MORE THAN JUST FOOD: MUSSELS AS BIOMONITORS OF MICROPLASTIC POLLUTION IN THE KWAZULU-NATAL COASTAL ENVIRONMENT

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A research dissertation submitted in fulfilment of the academic requirements for the degree of
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PREFACE

The research contained in this dissertation was completed by the candidate while based in the Discipline of Biological Sciences, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, South Africa. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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DECLARATION 1: PLAGIARISM

I, **Gemma Gerber**, declare that:

(i) the research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii) this dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii) this dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

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a) their words have been re-written but the general information attributed to them has been referenced;

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(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) this dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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Signed: Gemma Gerber

Date: 4 December 2017

DECLARATION 2: PUBLICATIONS

Details of contribution to publications that form part and/or include research presented in this dissertation:

Publication 1: Gerber, G., Kunnen, T.H., Coote, M.W., Moodley, G.K., and Robertson-Andersson, D.V. A novel macro-based methodology for accurate estimation of microplastic fibre uptake in mussels under laboratory conditions (In preparation).

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Publication 2: Gerber, G., Moodley, G.K., and Robertson-Andersson, D.V. Microplastic pollution distribution in selected KwaZulu-Natal temporarily open/closed estuaries during an open mouth phase (In preparation).

Author contributions: Study conception and design: Gerber, Moodley, and Robertson-Andersson. Acquisition of data: Gerber. Analysis and interpretation of data: Gerber. Drafting of manuscript: Gerber. Critical revision: Gerber, Moodley, and Robertson-Andersson.

Publication 3: Gerber, G., Moodley, G.K., and Robertson-Andersson, D.V. Microplastic pollution in beach sediment near three temporarily open/closed estuaries during an open mouth phase, KwaZulu-Natal (In preparation).

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Presentation 1: Gerber, G.*, Mkhize, M., Moodley, G.K., and Robertson-Andersson, D.V. 2016. The potential for biomonitoring microplastic pollution along the KwaZulu-Natal coastline using marine invertebrates: implications for human health. eThekwini University Research Symposium (EURS) 2016. Durban, South Africa. (Oral presentation).

Presentation 2: Gerber, G.*, Moodley, G.K., and Robertson-Andersson, D.V., 2016. Microplastics in mussels: what should the consumer know? Symposium of Contemporary Conservation Practices (SCCP). Howick, KwaZulu-Natal, South Africa. November 2016. (Oral presentation)
Nominated for KwaZulu-Natal Premier's Award
First place for KwaZulu-Natal Premier's Award

Presentation 3: Gerber, G.*, Moodley, G.K., and Robertson-Andersson, D.V., 2016. Ingestion of microplastics by *Perna perna* (L.): implications for human health? College of Agriculture, Engineering and Sciences Postgraduate Research Day. University of KwaZulu-Natal. Westville Campus, South Africa. October 2016. (Poster presentation)
First place award for School of Life Sciences student poster presentation

Presentation 4: Gerber, G.*, Moodley, G.K., and Robertson-Andersson, D.V., 2016. Microplastics in mussels: what should the consumer know? South African Marine Science Symposium (SAMSS). Port Elizabeth, South Africa. July 2017. (Poster presentation).

Presentation 5: Gerber, G.*, Moodley, G.K., and Robertson-Andersson, D.V., 2017. More than just food: Mussels as biomonitors of microplastic pollution in the KwaZulu-Natal coastal environment. College of Agriculture, Engineering and Sciences Postgraduate Research Day. University of KwaZulu-Natal. Westville Campus, South Africa. October 2017. (Poster presentation)

Presentation 6: Gerber, G.*, Moodley, G.K., Robertson-Andersson, D.V. 2017. More than just food: Mussels as biomonitors of microplastic pollution in the KwaZulu-Natal coastal environment. Symposium of Contemporary Conservation Practices (SCCP). Howick, KwaZulu-Natal, South Africa. November 2017. (Oral presentation)

ABSTRACT

Microplastics are small plastic particles (< 5 mm in longest dimension) and originate as manufactured small particles and from the fragmentation of larger plastic items. Microplastic pollution has recently become the subject of a large body of research due to the ubiquity throughout the marine environment and potential devastating ecosystem-wide impacts. As microplastic pollution theoretically cannot be totally eliminated from marine environments, one of the few available options is to monitor the scope and extent of microplastic pollution. Coastal marine microplastic pollution in South Africa is thought to originate from point sources such as estuaries. To date, there are no standardised protocols for microplastic pollution monitoring and limited information regarding microplastic pollution in South African estuaries and coastal environments. A recent development of microplastic pollution monitoring is using rocky shore invertebrate mussels as biomonitors of microplastic pollution in a particular area. Mussels are already used to successfully monitor heavy metal pollution along the South African coastline (SANCOR Mussel Watch Programme).

