical superscript indicate significant differences in OCPs between the harbours and common alphabetical superscript indicate significant differences within the harbour. An asterisk indicates a significant temporal difference with samples collected during the dry season (Table 3.2). Significance was taken as $p < 0.05$. Limits of detection (LOD) and limits of quantification (LOQ) are reported in Table 3.3.

	Sites						
	Reference mussels	**RBH resident**	**RBH transplanted**	**RBH SPMD**	**DH resident**	**DH transplanted**	**DH SPMD**
Alpha-HCH	ND	ND	ND	<LOD	ND	ND	36±5
Gamma-HCH	ND	ND	ND	ND	ND	ND	ND
Beta-HCH	ND	ND	ND	ND	ND	<LOD	ND
Delta-HCH	ND	ND	ND	ND	ND	ND	ND
p,p'DDE	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	20±3
o,p'DDT	ND	ND	ND	ND	ND	ND	ND
p,p'DDD	<LOD	<LOD	ND	ND	ND	ND	ND
p,p'DDT	ND	ND	<LOD	ND	<LOD	ND	ND
ΣDDs	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	66±10
CB 28	3.6±0[1]	3.6±0[2]	2±1[3]	31±0.3[4]	37±21[a12]	32±18[a123]	53±13[4]
CB 52	2.16±0[1]	3.86±0.2[2]	5.5±1	15.5±0.1[3]	37±13[12]	23±7	72±18[3]
CB 101	2.58±0[1]	<LOQ	<LOQ	10±0.7[2]	23±11[1]	18±7	74±21[2]
CB 118	3.48±0[1]	3.48±0[2]	4.9±1[3]	<LOD	35±7[123]	30±7[123]	<LOQ

Table 3.1. Continued.

	Reference mussels	RBH resident	RBH transplanted	RBH SPMD	DH resident	DH transplanted	DH SPMD
CB 153	8.7±0[1]	8.7±0[2]	15.64±3	<LOD	56±34[12]	52±33[1]	59±19
CB 138	18.24±0[1]	18.24±0[2]	25.33±1.2[3]	<LOD	75±24[12]	22±8	51±6
CB 180	28.92±0[1]	28.92±0[2]	28.92±0[3]	ND	85.12±2[123]	<LOQ	43.5±2
ΣPCBs	<LOD	66.8±0.2[2]	82.29±7.2[1]	56.5±1.1[3]	263±112[2]	177±80[1]	282±79[3]
Naphthalene	510±2001	2860±1120[1]	1730±690	410±220	1000±450	1220±470	480±320
Acenapthylene	<LOD	<LOQ	<LOD	610±270	<LOD	<LOD	770±330
Acenapthene	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD
Fluorene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Phenanthrene	<LOD	<LOD	<LOD	360±160	<LOD	<LOD	<LOD
Anthracene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
Fluoranthene	<LOD	<LOD	<LOD	1834±430	<LOD	<LOD	<LOQ
Pyrene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
Benz[a] anthracene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
Chrysene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
Benzo[b] fluoranthene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
Benzo[a]pyrene	<LOD	<LOD	<LOD	ND	<LOD	<LOD	ND
ΣPAHs	510±200[1]	2860±1120[12]	1730±690	3214±1080	1000±450[2]	1220±470[2]	1250±650

Table 3.2. Organochlorine (OCP), polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbon (PAH) concentrations in resident and transplanted Brown mussels (ng/g lipid) and semi-permeable membrane devices (SPMD) (ng/SPMD triolein) from Richards Bay Harbour (RBH) and Durban Harbour (DH) during the dry season (June 2014). Values represent mean ± standard error of the mean and not detected (ND) compounds. Rows with common numerical superscript indicate significant differences in OCPs between the harbours and common alphabetical superscript indicate significant differences within the harbour. An asterisk indicates a significant temporal difference with samples collected during the rainy season (Table 3.1). Significance was taken as $p < 0.05$. Limits of detection (LOD) and limits of quantification (LOQ) are reported in Table 3.3.

	Sites						
	Reference mussels	**RBH resident**	**RBH transplanted**	**RBH SPMD**	**DH resident**	**DH transplanted**	**DH SPMD**
Alpha-HCH	ND	ND	ND	<LOD	ND	ND	618±81
Gamma-HCH	ND	ND	ND	ND	ND	<LOD	ND
Beta-HCH	ND	ND	ND	ND	ND	ND	ND
Delta-HCH	ND	ND	ND	ND	ND	ND	ND
p,p'DDE	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	17
o,p'DDT	ND	ND	ND	ND	ND	ND	ND
p,p'DDD	ND	ND	<LOD	ND	ND	ND	ND
p,p'DDT	ND	ND	ND	ND	ND	ND	160±46
ΣDDs	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	675
CB 28	<LOD	<LOD	<LOD	<LOD	12±6	<LOQ	<LOQ
CB 52	<LOD	<LOD	<LOD	6.69±2	18±6	17±6	51±8
CB 101	$3.57±0.2^{1}$	$2.58±0^{2}$	$2.58±0^{3}$	8±2	$24±10^{123}$	$24±10^{23}$	43±10
CB 118	$3.48±0^{1}$	$3.48±0^{2}$	<LOD	23±0.83	$26±6^{12}$	18±6	$71±0.3^{3}$

Table 3.2. Continued.

	Reference mussels	RBH resident	RBH transplanted	RBH SPMD	DH resident	DH transplanted	DH SPMD
CB 153	<LOD	<LOD	<LOD	<LOQ	35±20	23±11	44±8
CB 138	<LOD	<LOD	<LOD	<LOD	12±4	<LOQ	46±6
CB 180	<LOD	<LOD	<LOD	ND	<LOQ	<LOQ	<LOQ
ΣPCBs	7.05±0.2	6.06±0	2.58±0	37.69±4.8	127±40	82±33	255±32.3
Naphthalene	1620±540	1420±560	2750±1090	1240±580	580±230	300±120	1080±300
Acenapthylene	<LOD	<LOD	<LOD	610±270	<LOD	<LOD	<LOD
Acenapthene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Fluorene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Phenanthrene	<LOD	<LOD	<LOD	680±350	<LOD	<LOD	<LOD
Anthracene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	30±6
Fluoranthene	<LOD	<LOD	<LOD	889.5±135	<LOD	<LOD	962±350
Pyrene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
Benz[a] anthracene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Chrysene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
Benzo[b] fluoranthene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Benzo[a]pyrene	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	ND
ΣPAHs	1620±540	1420±560	2750±1090	3409.5±850	580±230	300±120	2077±656

Table 3.3. Limits of detection (LODs) and limits of quantification (LOQ) for organic pollutant compounds (ng/g) in Table 3.1 and 3.2.

	LOD mussels	LOQ mussels	LOD SPMDs	LOQ SPMDs
Alpha-HCH	5	15	5	15
Gamma-HCH	6.96	N/A	ND	ND
Beta-HCH	11.32	N/A	ND	ND
Delta-HCH	11.62	N/A	ND	ND
p,p'-DDE	4.54	N/A	5	17
o,p-'DDT	5.72	N/A	ND	ND
p,p'-DDD	10	34	ND	ND
p,p'-DDT	7	22	7	22
CB 28	0.6	1.9	4	15
CB 52	0.36	1.21	2	5
CB 101	0.43	1.42	1	4
CB 118	0.58	1.94	4	14
CB 153	1.45	4.84	1	5
CB 138	3.04	10.13	2	6
CB 180	4.82	16.06	2	5
Naphthalene	50	165	50	165
Acenapthylene	150	500	150	500
Acenapthene	150	330	150	330
Fluorene	110	350	110	350
Phenanthrene	60	200	60	200
Anthracene	5	15	5	15
Fluoranthene	68	230	68	230
Pyrene	154	512	154	512
Benz[a] anthracene	18	62	18	62
Chrysene	30	110	30	110
Benzo[b] fluoranthene	50	180	50	180
Benzo[a]pyrene	6	21	ND	ND

3.1.2. Semi-permeable membrane (SPMD) analysis dry season survey 2014

For spatial comparison of OCPs, PCBs and PAHs, the results and significant differences (significance taken at $p < 0.05$) are presented in Table 3.2, and the fraction of total value for each chemical compound is illustrated in Figure 3.1A-B. As with the wet season the only OCPs detected were Alpha-HCH and *p,p'*-DDE. The

concentrations of Alpha-HCH and *p,p'*-DDE were higher in DH than at RBH site ($p > 0.05$). The concentrations of CB-52, 101 and 138 were higher in DH than at RBH site ($p > 0.05$). The concentrations for CB 118 were significantly different between sites ($p < 0.05$), and CB 180 was not detected in RBH samples. There were no significant differences in PAHs between the sites. Acenapthylene and phenanthrene were higher in RBH than in DH ($p > 0.05$), and the concentrations of anthracene lower in RBH site. Benzo[a]pyrene was not present in Durban Harbour. The ΣPAHs in RBH were higher than the concentrations present in DH site ($p > 0.05$).

3.1.3. Semi-permeable membrane (SPMD) analysis temporal comparison

There were no significant differences in total OCP concentrations between the two exposure periods. The total concentrations of OCPs in DH from the dry season were however higher than the concentrations of the rainy season survey ($p > 0.05$). There were no significant differences in ΣPCB concentrations between the rainy season and dry season surveys. The ΣPCB concentrations in DH from the dry season were higher than the concentrations from the rainy season ($p > 0.05$). There were also no significant differences in ΣPAH concentrations between the rainy and dry season surveys. There was a slight increase in ΣPAH concentrations in DH dry season in comparison with the concentrations in DH rainy season ($p > 0.05$).