Building on these principles, this study aimed to (1) Determine if a novel, macro-based automated counting feature could be used as a viable time-saving alternative to manual counting of microplastic fibres (microfibres) ingested by the rocky shore bivalve, *Perna perna* under laboratory conditions; and to assess microplastic pollution in (2) three temporarily open/closed KwaZulu-Natal estuaries, (3) beach sediment at sites up to 2 km North and South of each estuary mouth on the adjacent coastlines and (4) *Perna perna* (L.) at rocky shore sites up to 2 km North and South of each estuary mouth on the adjacent coastlines. The results of the novel, macro-based automated counting feature showed that the time taken to count microfibres in images was significantly reduced using the automated counting and measurement method (1.00 ± 0.14 minutes) as opposed to the manual counting and measuring method (23.91 ± 7.68 minutes). The findings showed that this novel counting methodology for microfibre uptake in mussels under laboratory conditions is as effective and reliable as manual microscopy, but resulted in significant reductions in microscopy time analysis. The environmental studies found that that Bilanhlolo Estuary had the highest microplastic pollution levels of the studied estuaries in both surface water (surface water (5.98 ± 0.46 microplastics.m^{-2}) and sediment (4.22 x 10^4

± 2.17 x 10^3 microplastics.m^{-2}). Mhlangeni Estuary and Kongweni Estuary displayed lower levels of microplastic pollution in surface water (Mhlangeni Estuary: 4.50 ± 0.59 microplastics.m^{-2}; Kongweni Estuary: 2.34 ± 0.23 microplastics.m^{-2}) and in sediment (Mhlangeni Estuary: 1.33 x 10^4 ± 1.52 x 10^3 microplastics.m^{-2}; Kongweni Estuary: 1.89 x 10^4 ± 2.31 x 10^3 microplastics.m^{-2}). The study investigating microplastic pollution in beach sediment adjacent to each estuary mouth showed that microplastic abundances (microplastics.m^{-2}) were greater at sites nearer to each estuary mouth than at beach sites further away. *Perna perna* in the sampled areas contained an average of 2.22 ± 0.79 microplastics.g^{-1} tissue w/w. Mussels nearer to each estuary mouth contained greater quantities of microplastics than sites further away. The results showed that microplastics were abundant in all sampled estuaries, beach sediment sites, and mussels. Microplastic fibres were the most dominant microplastic type in all samples. This study provides baseline data for the selected estuaries and adjacent coastal environments. The uptake of microplastic in *P. perna* in marine environments indicates that mussels may be used as biomonitors of marine microplastic pollution. The application of the results in our country will eventually build a clearer picture of microplastic pollution along our coastline, its threats to ecosystem health, and how we could potentially mitigate the impacts to ensure marine conservation.

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LIST OF ABBREVIATIONS

3D	Three Dimensional
ANOVA	Analysis of Variance
DAPI	4’6-diamidino-2-phenylindole
DTI	Department of Trade and Industry
DWA	Department of Water Affairs
E	East
EC	Ecological Categories
EDF	Extended Depth of Field
FTIR	Fourier Transform Infrared spectroscopy
GDP	Gross Domestic Product
GESAMP	Joint Group of Experts on Scientific Aspects of Marine Environment Protection
$HClO_4$	Perchloric acid
HNO_3	Nitric acid
ICES	International Council of the Exploration of the Sea
IPP	Image Pro Plus
IR	Infrared
KOH	Potassium hydroxide
KZN	KwaZulu-Natal
LADI	Low-Tech Aquatic Debris Instrument
MPA	Marine Protected Area

MPRP	Marine Pollution Research Programme
MSFD	Marine Strategy Framework Directive
MWP	Mussel Watch Program
N	North
NaCl	Sodium chloride
NaI	Sodium iodide
NDF	Neutral Density Filter
NOAA	National Oceanic and Atmospheric Administration
NR	Nile Red
PA	Polyamide
PAH	Polycyclic Aromatic Hydrocarbon
PBDE	Polybrominated Diphenyl Ether
PCB	Polychlorinated Biphenyl
PE	Polyethylene
PERMANOVA	Permutational analysis of variance
PES	Present Ecological State
PET	Polyethylene Terephthalate
pMAR	Present day Mean Annual Runoff
POP	Persistent Organic Pollutant
PP	Polypropylene
PRIMER	Plymouth Routines in Multivariate Ecological Research

PS	Polystyrene
PVA	Polyvinyl Alcohol
PVC	Polyvinylchloride
S	South
SANCOR	South African Network for Coastal and Oceanic Research (post 1993)
SD	Standard deviation
SIMPER	Similarity Percentage Analysis
SOP	Standard Operating Procedure
TOCE	Temporarily Open/Closed Estuary
UNEP	United Nations Environment Programme
UV	Ultra-violet
VBA	Visual Basic for Application
WWTW	Waste Water Treatment Works