3.1.4. Mussel analysis rainy season survey 2014

For spatial comparison between OCPs, PCBs and PAHs the significance was taken at $p < 0.05$ and results are illustrated in Table 3.1. The fraction of total value for each chemical compound is illustrated in Figure 3.2A-C. Most OCPs were not detected at any of the sites, with the exception of Beta-HCH in transplanted mussels from DH, *p,p'*-DDE at all the sites, *p,p'*-DDD in SB reference mussels and resident mussels from RBH and *p,p'*-DDT in transplanted mussels from RBH and resident DH mussels. The CB-28 concentrations in SB reference mussels and resident RBH mussels were significantly lower ($p < 0.05$) than the concentrations in resident and transplanted DH mussels. Transplanted mussels from DH had significantly higher concentrations ($p < 0.05$) of CB-28 than transplanted RBH mussels. Reference SB mussels and resident RBH mussels had significantly lower concentrations ($p < 0.05$) of CB 52 present than resident DH mussels. Higher concentrations of CB 101 were

present in resident and transplanted DH mussels than in SB and RBH mussels, with significant differences between resident DH mussels and SB reference mussels ($p < 0.05$). Resident and transplanted mussels from DH has significantly higher concentrations ($p < 0.05$) of CB 118 than SB and RBH mussels.

Resident and transplanted DH mussels had significantly higher concentrations ($p < 0.05$) of CB 153 than SB and RBH resident mussels, respectively, and transplanted RBH mussels had significantly higher concentrations ($p < 0.05$) than SB reference mussels. Resident DH mussels had significantly higher concentrations ($p < 0.05$) of CB 138 than SB reference and RBH resident mussels. The ΣPCB concentrations in DH was significantly higher ($p < 0.05$) than the total concentrations in RBH mussels. The ΣPAH concentrations in resident RBH mussels were significantly higher ($p < 0.05$) than the concentrations in SB, resident DH and transplanted DH mussels, with significantly higher ($p < 0.05$) naphthalene concentrations.

3.1.5. Mussel analysis dry season survey 2014

For spatial comparison between OCPs, PCBs and PAHs the significance was taken at $p < 0.05$ and results are illustrated in Table 3.1. The fraction of total value for each chemical compound is illustrated in Figure 3.2A-C. Gamma-HCH was detected in transplanted DH mussels and *p,p'*-DDE was present in mussels at all the sites. Only the transplanted RBH mussels had measurable concentrations of *p,p'*-DDD. Concentrations of CB 28 and 52 were present at all sites, with higher concentrations ($p > 0.05$) in DH resident and transplanted mussels. Significantly higher concentrations ($p < 0.05$) of CB 101 were present in DH resident and transplanted mussels. Resident DH mussels had significantly higher ($p < 0.05$) CB 118 concentrations than SB reference and RBH resident mussels. Higher concentrations ($p > 0.05$) of CB 153, 138 and 180 were present in DH mussels, therefore the ΣPCB concentrations were higher in DH than in the other exposure sites. The PAH concentrations did not differ significantly, but concentrations were higher in SB and RBH mussels than in DH mussels ($p > 0.05$).

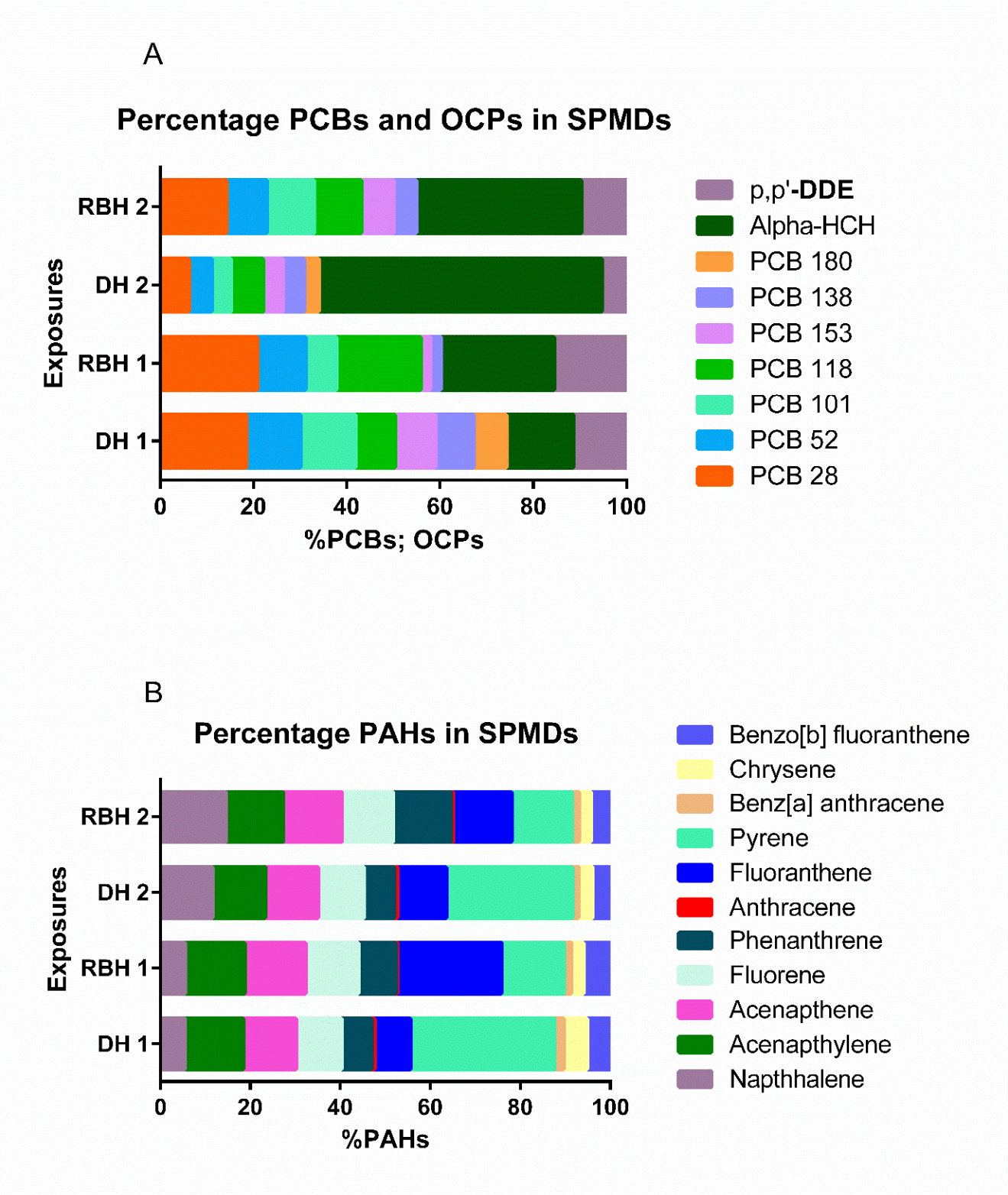

Figure 3.1. Percentage contributions of OCPs, PCBs and PAHs in semi-permeable membrane devices for rainy (1) and dry season exposures (2) at DH and RBH.