LIST OF UNITS AND SYMBOLS

%	Percentage
<	Less than
>	Greater than
±	Plus/minus
≈	Approximately
⌀	Diameter
°C	Degree(s) Celsius
μm	Micrometre
¼	Quarter
articles.L^{-1}	Article(s) per Litre
cm	Centimetre
g	Gram
g.cm^{-3}	Grams per cubic centimetre
items/g	Items per gram
km	Kilometre
km^2	Square kilometre
L	Litre
m	Metre
M	Moles
m^2	Square metre

m^3	Cubic metre
$mg.L^{-1}$	Milligram(s) per litre
$microfibres.mussel^{-1}$	Microfibres per mussel
$microplastics.g^{-1}$ tissue	Microplastics per gram of soft tissue
$microplastics.m^{-2}$	Microplastics per square metre
mL	Millilitre
mm	Millimetre
nm	Nanometre
$particles.m^{-2}$	Particles per square metre
$particles.m^{-3}$	Particles per cubic metre
psu	Practical Salinity Units
s	Second(s)
v:v	volume/volume
w/w	Wet weight

CHAPTER 1: INTRODUCTION

1.1. Preamble

Plastics can be defined as synthetic organic polymers which are derived from monomers extracted from oil, coal, and gas (Thompson *et al*., 2009) and are used in every sector within South Africa (Verster *et al*., 2017). The plastic manufacturing industry contributes 16.5 % to South Africa's total manufacturing industry (Plastics SA, 2016) and a total of 1.9 % to South Africa's gross domestic product (GDP) (Plastics SA, 2016). The economic contribution to South Africa via import profits and increase in local employment rates has caused the South African government to identify the national plastic industry as a priority sector (Plastics SA, 2016). The increase of South Africa's production and consumption of plastic products has unfortunately led to large quantities of plastic waste, of which 72 % is not recovered (Department of Trade and Industry (DTI), 2016). Lack of maintained infrastructure and inadequate waste disposal methods largely contribute to the increasing plastic waste accumulation in aquatic environments. Whilst the majority of research has, in the past, focused on larger plastic items and their negative impacts on environmental health (Andrady, 2011; Setälä *et al*., 2014), comparatively less attention has been placed on microplastics and microplastic pollution, both globally and in South Africa (Andrady, 2011; Naidoo *et al*., 2015).

Microplastics are plastic particles smaller than 5 mm in maximum size dimension (Lusher *et al*., 2017) and have recently become the focus of a large amount of research. Microplastics can be classified based on the origin: primary microplastics are manufactured to be of a small size (Andrady, 2011), and secondary microplastics are as the result of fragmentation of larger plastic items in the environment (Carr *et al*., 2016). Due to the small size of microplastics, as well as their longevity and ubiquity through the marine environment, they become available for ingestion to a variety of marine organisms (Van Cauwenberghe *et al*., 2015). Ingested microplastics may cause physical damage to the organisms, such as gut blockage/damage, false sense of satiation, malnutrition, and even death (Wright *et al*., 2013). Toxicological damage may occur from adsorbed toxicants transferring from the microplastics to the

organism (Chua *et al*., 2014). These organisms may be consumed by larger, predator organisms, leading to a potential bioaccumulation of toxicants along the food web (Ivar do Sul and Costa, 2014). These potential impacts of microplastics on marine organisms may be an issue for humans, as not only may marine food resources decline as a result, but this also opens up the possibility of toxicant transfer from organisms to humans (Vandermeersch *et al*., 2015). It is therefore of paramount importance to monitor the scope of microplastic pollution in the environment as well as determine any remediation methodologies available to curb the widespread impact of microplastic pollution.

1.2. Problem Statement

There have been a large number of recent publications highlighting the global distribution of microplastic pollution in freshwater environments, marine environments, and within organisms (Mahon *et al*., 2017). Despite the fact that approximately 80 % of plastic and microplastic pollution in marine environments is derived from land-based sources (Andrady, 2011), there are still enormous knowledge gaps regarding the impacts on ecological and human health of freshwater microplastic pollution and consequently transport to marine environments via estuaries (Eerkes-Medrano *et al*., 2015; Cheung *et al*., 2016). As microplastic pollution is a relatively new threat to environmental and human health, methodology is limited and unharmonized. The limitations and disharmony of methodology does not allow for the accurate reporting of microplastic pollution loads, nor the accurate comparison of microplastic reporting between studies (Eerkes-Medrano *et al*., 2015).