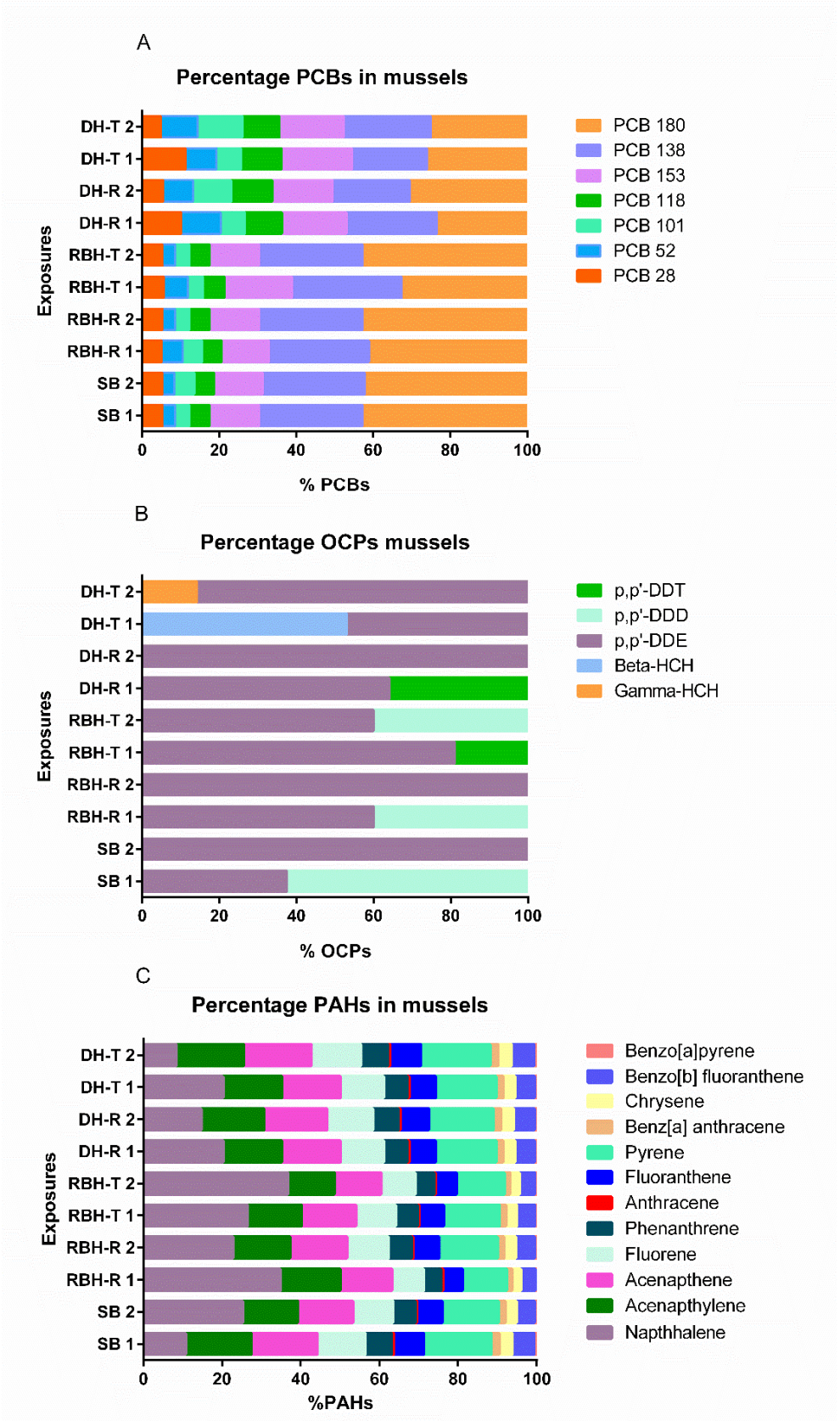

Figure 3.2. Percentage contribution of OCPs, PCBs and PAHs in resident (R) and transplanted (T) mussels for the rainy (1) - and dry (2) season exposures at DH and RBH.

3.1.6. Mussel analysis temporal comparison

There were no significant differences between the rainy season and dry season surveys for OCP, PCB or PAH concentrations. The ΣOCP concentrations during the dry season survey were slightly higher ($p > 0.05$) than the rainy season survey (Table 3.1 and 3.2). The ΣPCB concentrations in DH and RBH decreased ($p > 0.05$) during the dry season survey. The ΣPAH concentrations in SB for the dry season were higher ($p > 0.05$) than the concentrations for the rainy season. The total PAH concentrations decreased ($p > 0.05$) in resident mussels from RBH dry to the wet seasons, while PAHs in transplanted mussels showed increases ($p > 0.05$) in the wet sampling season. Both resident and transplanted mussels from DH dry season had decreased concentrations ($p > 0.05$) of ΣPAHs compared to the rainy season. The concentrations of CB 52 and 180 were higher ($p > 0.05$) during the two seasonal surveys when compared to the other PCBs (Figure 3.2A). The *p,p;*-DDE was present in most sites (Figure 3.2B) and Naphthalene mostly present in RBH mussels (Figure 3.2C)

3.2. BIOMARKER ANALYSES

Biomarkers of exposure and effect are represented in Figures 3.3 and 3.4 for both exposure surveys during 2014. All data reported represent a sample size of 30 mussels per treatment for both transplanted and resident mussels, a total of 150 mussels for each exposure period, respectively. The relationships between biomarker responses and OCP, PCB and PAH exposures are presented in an RDA triplot in Figure 3.5.

3.2.1. Rainy season survey 2014

The AChE activities showed no significant differences between sites, although there is an increase ($p > 0.05$) in activity in both RBH resident and transplanted mussels, respectively (Figure 3.3A). The CYP450 levels showed no significant differences, but levels in resident and transplanted mussels from DH were lower ($p > 0.05$) than the levels in SB reference mussels (Figure 3.3B). The CAT activities were not significantly different but the levels in transplanted RBH mussels were higher ($p > 0.05$) than the activity levels in the mussels from the other sites (Figure 3.3C). The SOD levels in resident mussels from DH were significantly higher ($p < 0.05$) than the

levels in both resident mussels from RBH and SB reference mussels (Figure 3.3D). The SOD levels in transplanted mussels from both RBH and DH were also higher than the levels in SB reference mussels ($p > 0.05$). The MDA levels (Figure 3.3E) did not differ significantly with a slight decrease in transplanted mussels from DH exposure site ($p > 0.05$). The PC levels in resident and transplanted mussels from DH were significantly lower ($p < 0.05$) than the levels in resident mussels from RBH and SB reference mussels (Figure 3.3F). However, although lower than the levels in transplanted mussels from RBH it was not significant ($p > 0.05$).

3.2.2. Dry season survey 2014

The AChE activities in SB and resident RBH mussels were significantly lower ($p < 0.05$) than the activity levels in transplanted RBH mussels (Figure 3.3A). The AChE activity levels in both resident and transplanted mussels from DH were significantly lower ($p < 0.05$) than the activity levels in transplanted RBH mussels. The CYP450 levels did not differ significantly between the sites but, as illustrated in Figure 3.3B, the levels in resident and transplanted mussels from RBH were higher than the levels in resident and transplanted mussels from DH and SB reference mussels ($p > 0.05$). The CAT activities in both RBH and DH transplanted mussels were significantly higher ($p < 0.05$) than the CAT activities in SB reference mussels (Figure 3.3C). The SOD levels in all mussels did not differ significantly between sites (Figure 3.3D), but SOD levels were higher in the resident and transplanted mussels from RBH than the levels in SB reference mussels and lower levels were observed in resident and transplanted mussels from DH than in both RBH and SB mussels ($p > 0.05$). The MDA levels in both resident and transplanted mussels from DH were significantly higher ($p < 0.05$) than the levels in SB reference mussels (Figure 3.3E). The PC levels indicated no significant differences between sites ($p > 0.05$), but an increase of levels was observed in transplanted mussels from RBH, and slight decreases in levels were observed in transplanted and resident mussels from DH exposure site (Figure 3.3F).

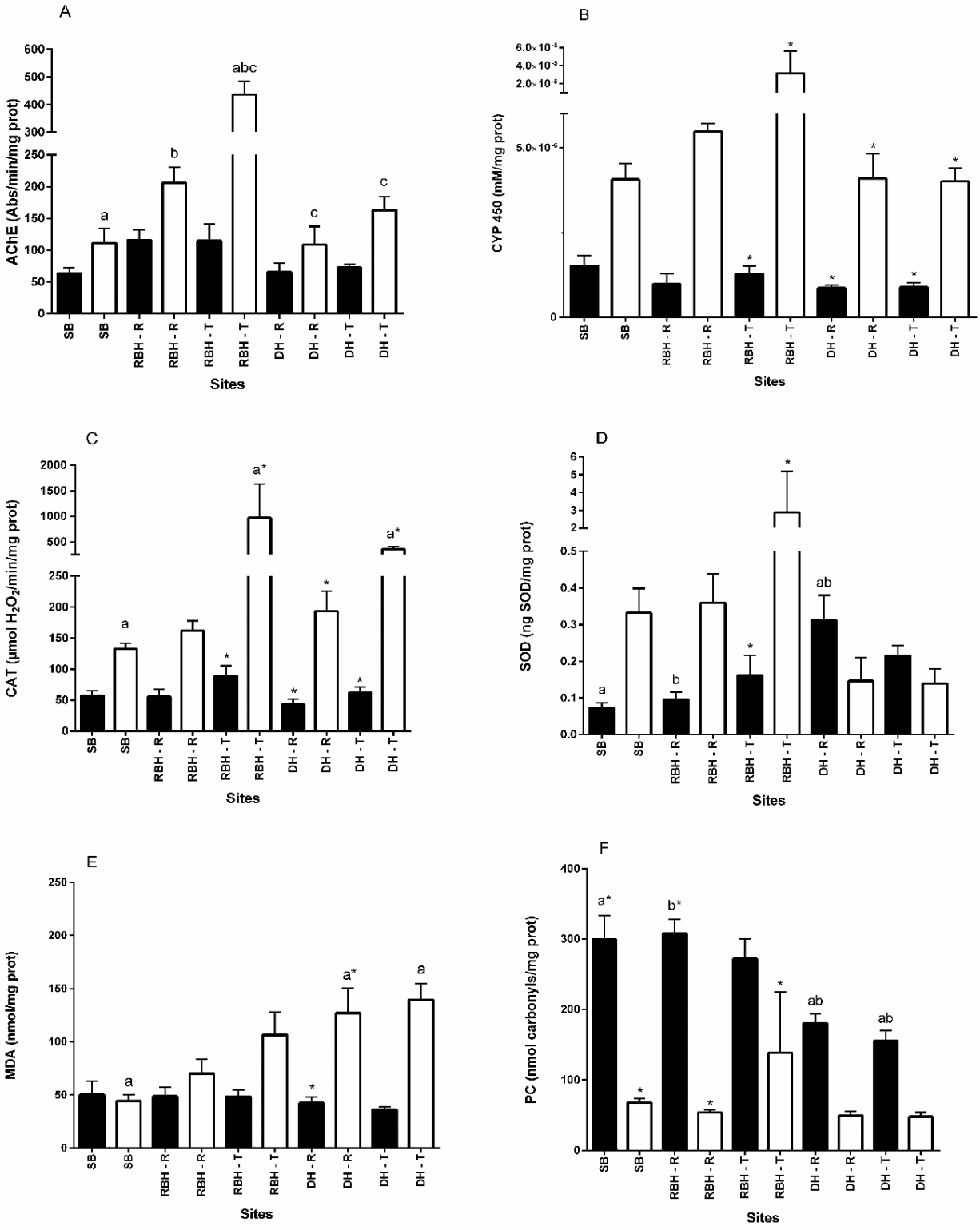

Figure 3.3. Mean + standard error of biomarkers of exposure (A-B) and effect (C-F) in digestive gland tissue of *P. perna* from three locations along the KwaZulu-Natal coastline. Spatial significant differences between sites within each exposure ($p < 0.05$) indicated by common script, and temporal differences by an asterisk (*). Solid black bars represent the rainy season exposure and open bars represent the dry season exposure.