South Africa, as a developing country, has a slow economic growth and as such, development, growth and poverty reduction receive prioritization ahead of ecological issues such as microplastic pollution (Verster *et al*., 2017). With the plastic industry greatly stimulating the economy (Verster *et al*., 2017), it is unlikely that plastic production and plastic waste generation will decrease in the near future. In South Africa, there are currently few published reports of microplastic pollution (Ryan, 1988; Ryan and Moloney, 1990, Naidoo *et al*., 2015; Nel and Froneman, 2015, Nel *et al*., 2017; Nel *et al*., 2018). The lack of knowledge of the status of microplastic pollution in South Africa is worrying. Whilst the prioritization of economic and social

development is vital for the well-being of the people of South Africa, the potential risks posed by microplastics to human health and ecological integrity cannot be ignored. From an ecological perspective, South Africa is considered to be one of the most bio diverse regions in the world. The potential impacts of microplastic pollution on biota may decrease the natural biodiversity of South Africa, further negatively impacting the ecological integrity of the country.

1.3. Purpose and significance of study

The purpose of the study is to investigate microplastic pollution in selected temporarily open/closed estuaries (TOCEs) along the KwaZulu-Natal (KZN) coastline, the inputs of microplastics into the nearby coastal environment from these identified estuaries, and the microplastic pollution present in the indigenous rocky shore bivalve, *Perna perna* (L.), at rocky shore sites near these identified estuaries. The significance of the study includes the presentation of microplastic pollution in previously unstudied estuarine and beach environments, as well as a novel methodology of microplastic pollution estimation along South African coastlines by using *P. perna* mussels as biomonitors. As there are many different types of microplastics, biomonitoring allows for the identification of microplastics that are most likely to be ingested by mussels and therefore, pose the most threat to the mussels. The identification of microplastics in mussels is not only useful from a microplastic monitoring perspective, but as mussels are an important subsistence food source for a large social sector (Richir and Gobert, 2016), it is important to quantify the microplastics in *P. perna* in order to identify the relevant risks they may pose to an already vulnerable population. The information presented in this study has the potential to add significant value to the knowledge of microplastic pollution in South Africa, as well as providing new insight in the field of microplastic pollution analysis and quantification on a global scale.

There are currently limited methodologies available for sampling, processing and analysis of microplastics within samples. As microplastic pollution is an enormous threat to global ecosystems, it is imperative that microplastic pollution is rapidly reported and published. To date, the microscopy techniques used to identify microplastics in samples frequently involve manual counting of particles. Manual

counting is slow and is incredibly sensitive to human errors due to lack of skills, fatigue, and underlying physiological issues. The study also aimed to create a novel methodology to count and measure microplastics within samples using an automated macro-based computer technique.

1.4. Research aims and objectives

The aims and objectives for each study chapter are outlined below:

Chapter 4

Aim: Determine if a novel, macro-based automated counting feature could be used as a viable time-saving alternative to manual counting of microplastic fibres (microfibres) ingested by the rocky shore bivalve, *Perna perna* under laboratory conditions.

Objective 4.1: Compare microfibre counts and measurements of microfibres ingested by mussels between data captured manually by volunteers and data captured using the automated macro-based methodology.

H_A: There is a significant difference in microfibre counts and measurements between manual and automated macro-based methodologies.

Objective 4.2: Compare time taken to count and measure microfibres between manual data capture and automated macro-based methodologies.

H_A: There is a significant difference in data capture time between the manual and automated macro-based methodologies.

Chapter 5

Aim: Determine and compare spatial differences in microplastic pollution between selected TOCEs (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) in open mouth phases during a summer season (wet season).

Objective 5.1: Compare microplastic abundances (microplastics.m^{-2}) in surface water and sediment between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary in open mouth phases during a summer season.

H_A: There is a significant difference in microplastic abundance (microplastics.m^{-2}) in surface water and sediment between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary in open mouth phases during a summer season.

Objective 5.2: Compare microplastic type composition (%) in surface water and sediment between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary in open mouth phases during a summer season.

H_A: There is a significant difference in microplastic type composition (%) in surface water and sediment between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary in open mouth phases during a summer season.

Objective 5.3: Compare microplastic size class (μm) distribution (%) in surface water and sediment between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary in open mouth phases during a summer season.

H_A: There is a significant difference in microplastic size class (μm) distribution (%) in surface water and sediment between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary in open mouth phases during a summer season.

Chapter 6

Aim: Determine and compare spatial differences in beach sediment microplastic pollution originating from selected TOCEs (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) during open mouth phases at increasing distance 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth.

Objective 6.1: Compare microplastic abundances (microplastics.m^{-2}) in beach sediment at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

H_A: There is a significant difference in microplastic abundances (microplastics.m^{-2}) in beach sediment at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

Objective 6.2: Compare microplastic type composition (%) in beach sediment at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

H_A: There is a significant difference microplastic type composition (%) in beach sediment at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

Objective 6.3: Compare microplastic size class (μm) distribution (%) in beach sediment at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

H_A: There is a significant difference in microplastic size class (μm) distribution (%) in beach sediment at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

Chapter 7

Aim: Determine and compare spatial differences in microplastic pollution in the mussel species, *Perna perna*, originating from selected TOCEs (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) during an open mouth phase at increasing distance 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth.

Objective 7.1: Compare microplastic abundances in *Perna perna* at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

H_A: There is a significant difference in microplastic abundances (microplastics.g^{-1} tissue w/w) in *Perna perna* at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

Objective 7.2: Compare microplastic type composition (%) in *Perna perna* at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

H_A: There is a significant difference in microplastic type composition (%) in *Perna perna* at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

Objective 7.3: Compare microplastic size class (*µ*m) distribution (%) in *Perna perna* at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

H_A: There is a significant difference in microplastic size class (*µ*m) distribution (%) in *Perna perna* at stations 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth during an open mouth phase.

1.5. Scope and limitations

The research undertaken aimed to investigate the microplastic pollution in three selected KZN estuaries during a single wet season and subsequent open mouth phase, as well as the distribution of microplastics in sediment and *P. perna* along coastlines up to and including 2 km North and South away from each estuary mouth.

While sources of microplastics may be inferred by the results, the study does not investigate sources of microplastic pollution into the studied estuaries.

1.6. Ethical considerations

The marine invertebrate mollusc *P. perna* is not defined as an “experimental animal” in the National Ethics Guidelines Act of 1990; therefore no ethical clearance for this study was required. Mussel samples were collected in accordance with the field permit [RES2017/71] for the purposes of scientific investigations or practical experiment in terms of Section 83 of the Marine Living Resource Act (Act No. 18 of 1998) issued by the Department of Agriculture, Forestry and Fisheries of the Republic of South Africa (**Appendix B**).

1.7. Chapter overviews

This dissertation comprises of eight chapters. This current chapter (Chapter 1) presents a brief topic background of microplastic pollution in a South African context, identifies the problems which motivated the study, places the motivation (rationale) and approach of the study into context for the study, states the aims and objectives of the study, states the underlying assumptions, limitations, and scope of the study.

Chapter 2 is a review of the relevant literature regarding microplastic pollution, the current scope of microplastic pollution research in South Africa, the effects of microplastics on organisms, and a special focus on *P. perna* as potential biomonitors of microplastic pollution in marine environments.

Chapter 3 is a secondary literature review of the current global methodological approaches and limitations of microplastic pollution research in estuaries, marine environments and within organisms. The information presented in Chapter 3 was largely used to derive the overall methodological approach of the study.

Chapter 4 presents a manuscript of a novel macro-based methodology for accurate estimation of microplastic fibre uptake in mussels under laboratory conditions using automated macros as compared to manual microscopy methods.

Chapter 5 presents a manuscript of a baseline study of microplastic pollution in three temporarily open/closed estuaries in KwaZulu-Natal during an open mouth phase.

Chapter 6 presents a manuscript on microplastic pollution in beach sediment near three selected temporarily open/closed estuaries during an open mouth phase.

Chapter 7 presents a manuscript on the rapid assessment of microplastic pollution in marine environments using the brown mussel, *P. perna*, as biomonitors.

Chapter 8 concludes the study, describing major findings, discusses the challenges and limitations of the study findings, and presents the recommendations for future research of microplastic pollution.

1.8. Study sites

Three study sites were selected for investigation in this study (Chapter 5, 6, and 7) (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) (**Figure 1.1**). The study sites were selected based on the following four criteria: (1) Described as a TOCE and open for approximately 50 % of the year, (2) The presence of rocky shores along the adjacent coastline of each estuary mouth for at least 2 km, (3) study sites needed to be geographically close, but not overlap in distance, allowing differences in climatic conditions be similar between study sites, and (4) Present Ecological State (PES) as described by Department of Water Affairs (DWA), 2013 (**Appendix A: Table A 1**). Estuaries are all located within the Ugu District Municipality, KZN. Geographic co-ordinates of individual sample sites are listed in **Table A 2** (**Appendix A**).

Mhlangeni Estuary

The Mhlangeni Estuary (30°49’06’’ S; 30°24’22’’ E) (**Figure 1.1**) is a TOCE in the Ugu District Municipality, KZN (DWA, 2013) near the coastal town of Margate. The Mhlangeni Estuary mouth has an average depth of 1 m (DWA, 2013) and is open to the sea approximately 47 % of the year (DWA, 2013). Recreational activities that

take place in the Mhlangeni Estuary include boating and recreational fishing (DWA, 2013). The Mhlangeni river has a catchment area of 37.2 km^2 (DWA, 2013). The present day Mean Annual Runoff (pNAR) is 9.6 million m^3 per annum (DWA, 2013). The PES of Mhlangeni Estuary is characterised as C (moderately modified) (**Appendix A: Table A 1**). The adjacent coastline is characterised by rocky shores up to 2 km north and south of the Mhlangeni Estuary mouth. Mhlangeni Estuary is surrounded by dense residential areas (DWA, 2013). Several restaurants are located in close proximity to the Mhlangeni Estuary mouth. During the sampling period, very few macroplastic items were observed in the near vicinity, except for a few plastic bottle tops and one polystyrene cup.