3.2.3. Temporal comparisons

The AChE activity in transplanted mussels from DH dry season exposure was significantly higher ($p < 0.05$) than the activity reported in transplanted mussels from DH rainy season exposure (Figure 3.3A). The AChE activity levels in resident and transplanted mussels from RBH dry season exposure was higher than the levels in resident and transplanted mussels from RBH rainy season exposure, but not significant ($p > 0.05$). The levels in resident mussels from DH dry season exposure was also higher than the levels in resident mussels from DH rainy season exposure ($p > 0.05$). The CYP450 levels in transplanted mussels from RBH dry season exposure were significantly higher ($p < 0.05$) than the levels in transplanted mussels from RBH rainy season (Figure 3.3B). The CYP450 levels in resident and transplanted mussels from DH dry season exposure were both significantly higher ($p < 0.05$) than the levels in resident and transplanted mussels from DH rainy season exposure (Figure 3.3B). The CYP450 levels in SB reference mussels and resident mussels from RBH dry season exposure were higher, but not significant, than the levels in SB reference mussels and resident mussels from RBH rainy season exposure, respectively ($p > 0.05$).

The CAT activities during the dry season exposure in transplanted mussels from RBH were significantly higher ($p < 0.05$) than the activity levels in transplanted mussels from RBH during the rainy season exposure (Figure3.3C). This was the same observation for resident and transplanted mussels from DH during the dry season and rainy season exposures. The CAT activities in SB reference mussels' dry season exposure were higher than the activity levels in SB reference mussels during the rainy season exposure ($p > 0.05$). The SOD levels in transplanted mussels from RBH during the dry season exposure were significantly higher ($p < 0.05$) than the levels in transplanted mussels from RBH during the rainy season (Figure 3.3D). No other significant differences were observed, but it is noted that the levels in SB reference mussels and resident mussels from RBH during the dry season were higher than the levels in SB reference mussels and resident mussels from RBH during the rainy season ($p > 0.05$). The opposite is true for resident and transplanted mussels from DH exposure site.

The MDA levels in resident mussels from DH dry season exposure were significantly higher ($p < 0.05$) than the levels in resident mussels from DH rainy season exposure (Figure3.3E). No other significant differences were observed, but levels during the dry season exposure for resident and transplanted mussels from RBH and transplanted mussels from DH were higher than the levels during the rainy season exposure ($p > 0.05$). The SB reference mussels and both transplanted and resident mussels from RBH were all significantly higher ($p < 0.05$) than the levels for the similar groups during the dry season exposure (Figure 3.3F).

3.3. RELATIONSHIP BETWEEN BIOMARKER RESPONSES AND ORGANIC EXPOSURE

The relationships between the temporal and spatial biomarker responses and the exposure of the mussels to OCPs, PAHs and PCBs are presented in a RDA triplot (Figure 3.4). The data were log transformed. On the first axis (representing 72 % of the total variation) the biomarkers in transplanted mussels during the dry season survey in RBH separated from the other sites based on increased SOD and CYP450 activity. These were associated with higher naphthalene bioaccumulation in the mussels. During the rainy period mussels (both transplanted and resident) were associated with increased CB 28 and Beta-HCH concentrations and the PAHs in resident mussels. The DH mussels showed increased *p,p*'-DDD and *p,p*'-DDT bioaccumulation. The second axis (24 % of the total variation explained) showed that there were distinct differences in the bioaccumulation patterns between the rainy and dry seasons. The only OCP associated with the dry season was high concentrations of Alpha-HCH in transplanted mussels in DH.

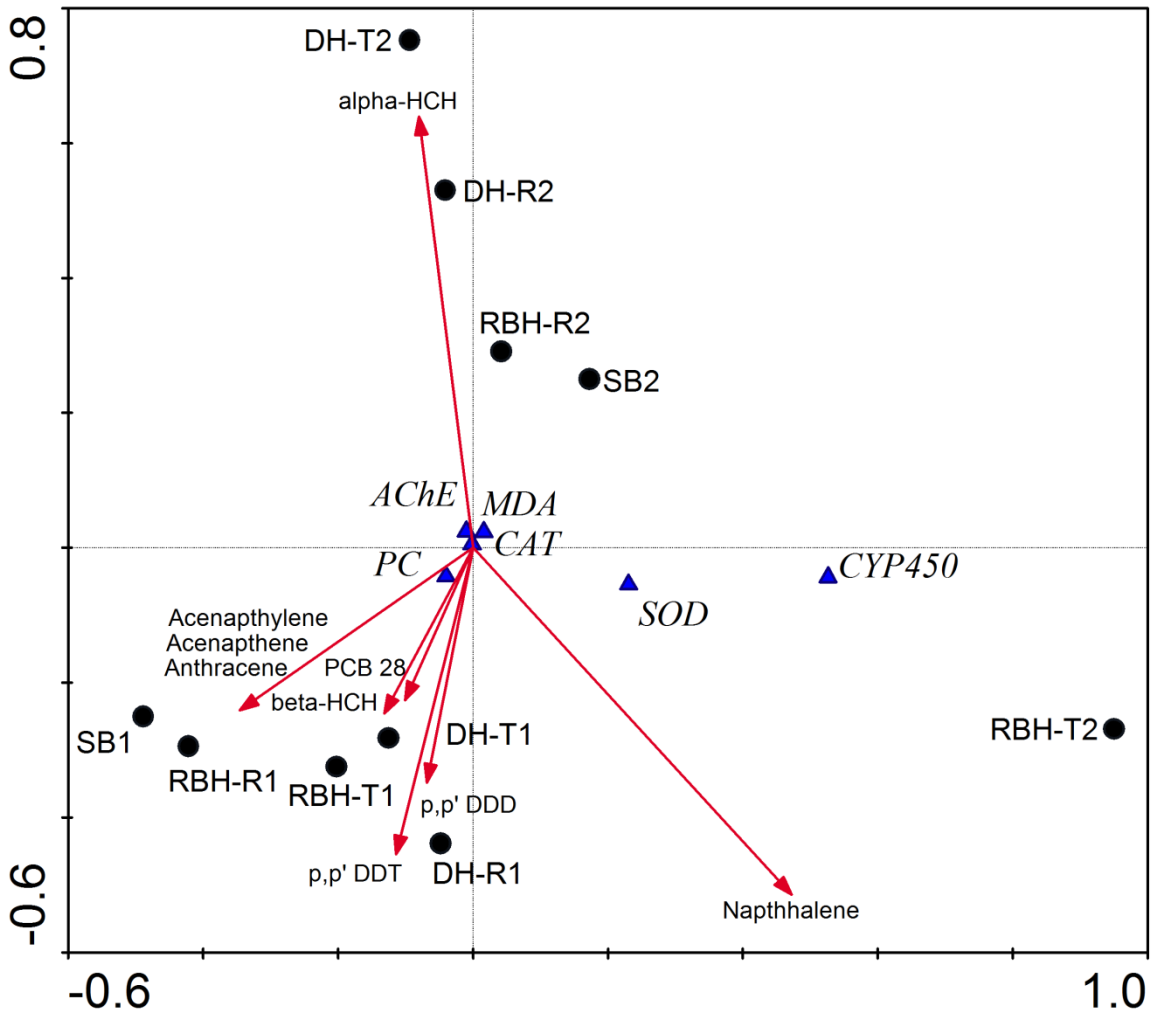

Figure 3.4. Redundancy analysis triplot representing the biomarker data at the selected sampling sites with OCP, PCB and PAH concentrations overlain as environmental (descriptive) variables. The first two axes represent 96% of the total variation (72% on axis 1 and 24% on axis 2). The descriptive variables account for 98.5% of the variation in the data with those OCPs, PCBs and PAHs responsible for significant interactions being displayed on the triplot.

3.3.1. Integrated biomarker response index (IBR)

The lowest calculated IBR/N values were in the transplanted mussels from RBH during the dry exposure period and in the dry season reference mussels, with IBR/N values of 3.86 and 4.73 respectively (Figure 3.5). The strongest contributing biomarkers to the IBR index for transplanted mussels from RBH during the dry season were AChE and anti-oxidant enzymes CAT, and PC (Figure 3.6), for reference SB mussels during the dry season the IBR index was mostly affected by AChE, and antioxidant enzymes MDA and PC levels (Figure 3.6). Reference SB mussels, resident and transplanted DH mussels during the rainy season, and transplanted DH mussels during the dry season presented with the highest IBR

indexes (Figure 3.5). The recorded values were 7.27, 6.96, 7.43 and 7.56 respectively. The IBR index of reference SB mussels (rainy season) was influenced by AChE and antioxidant biomarkers CAT, MDA and PC (Figure 3.6), the IBR index of resident DH mussels were influenced by antioxidant biomarkers MDA and SOD (Figure 3.6), the IBR index of transplanted DH mussels (rainy season) were influenced by CYP450, MDA and PC (Figure 3.6) and the IBR index of transplanted DH mussels (dry season) were influenced by CYP450, AChE and PC levels (Figure 3.6). The IBR index of the transplanted RBH mussels (dry season) was the lowest in comparison to all other groups (Figure 3.5). The biomarkers that contributed to the IBR index of resident RBH mussels (rainy season) were AChE and antioxidant biomarkers CAT and PC (Figure 3.6) and for resident RBH mussels (dry season) it were AChE, CAT, SOD and PC levels (Figure 3.6). The PC levels in transplanted RBH mussels (rainy season) were the most contributing biomarker levels to the IBR index (Figure 3.6). Biomarkers that contributed to the IBR index in resident DH mussels (dry season) were antioxidant biomarkers MDA and PC. The IBR index of transplanted DH mussels (dry season) were mostly influences by biomarkers CYP450 and PC levels.