Kongweni Estuary

The Kongweni Estuary (30°51’39’ ’S 30°22’19’’ E) (**Figure 1.1**) is a TOCE on the KZN coastline (Whitfield and Baliwe, 2013) near the coastal town of Margate. Kongweni Estuary mouth has an average depth of 2 m (DWA, 20113), and is open approximately 49 % of the year (DWA, 2013). Kongweni Estuary serves a catchment area of 7.9 km^2 and displays a pMAR of 2.95 million m^3 per annum (DWA, 2013). Kongweni Estuary receives a daily volume of approximately 4998 m^3 of sewage effluent (approximately 1.825×10^6 m^3 per year) from the nearby Margate waste water treatment works (WWTW) (DWA, 2013). Despite the direct input of treated sewage, recreational activities still take place in Kongweni Estuary which include swimming and paddle-boating (DWA, 2013). Developmental pressures have resulted in the loss of mangroves from the Kongweni Estuary (Van Niekerk and Turpie, 2012). The condition of the estuary has been described as poor as a result of the dense surrounding urban area and being highly degraded (Whitfield and Baliwe, 2013). The PES has been categorized as D (Largely modified. A loss and change of natural habitat, biota and ecosystem functions and processes have occurred) (**Appendix A: Table A 1**) (DWA, 2013). The surrounding habitats are characterized as sandy shores for approximately 600 m north of the estuary mouth, and as rocky shores 2 km north and south of the estuary mouth. At the time of sampling the estuary water was heavily silted, and had a foul smell. Observed commercial activities that surround Kongweni Estuary include restaurants and accommodation venues. No large plastic items were visible during the sampling period.

Bilanhlolo Estuary

The Bilanhlolo Estuary (30°53’21’’S 30°20’58’’E) is a TOCE (**Figure 1.1**) on the KZN coastline. The Bilanhlolo Estuary mouth is approximately 1 m in depth (DWA, 2013) and is open to the sea usually 47 % of the year (DWA, 2013). Bilanhlolo Estuary serves a catchment area of 19.8 km^2 (DWA, 2013) and has a pMAR of approximately 4.98 million m^3 per annum (DWA, 2013). The Bilanhlolo Estuary is cited as having important recreational value (DWA, 2013) and is frequently used for leisure activities such as swimming, angling and boating (DWA, 2013). The PES of Bilanhlolo Estuary is characterized as C (moderately modified) (**Appendix A: Table A 1**) (DWA, 2013). The surrounding coastal habitats are characterized as rocky shores for over 2 km north and south of the estuary mouth. Several popular restaurants and accommodation facilities surround Bilanhlolo Estuary. Various large plastic items were observed floating in the water, including plastic bags and pieces of unidentified fragmented plastic.