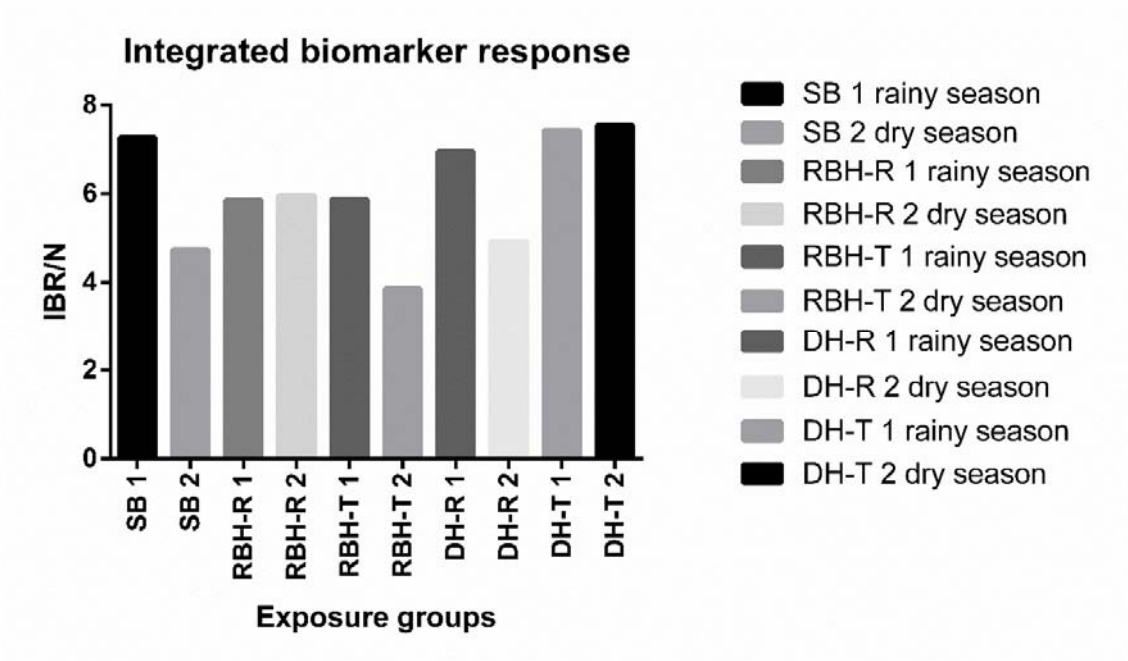

Figure 3.5. Integrated biomarker response index (IBR) for spatial and temporal comparison

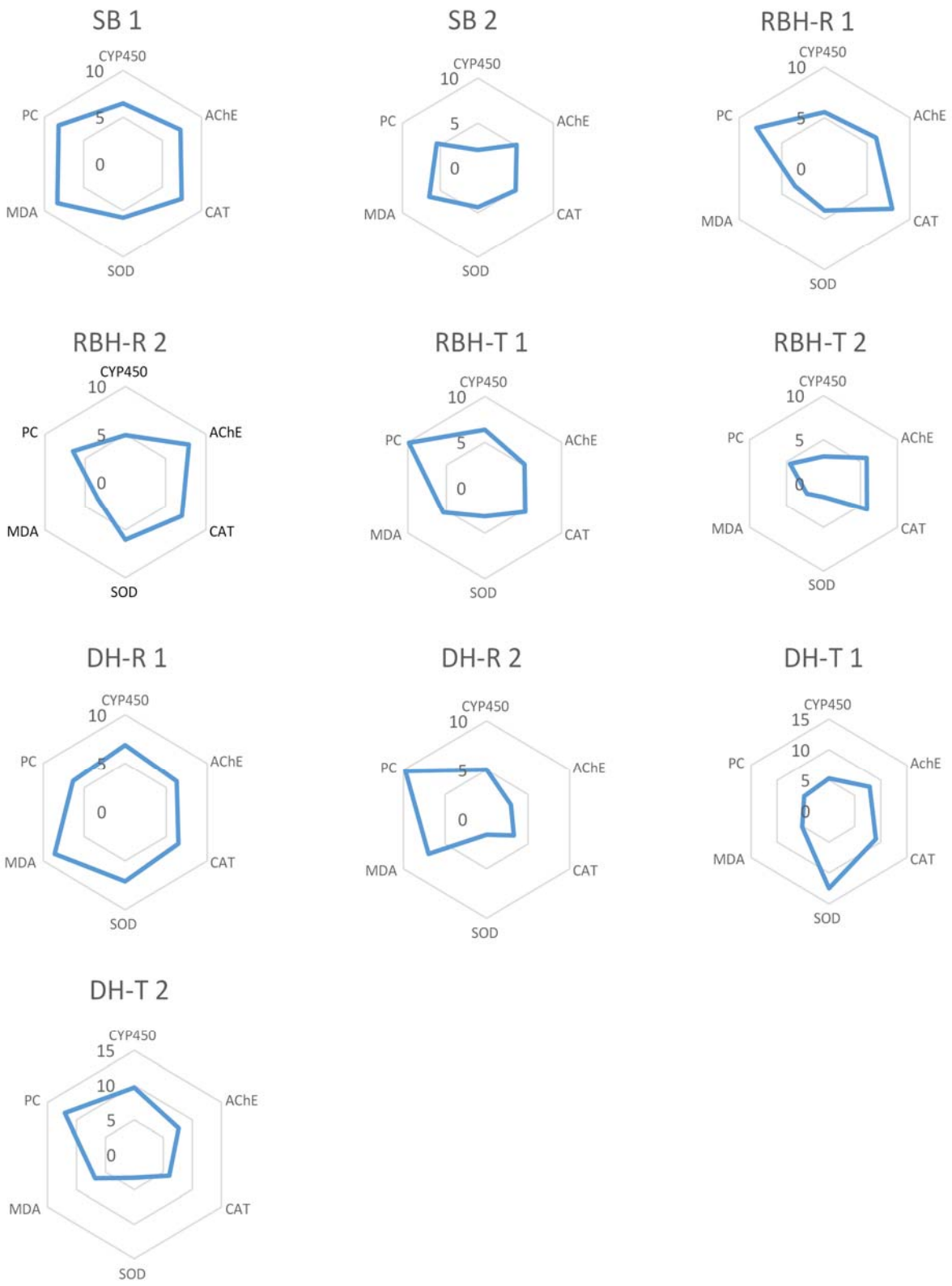

Figure 3.6. Integrated biomarker responds index (IBR) star plots for resident (R) and transplanted (T) mussel exposure groups during the rainy season (1) and dry season (2) surveys at Sheffield Beach (SB), Richards Bay Harbour (RBH) and Durban Harbour (DH).

CHAPTER 4

DISCUSSIONS

4.1. CHEMICAL ANALYSIS

Semi-permeable membrane devices have been successfully implemented as a monitoring tool of organic pollution in the marine environment on a worldwide scale, including in South-Africa (Prest et al. 1995; Söderström and Bergguist 2004; Augulyte and Berggoist 2007; Degger et al. 2011). This current study also demonstrated the effectiveness of defining and detecting levels of organic pollutants which included OCPs, PCBs and PAHs in the harbour sites. The use of OCPs were banned globally almost 20 years ago, but are still of concern in South-Africa since DDT is still used as pest control for controlling malaria. A study by Wepener et al. (2012) showed definite OCP exposure and bioaccumulation in the Phongolo catchment, which motivated the effort of this study to determine the current levels in the eastern South African coastal waters. Studies by Barnhoorn et al. (2009; 2010) showed definite OCP exposure, bioaccumulation and effects on biota in the Luvuvhu River in Limpopo Province South Africa, and a study by Sibali et al. (2008) also showed OCP exposure in the Jukskei River catchment. With these levels found in the aforementioned studies, the possibly high OCP levels along the South African east coast are of concern due to freshwater runoff from these river systems into the Indian Ocean. There are limited published data on OCP levels in biota from the South African coastline, e.g. OCPs in sharks (Schlenk et al. 2005) and penguin eggs (Bouwman et al. 2015). The majority of results are presented in the National Marine Pollution Monitoring Programme report series (e.g. SANCOR 1976; 1981; 1983; 1985 and Van Dyk et al. 1982)

PCBs, which were globally distributed and harmful to both humans and the environment, have been well studied worldwide (Breivik et al. 2004; Cimenci et al. 2013) and in the South African marine environment (Degger et al. 2011; Kampire et al. 2015). These contaminants can resist chemical breakdown, giving them long half-lives in the environment (Okay et al. 2009; Yang et al. 2010). PAHs have carcinogenic, immunotoxic and neurotoxic properties, therefore they have been well studied in the environment over the past decade (Casale et al. 2000; N'Diaye et al. 2006, Echevese et al. 2010; Degger et al. 2011, Wolska et al. 2012).

Active biomonitoring (ABM) has been successfully tested as a monitoring tool of organic marine pollution in the past (Nasci et al. 1999; Damiens et al. 2007;

Wepener et al. 2008, Brooks et al. 2009). According to Wepener (2013), ABM is defined as the translocation of a bio-indicator organism from a non-contaminated reference site to a contaminated study site, after which organismal responses are measured. In this study, resident mussels from the contaminated sites were also studied, and the chemical levels of the organic contaminants (OCPs, PCBs and PAHs) measured. These measurements have also been successful in recent studies in South Africa (Degger et al. 2011; Kampire et al. 2015a). Greenfield et al. (2014) used the same mussel ABM protocol, i.e. the transplantation of *P. perna* from a pristine environment to a contaminated environment to monitor both bioaccumulation and effects of contaminants in the marine environment. The study showed the success in using transplanted mussels as a monitoring tool for marine pollution, as the transplanted mussels showed higher accumulation of contaminants than the resident mussels, indicating that the exposure period of six weeks is short enough to prevent the regulation of pollutants. Since contaminant levels are regulated within the resident mussels due to long exposure, the transplanted mussels can give another insight to the bioaccumulation and physiological effects of contaminants.

4.1.1. Spatial and temporal analyses of semi-permeable membrane devices (SPMDs)

The results showed that OCPs were mostly not quantifiable in the SPMDs, with the exceptions of Alpha-HCH and *p,p'*-DDE. Other marine and freshwater studies in South Africa also showed higher concentrations and effects of OCPs, but these studies did not use SPMDs, but rather other artificial methods such as the international pellet watch studies (Ogata et al. 2009; Ryan et al. 2012; Jayasiri et al. 2015). There are limited studies monitoring the OCP levels using artificial devices along the east coast of South Africa, making it difficult to compare and conclude if levels are higher, lower or stable at these sites. The international pellet watch study by Ogata et al. (2009) showed low concentrations of DDTs (0.89 ng/g pellet) in Durban in comparison to international levels. However the HCH concentrations in the pellets during the 2009 study were extremely high (34 ng/g pellet). The OCP results in the SPMDs of the current study were similar, with high concentrations of HCH (Table 3.1 and 3.2). In contrast higher concentrations of DDTs were observed

than found in the pellets. In a pellet study by Ryan et al. (2012) of the South African coastal waters, lower concentrations of DDTs and HCHs were detected (8 – 31 ng/g pellet and 2 – 5 ng/g pellet, respectively). The historical use of DDT and therefore persistence is evident in the consistent concentrations of *p,p'* DDE measured during the two surveys in DH. The high *p,p'* DDT concentrations recorded during the dry season in DH can only be attributed to a recent accidental spills of a stockpile since Durban is not regarded as a malaria risk region and would therefore not be subjected to spraying. On an international scale, OCP analyses in DH found higher concentrations than the levels found near a Turkish shipyard (Karacik et al. 2013). It should be noted that Karacik et al. (2013) reported SPMD concentrations as ng/g triolein, whereas the current study reports SPMD concentrations as ng/SPMD.

The PCB results showed a persistence of these contaminants at both harbours, with the exception of CB-180 which was not quantifiable in RBH which is in accordance with the PCBs found by Degger et al. (2011a). In contrast with the results of Degger et al. (2011a) CB-52 was detected in RBH during the dry season at a higher concentration than the rainy season. All PCB congeners were present in DH, which may be due to high intensity harbour activities (Greenfield et al. 2011). The PCB concentrations of the rainy season survey in DH are of higher levels than in the dry season. This may be caused by temperature differences having an impact on partitioning and lipophilic characteristics of the PCB congeners (Adams et al. 2007). Ogata et al. (2009) monitored a total of 13 CBs (66, 110, 101, 105, 118, 149, 128, 153, 138, 180, 187, 170 and 206) with a total concentration of 41 ng/g pellet in the Durban catchment. The total PCB concentration of the seven PCBs (Table 3.1 and 3.2) monitored in this study for DH were 282 ng/SPMD, which would indicate an increase in PCB concentrations since 2009. The ΣPCB concentrations in Port Elizabeth had shown a decrease (41 ng/pellet to 27 ng/pellet) over the last two decades (Ryan et al. 2012).

The SPMD results from RBH showed higher concentrations of PAHs than found in DH, which is in contrast with what was expected, since DH is a larger and more concentrated harbour (less of a tidal influence) than RBH. This study showed various PAHs (Table 3.1 and 3.2) present in RBH, some of which were not present during the Degger et al. (2011a) study, and included naphthalene, acenaphthene,

benzo[b]fluoranthene and benzo[a]pyrene. The temporal analysis showed that PAH concentrations for the dry season were higher than for the rainy season, which may be due to the lack of rain and less storm water runoff that may cause dilution effects as is the case with metal pollutants (Rate et al. 1999) making the PAHs formed by combustion and petroleum activities more concentrated.

According to Irwin et al. (1997), the presence of naphthalene and phenanthrene are good indicators of petroleum and not combustion as the source of contamination. As both these PAHs are present in RBH, it could be concluded that the source is petroleum based. The higher concentrations of these two PAHs during the dry season survey could be attributed to an oil spill (Figure 4.1) from an underwater oil pipeline that occurred between the two exposure periods (Mkhize 2014). Similar results were obtained by Degger (2010) who also observed increases in petroleum derived contaminants after an oil spill in Port Elizabeth Harbour. Proper clean-up of RBH had been implemented before the dry season exposure commenced, but traces of this environmental disturbance may still be present. The PAHs in DH showed high concentrations of naphthalene and fluoranthene during the dry season survey. These high concentrations indicate the sources to be combustion activities and petroleum derived (Sporstol et al. 1983). With higher concentrations found in RBH than in DH for both seasonal surveys, it may indicate more petroleum derived activities in RBH than in DH, which was not expected as DH is a larger and busier port. The PAH concentrations from SPMD analyses along the South African east coast are higher than the concentrations found in the Istanbul strait and the Marmara Sea (Karacik et al. 2013). These sampling sites are situated near the main shipyard area of Turkey, therefore indicating that according to SPMD analyses the South African marine environment is more polluted, as well as when compared with the mussel tissue analyses.

Figure 4.1. Underwater oil pipe spill during April 2014 (van der Sandt 2014; www.iol.co.za)

4.1.2. Spatial and temporal analyses of resident and transplanted mussels

The OCPs, PCBs and PAHs found in the mussels were similar to the contaminants detected in the SPMDs from both harbour sites. The presence of *p,*p'-DDE were detected at all sites (Figure 3.2A) during the rainy season survey, which was also found in the SPMDs, but with higher concentrations in DH (Figure 3.1A). The presence of other OCPs (Figure 3.2A) were detected in the mussels, but not in the SPMDs from either exposure sites. The mussels could be more able to bioaccumulate small traces of OCPs which may not have been able to biodegrade fully. Most OCPs found in mussels during both exposures were not detected by the SPMDs, they may not have been able to fully partition into the triolein lipid due to the OCPs' partition factors (Adams et al. 2007) or biofouling of the SPMD membranes (Prest et al. 1992) which was a factor in this study. Regarding the OCP analyses, few differences in concentrations were observed between resident and transplanted mussels, with the exceptions of the presence of *p,p'*-DDT in transplanted mussels from RBH during the rainy season survey which were not present in the resident mussels (Table 3.1) and the presence of *p,p'*-DDD during the dry season survey. The changes during the rainy season survey may be attributed to the inability of the transplanted mussels to successfully biotransform the *p,p'*-DDT in the time of exposure (Greenfield et al. 2014). The temporal results did not show differences between the two exposure periods. Schlenk et al. (2005) recorded ΣDDs of 200 – 3600 ng/g lipid in male and female sharks (*Carcharodon carcharias*) in the Durban area. In Durban Bay fish (not specified), had high levels of DDE (40 – 150 ng/g) and DDT (30 – 70 ng/g) between 1979 and 1982 (SANCOR 1985). It was also reported that these concentrations were higher than the concentrations in RBH fish (not

specified), with a slight dominance of DDT in RBH. From these reports it is clear that there has been a decrease in current total OCP concentrations, in particular the DDs.

Higher concentrations of PCBs were found in DH than in any of the other sites (Figure 3.2B), with a general increase in accumulation with increased molecule size, i.e. CBs 153 and 138 are larger molecules and more readily available in the water than CBs 28 and 52 which are smaller molecules and more readily partition to air (Figure 3.2B). The higher levels in DH would reflect more industrial discharge and waste water runoff (Kennish 1997). These higher PCB concentrations are also in conjunction with the higher concentrations found in the SPMDs from DH site (Figure 3.1A) and the temporal analyses showed lower concentrations during the dry season than the rainy season survey. The PCB concentrations along the Hong Kong coast are also much higher in the mussel tissues than for South Africa, with levels ranging from 900 – 4000 $ng.g^{-1}$ and South Africa ranging from 80 – 270 $ng.g^{-1}$. In comparison with international studies, RBH and DH are not as contaminated then most other harbours are by organic pollution.