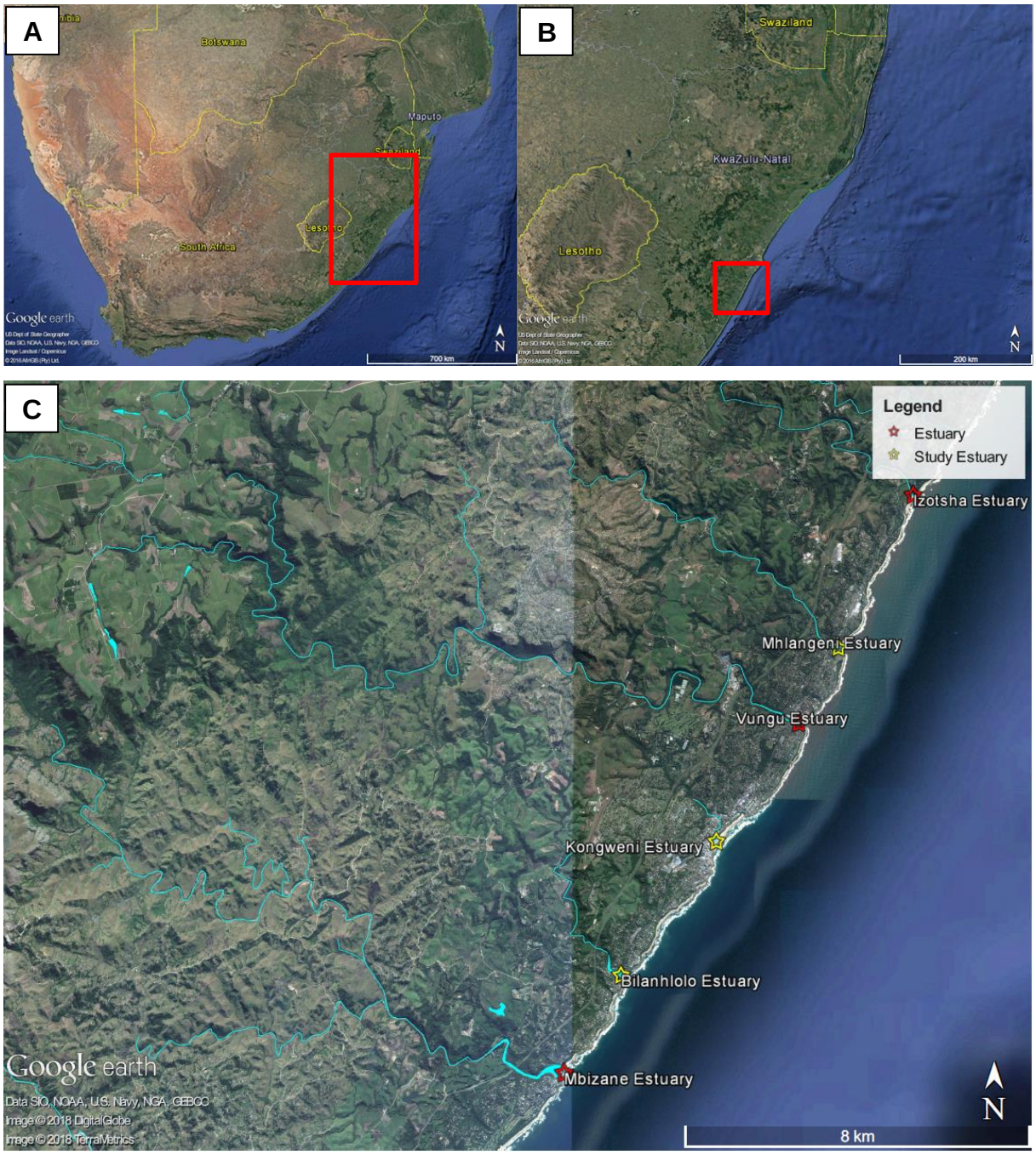

Figure 1.1: Location of KwaZulu-Natal province in South Africa (A) and Ugu District Municipality (B). Locations of Mhlangeni Estuary, Kongweni Estuary and Bilanhlolo Estuary (C). (Source: Google™ Earth Pro; adapted by Gerber, 2017)

CHAPTER 2: LITERATURE REVIEW

2.1. Microplastics: definitions and sources

The National Oceanic and Atmospheric Administration (NOAA) describe microplastics as plastic particles smaller than 5 mm in their longest dimension (Rocha-Santos and Duarte, 2015; Lusher *et al*., 2017) and can be classified as either primary or secondary microplastics according to their origin (Cole *et al*., 2011). Primary microplastics are manufactured to be smaller than 5 mm in maximum dimension, commonly used as virgin material in plastic injection moulding, domestic uses such as exfoliants in face washes, industrial uses such as 'sand-blasting', as well as vectors for drug delivery (Ivar do Sul and Costa, 2014; Luís *et al*., 2015). These particles directly enter the marine environment via rivers, terrestrial runoff as well as domestic and industrial waste effluents (Lima *et al*., 2015; Luís *et al*., 2015; Gallagher *et al*., 2016). Secondary microplastics are those which are derived from breakdown of larger plastic items through a number of degradation processes (Ivar do Sul and Costa, 2014; Carr *et al*., 2016). These include fragmentation via wave and tidal action (Ivar do Sul and Costa, 2014), photodegradation due to exposure of ultraviolet (UV) rays from the sun (Syberg *et al*., 2015) as well as biodegradation by fouling organisms and other biological pathways (Barnes *et al*., 2009). Another prominent source of secondary microplastics results from synthetic polymer clothing being washed in washing machines (Rocha-Santos and Duarte, 2015). Microplastic fibres are stripped from the clothing items, enter waterways and eventually the marine environment. According to Thompson *et al*. (2004), a single piece of synthetic polymer clothing can release as many as 1900 microplastic particles per washing machine cycle. Considering that the majority of manufactured clothing is composed of synthetic polymer blends (Napper and Thompson, 2016), this can translate to continually increasing inputs of large amounts of microfibres into the marine environment.