The mussel analyses results also showed higher concentrations of PAHs in RBH than in the other sites (Figure 3.2C). As mentioned in Section 4.1.1, there was an oil spill in RBH between the two exposure periods, which may account for the higher PAH levels in mussels collected during the dry season survey. As for the concentrations from the rainy season survey, it would be explained by increased storm water run-off from adjacent activities, as the naphthalene concentrations were higher than the other PAH concentrations (Irwin et al. 1997). Phenanthrene was only detected at very low concentrations (Figure 3.2C), but it could be possible that the mussels were able to eliminate this compound as it is also a lower molecular weight PAH (Baumard et al. 1998). The presence of naphthalene may be too high to successfully eliminate through biotransformation. The naphthalene concentrations are in conjunction with the concentrations in the SPMDs (Figure 3.1B), but the lower phenanthrene concentrations are not. The PAH concentrations for RBH resident mussels during the dry season had a decrease, while transplanted mussels had an increase when compared to the rainy season transplanted mussels. The decrease in PAH concentrations in resident mussels may be from chronic exposure to high

concentrations where the mussels were able to metabolize and successfully eliminate some of the contaminant load (Baumard et al. 1998). As for the higher concentrations in transplanted mussels, this is in conjunction with the results from the SPMDs (Figure 3.1B), indicating increased environmental contamination. The transplanted mussels were translocated from a pristine environment to a contaminated environment, in this case contamination due to an oil spill, which accounts for the higher concentrations naphthalene which these mussels may not have been able to eliminate to lower concentrations in mussel tissue in the six week exposure time period (Greenfield et al. 2014). South African PAH naphthalene concentrations in bivalve *P. perna* tissues are higher than the concentrations found off-shore from Brazil (Lourenco et al. 2015) in local bivalves, which was studied near an oil and gas production site. The PAH concentrations along the Hong Kong coast are much higher in mussel tissues, with total concentrations ranging between 18 – 75 $\mu g.g^{-1}$ lipid weight (Richardson et al. 2003) and South African concentrations ranging between 1 – 4 $\mu g.g^{-1}$ lipid weight.

4.2. BIOMARKER ANALYSES

As part of the ABM method, biochemical biomarkers (also known as assessment endpoints) (Wepener 2013), are used to assess the physiological responses to environmental stress caused by organic pollutant exposure. Spatial differences may vary not only in responses to contaminant levels, but also the different environmental factors of each exposure site, but temporal differences are variances within the same exposure site at different times/in different seasons (Petrović et al. 2004). The interpretive value of biomarker changes are either whether they are induced or inhibited. These are presented in Table 4.1.

Table 4.1. Inhibition/induction tendencies of biomarkers of exposure and effect (adapted from van der Oost et al. 2003).

Biomarker	Induction/Inhibition	Interpretation
Acetylcholine esterase (AChE)	Inhibition	Organophosphate and carbamate pesticide (all OCPs) exposure
Cytochrome P450 (CYP450)	Induction	Stimulated in the presence of OCPs, PCBs and PAHs
Superoxide dismutase (SOD)	Induction	Decomposition of super oxides in the presence of ROS
Catalase (CAT)	Induction	In response to SOD in the presence of ROS
Malondialdehyde (MDA)	Induction	Liver peroxidation/lipid peroxidation in the presence of ROS
Protein Carbonyl (PC)	Induction	Oxidative damage to proteins by ROS

4.2.1. Spatial analysis of biomarkers

As seen in Table 4.1 most of the biomarkers, except for AChE, are functional measurements of environmental stress through induction, i.e. up-regulation of the biomarker levels. There were no significant differences between sites during the rainy season survey, but transplanted mussels had significant increases in AChE activity during the dry season survey. This would imply that the mussels from SB reference site became less stressed when translocated to the RBH site. As the inhibition of AChE activity is caused by pesticide/organochlorine (OCPs) exposure (van der Oost et al. 2003, Wepener et al. 2011), the activation of AChE would suggest that OCP exposure was not significant enough to cause physiological stress, which is also in conjunction with the OCP levels found in mussel tissue (Table 3.1 and 3.2). According to Natalotto et al. (2015), the AChE activities in the gills of

Pinna nobilis did decrease with increased levels of organophosphate contaminant exposure, therefore also indicating that the OCP levels found in this study were not enough to inhibit AChE activity. The AChE activities between the two exposure groups indicated (Figure 3.3A) that during the dry season exposure, the effects of possible OCP exposure were non-existent, which is in conjunction with the OCP levels found in the mussel tissues.

The CYP450 levels had no significant differences between sites for either exposure periods. During the dry season, the mussels from RBH had higher levels of CYP450, which is stimulated in the presence chlorinated organic compounds (Wepener et al. 2011). With the increased levels of PCBs and PAHs found in mussels at RBH, CYP450 levels increased, especially for the higher PAH levels found during the dry season survey which could have been from the then recent oil spill (2014). According to Bebianno et al. (2007), the presence of PCBs and OCPs did not have an induction effect on CYP450 levels in *Mytilus galloprovincialis* digestive glands. Only in the presence of PAHs were CYP450 levels elevated, which is in conjunction with the results in the current study. The CYP450 levels in DH mussels were lower than for the SB reference site. The reference mussels may have been exposed to more harsh environmental elements, as the mussels are found in rocky intertidal shores, and stormy weather, increased freshwater runoff and seasonal fluctuations may have caused environmental stress (Kirby et al. 2004). The higher CYP450 levels at all the sites during the dry season exposure (Figure 3.3B), some significant, indicates that the presence of higher levels of PAHs and PCBs found in the mussel tissues (Table 3.1 and 3.2) induced CYP450 levels.

With increasing levels of ROS, an antioxidant defence system is activated to decompose superoxides into hydrogen peroxide (Storey 1996). The first step in this defence system is the activation of the SOD enzyme. During the rainy season survey, SOD levels in DH mussels were significantly elevated in comparison with the other sites (Figure 3.3D), indicating increased oxidative stress due to organic pollutant exposure. This is also in conjunction with the PCB and PAH levels found in mussel tissue (Table 3.1 and 3.2). The higher levels in transplanted mussels from RBH and DH in comparison with SB during the rainy season showed increased oxidative stress. The increased SOD levels in RBH mussels indicated increased

oxidative stress in comparison with the other sites, which is in conjunction with the levels of PAHs and PCBs found in RBH after the recent oil spill. Digestive glands and gills from *P. perna* have showed increased SOD levels during oxidative stress (Almeida et al. 2005; 2015). DH mussels showed decreased oxidative stress during the dry season survey, which is unexpected when compared with the PCB and PAH levels found in mussel tissue, but the significantly increased levels of CAT activity may be the reason as CAT is the second line of defence in the antioxidant defence system (Storey 1996). The higher SOD levels in DH during the rainy season are indicative of higher oxidative stress than in the dry season.

The function of the CAT enzyme is to convert hydrogen peroxide, which is the product from hydrolysis by SOD, to unreactive water and oxygen (Limón-Pacheco and Gansebatt 2009). The significantly high CAT levels in DH mussels indicate that the antioxidant defence system works and may account for the lower levels in SOD activity as most ROS could have been converted to hydrogen peroxide. The higher CAT levels (Figure 3.3C) in RBH mussels during the rainy and dry season surveys are also in conjunction with the SOD levels (Figure 3.3D) and organic pollutant levels (Table 3.1 and 3.2) found in the mussel tissues. The CAT activity in *M. galloprovincialis* had increased with increased levels of organic pollutant related oxidative stress (Khessiba et al. 2005). The lower SOD levels in relation to the higher CAT levels could be the completion of the antioxidant defence reaction. The significant CAT levels (Figure 3.3C) during the dry season in RBH are also indicative of higher oxidative stress, of which the chemical levels of PCBs and PAHs also correspond with these higher oxidative stress patterns and indicative of an oil spill (2014).

Lipid peroxidation has harmful physiological effects and is activated in the presence of harmful organic and metal substances (Ameur et al. 2015). Lipid peroxidation is a consequence of ROS, where MDA is formed during this process. During the dry season survey the significant higher levels in DH is in conjunction with the oxidative stress levels of CAT and the PAH and PCB concentrations found in the mussel tissues. The higher MDA levels (Figure 3.3E) during the dry season are indicative of higher oxidative stress. Ameur et al. (2015) showed increased MDA levels in fish (*Mugil cephalus*) with increased oxidative stress due to organic pollutant exposure.

With increases in oxidative stress, the production of ROS could overwhelm the antioxidant defence system, resulting in protein damage and PC formation (Requena et al. 2003). Generally an increase in PC levels would be an indicator, but severely oxidized proteins could become stable due to aggregation, enabling the proteins to crosslink and therefore decrease the solubility (Grune et al. 2003; Grune et al. 2004). This explains the significantly lower levels of PC found in DH mussels during the rainy season survey. Seasonal and environmental elements such as stormy weather and increased freshwater runoff could also affect oxidative stress, as mentioned previously, and would account for the levels in SB reference mussels. The PC levels during the dry season survey were mostly unchanged between the sites, indicating that even in the presence of the environmental stressors, they seem to have almost no effect on the protein structures within the organisms. Increased PC levels in *M. galloprovincialis* with increased organic pollutant exposure were observed in a study by Kasiotis et al. (2015). The PC levels for the dry season were lower, indicating that increased PAH and PCB levels in RBH did not have an effect on protein structure within the organism, but rather that environmental elements and seasonal changes might have more impact on protein structures in this study (Figure 3.3F).

The biomarker responses of the different sites and exposures to chemical bioaccumulation are represented in Figure 3.5. The results obtained from these surveys are similar to results from other biomarker studies involving bivalves and the effects of both organic and metal pollution in the world (Schiedeck et al. 2006; Sarkar et al. 2006; Gorbi et al. 2008; Faria et al. 2010), except for the increases in AChE activities observed in both surveys.

4.3. INTEGRATED BIOMARKER RESPONSE INDEX (IBR)

The IBR index (also known as the IBR/N value) is used to give a visual integration of the biomarker responses in mussels from selected sites. A higher IBR/N value indicates an increase in biological response to environmental stress and a poorer health status of the organism (Brooks et al. 2015). The position of the biomarkers in the starplot have great influence on the IBR/N value, therefore biomarkers that measure similar physiological responses are grouped together (Brooks et al. 2015). The biomarkers of exposure are AChE and CYP450. The biomarkers of effect are

CAT, SOD, MDA and protein carbonyls. The biomarkers were grouped accordingly to generate the IBR/N values (Figure 3.5) and the starplots (Figure 3.6).

The slight decrease in the IBR index of transplanted RBH mussels (dry season) is likely due to the translocation of the mussels to a slightly less stressed environment at that time, also indicated by the biomarkers (AChE, CAT and PC) that contributed, since it was during the dry season and mussels on the rocky shore were more exposed to the elements, less food resources and more competition than in the RBH estuarine environment. The high IBR index of SB may be due to environmental stress during the rainy season. The contributing biomarkers (AChE, CAT, MDA and PC) are likely to be a response to stormy weather on the rocky shores as well as freshwater runoff due to heavy rains at SB in 2014 (Figure 3.2A-C), since organic contamination does not occur and therefore is unlikely to be the cause of the magnitude of the effects measured (Wang et al. 2011; Turja et al. 2015). The high IBR indexes of resident and transplanted DH mussels (rainy season) and transplanted DH mussels (dry season) indicate an increase in biological responses of the mussels. The contributing biomarkers (Figure 3.6) and the high IBR indexes (Figure 3.5) for the mussels at DH indicate oxidative stress and poorer health status. Since it is also an estuarine environment, i.e. mussels are more sheltered from harsh environmental elements, the increased biological response may be due to organic pollutant exposure and some extent freshwater runoff from the canal during the rainy season.

The mussels from RBH, with the exception of transplanted RBH mussels (dry season), have very similar recorded IBR indexes which indicated the same level of biological response. The strongest contributing biomarkers (PC, SOD, CAT and AChE) indicate chronic exposure to organic phosphate pollutants (Wepener et al. 2011). This consistency, even though lower than DH, indicates higher levels than were expected. With the recent oil spill the levels during the dry season survey is expected due to the environmental stress and chemical levels in the mussel tissues (Chapter 4.1). The higher IBR indexes of DH mussel groups are in conjunction with the chemical analyses of the mussel tissues.

The results obtained from this study are similar to the results from Bebianno et al. (2015), who studied caged (*M. galloprovincialis*) mussels in different harbour areas

of Portimão Harbour. Damiens et al. (2007) also showed similar results for (*M. galloprovincialis*) mussels exposed to PAHs and PCBs in the Bay of Cannes, indicating higher IBR indexes in more contaminated and therefore stressed environments than the less contaminated sites. These studies did use different biomarkers such as glutathione S-transferase, metallothioneins, glutathione peroxidase and estrogenic effects, which demonstrated the effectiveness of the IBR index as a helpful tool to illustrate biomarker responses in a less complicated manner.

CHAPTER 5

CONCLUSIONS

5.1. CONCLUSIONS

The results in this study provided an indication of the current state of organic pollution in both Durban and Richards Bay harbours. The results from the chemical analyses are linked with the results from the biomarker analyses, and the SPMDs also show a relationship with the organic pollutant levels found in the mussel tissues. For chemical evaluation of the contaminated sites, SPMDs were deployed according to the methods discussed in Chapter 3. For bioaccumulation and biochemical evaluation ABM methods, discussed in Chapter 3, were successfully implemented. The brown mussel, *P. perna*, was successfully translocated from a reference site (SB) which was considered pristine, to two contaminated sites. Resident mussels were also successfully analysed.

The first study hypothesis was that the SPMDs and bio-indicator organisms (transplanted and resident mussels) will show similar bioaccumulation patterns for organic pollutants. The OCP results of the SPMDs and both resident and transplanted mussels had similar trends, with similar levels of *p,p'*-DDE detected during both surveys. The PCB results of the SPMDs and resident/transplanted mussels had similar levels, with higher levels found in DH during both surveys. In some cases the SPMDs did accumulate organic pollutants that were not quantifiable in the mussel tissues. Unfortunately a proper correlation study could not be done due to some triolein loss caused by biofouling and small tears in the SPMD membranes, but the hypothesis is accepted due to similar trends. The SPMDs are also able to accumulate certain contaminants more readily, without the biodegrading that occurs within organisms.

The second study hypothesis was that biomarker responses in ABM and resident mussels will provide an indication of oxidative stress due to organic pollutant exposure. With increasing OCP, PCB and PAH bioaccumulation, the biomarker results indicated increased levels of oxidative stress. The hypothesis is therefore accepted. The study also indicated that with increasing environmental stress due to heavy rains on the east coast during the rainy season survey 2014, seasonal changes, spawning and competition for food and survival, there are also higher levels of oxidative stress. It was also found that the mussels in some cases became less stressed when translocated from the pristine environment to the harbours, even

when bioaccumulation results indicated that organic pollutant levels at the reference site was at a minimal. This also indicated the physiological influences natural environmental occurrences have on the organisms.

The third study hypothesis was exposure to POPs and the ensuing biomarker responses are influenced by increased run-off during the high rainfall periods. The ABM method used during this study with biomarker analyses was a useful tool for monitoring the exposure and possible effects of organic pollutants and seasonal environmental stress on marine biota. The results however did not reflect influences due to increased runoff, but rather influences regarding an oil spill in RBH and other environmental stresses such as storms and freshwater runoff at the reference site. The hypothesis is therefore rejected in this study.

The fourth study hypothesis was both transplanted (ABM) and passive monitoring methods are efficient in monitoring organic pollutants along the KwaZulu-Natal coastline. The results for both ABM and passive monitoring methods in this study were efficient to accept this hypothesis. The results of both methods made it possible to distinguish whether physiological effects were indeed bioaccumulation related, and the SPMD analyses were able to evaluate the effectiveness of the passive monitoring method in South African harbours and the similarities in results obtained. The IBR index gave an adequate visual representation of the biomarker responses to environmental stress during this study, including the responses that are organic pollutant related. It was able to clearly identify oxidative stress in some cases where environmental stress, due to organic pollutant exposure, was evident. It was particularly the case with the levels of PAHs found in mussels, together with the physiological responses to the increased PAH exposure.

There is a distinct lack of organic pollutant monitoring in the South African marine environment, also discussed by Wepener and Degger (2012). The majority of studies have focussed on metal exposures and effects on sediment, benthic biota, higher marine biota, artificial devices for passive monitoring and water (Vermeulen and Wepener 1999; Wepener and Vermeulen 2005; Wepener et al. 2008; Degger et al. 2011b; Greenfield et al. 2011). This has made it difficult to compare the levels and effects of OCPs, PCBs and PAHs in the harbours. Degger et al. (2011a) did report concentrations of PCBs and PAHs in RBH using both SPMDs and mussels,

but no published records of DH are available to the public. Kampire et al. (2015) reported PCB concentrations in sediment and mussels in Port Elizabeth Harbour which can be used, together with Degger et al. (2011a) whom also reported PCB concentrations in Port Elizabeth Harbour, as a general reference to the current state of South African harbours. These results also provide sufficient motivation for the application of SPMDs and ABM methods for organic pollutant monitoring in South African marine environments.

5.2. RECOMMENDATIONS

The changes in physiological effects observed from biomarker responses indicate that seasonal variations and natural environmental stress have an influence on the biomarker status of mussels. This study has provided extra insight and data sets to be used for future monitoring programme development. For RBH, this is the second organic pollutant monitoring data set, which has given the opportunity to successfully compare the state of the harbour environment over a period of time. For DH, there was no pervious data sets that the results from this study could be compared with to assess the state of the harbour environment over a period of time.

This study, however, has opened the door for further organic pollutant monitoring at both DH and RBH, to successfully implement an ongoing monitoring program, and is also recommended to build a broader understanding of the bioavailable organic pollutants and their effects on marine biota. These studies will also give a broader understanding of the possible risks to human health with the consumption of marine biota.

For ABM purposes, it is recommended that biomarker responses to natural environmental stress and seasonal variations be determined. This could act as a control reference to ensure that these natural biomarker responses are eliminated in order to obtain a more accurate indication of pollution related stress. Spawning cycles also have to be considered, since spawning does have physiological effects. It is recommended that a broader range of biomarkers need to be studied to give a more detailed representation of physiological health in response to organic pollutant exposure.

During this study, four replicate SPMDs were used per exposure site during each exposure survey together with a field blank. More SPMD deployment sites are needed in a single harbour to identify possible point sources of organic pollutants. Future studies may also include genotoxic tests to assess the effects of OCPs, PCBs and PAHs at DNA level. Genotoxic analyses may include comet assay analyses and mRNA analyses using real time polymerase chain reactions (PCRs) to assess which genes are targeted by organic pollutant exposure.

CHAPTER 6

REFERENCES

6.1. REFERENCES

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