2.2. Microplastics in the marine environment

2.2.1. The role of polymer density in microplastic transport

The most commonly produced plastic polymer types include polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinylchloride (PVC), polyamide (PA), polyethylene terephthalate (PET), and polyvinyl alcohol (PVA) (Avio *et al*., 2015). It can therefore be inferred that the majority of microplastic debris found in marine environments will consist of a mosaic of these types of polymers. Once these microplastic particles are released into the marine environment, their fate will largely be determined by their inherently different density properties (**Table 2.1**) as well as retention time in the marine environment (Carr *et al*., 2016; Wang *et al*., 2016; Avio *et al*., 2017). Microplastic density plays a significant role in the transport of these particles as well as settlement and resuspension (Avio *et al*., 2017). Lower density microplastics, such as PP and PE, will often remain in the water column and higher density microplastics, such as PVC and PET will sink and accumulate in the sediment (Dekiff *et al*., 2014; Van Cauwenberghe *et al*., 2015). The density of microplastics in the marine environment may increase with an increase in residence time (Wang *et al*., 2016). The increase in density may be due to the accumulation of proteins and bacterial colonization (biofouling) on the microplastic surfaces, making microplastic particles less hydrophobic and more neutrally buoyant, allowing the particles to remain in the water column for longer periods of time (Lobelle and Cunliffe, 2011). The density changes allow for greater transport distances of the microplastics, and increased availability for ingestion by pelagic organisms (Lobelle and Cunliffe, 2011). Microplastic particles that have settled out of the water column into the sediment may be susceptible to ingestion by benthic organisms and thereafter resuspended into the water column via the production of faeces and pseudofaeces (Wright *et al*., 2013). In addition, settled microplastics are resuspended into the water column via events such as storms (Wegner *et al*., 2012). This benthic-pelagic coupling may result in the repeated exposure of microplastics and their associated toxicants to pelagic organisms (Wegner *et al*., 2012; Canesi *et al.*, 2015).

Table 2.1: Typical plastic polymer densities (g.cm^{-3}) compared to densities of water (g.cm^{-3}) at various salinities (GESAMP, 2015; Avio *et al*., 2017)

Matrix	Density range (g.cm^{-3})
Distilled water	1.000
Brackish water	1.005 – 1.012
Seawater	1.025 – 1.027
Polyethylene (PE)	0.91 – 0.98
Polypropylene (PP)	0.89 – 0.92
Polystyrene (PS)	1.01 – 1.11
Polyvinyl chloride (PVC)	1.16 – 1.45
Polyamide (PA)	1.13 - 1.5
Polyethylene terephthalate (PET)	1.34 - 1.39
Polyvinyl alcohol (PVA)	1.19 - 1.35
Cellulose acetate	1.22 – 1.24

2.2.2. Microplastic pollution effects in marine organisms and ecosystems

Due to their small size, microplastics are an environmental concern as they become available for ingestion to a large number of marine organisms (Van Cauwenberghe and Janssen, 2014) and can potentially be passed along the food web (Setälä *et al*., 2014). Filter feeders and organisms near the bottom of the food chain may be primarily affected by these microplastic particles (Zarfl *et al*., 2011). These organisms have limited selective capacity with regards to food selection and will therefore consume most particulate matter that is of an appropriate size (Wright *et al*., 2013). Previous laboratory experiments have shown that microplastics are ingested by a wide variety of benthic invertebrates, such as lugworms, barnacles, amphipods and mussels (Setälä *et al*., 2014). The uptake of microplastics by organisms is determined by a number of factors including: size, density and shape of the particles (Van Cauwenberghe *et al*., 2015). Microplastics have been shown to have numerous physiological effects on marine organisms, such as gut blockage, false satiation,

decrease of fitness and malnutrition (Luís *et al*., 2015). More worryingly, recent evidence has suggested that microplastics may act as vectors of chemical pollutants being transferred to organisms (Chua *et al*., 2014). Microplastics may contain toxic chemicals which are initially used as additives in the manufacturing process (Luís *et al*., 2015). These include, but are not limited to, polybrominated diphenyl ethers (PBDEs), a component of flame retardants, and phthalates which act as plastic softeners. Moreover, chemical pollutants may adhere and accumulate on the microplastic surface in quantities much greater than those detected in the surrounding environment (Avio *et al*., 2015). The relatively large surface area to volume ratio and hydrophobic nature of microplastic particles facilitates the formation of a biofilm on the microplastic surface, further enabling the adsorption of persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and heavy metals to the microplastic (Rochman *et al*., 2015). Organisms that ingest biofilmed microplastics may be consumed by larger predatory organisms, leading to a potential bioaccumulation of toxicants along the food web (Ivar do Sul and Costa, 2014). Persistent organic pollutants and heavy metals may result in fertility problems, stunted growth and possibly even death in marine invertebrates (Liu *et al*., 2012).

2.2.3. Potential implications for human health

The direct risks posed by microplastics to humans are as a result of the ingestion of microplastics by organisms which are utilized as marine food resources (Santana *et al*., 2016). As the majority of fisheries are located in microplastic 'hotspots' near coastal areas (Mathalon and Hill, 2014), microplastics are becoming an increasing concern for human health (Vandermeersch *et al*., 2015). Microplastics may be a vector for toxicant transfer from the marine environment to humans in concentrations much greater than those detected in the environment (Ziccardi *et al*., 2016). Studies reporting ingestion of microplastics by marine species (Moore, 2008; Van Cauwenberghe and Janssen, 2014) indicate that microplastics are indeed entering the food web, which may be a concern for human health (Van Cauwenberghe and Janssen, 2014). To date, there is limited published literature on *in vitro* and *in vivo* toxicity studies of human ingestion of microplastics (Vandermeersch *et al*., 2015). Not much is known on the physical and toxicological effects of microplastic ingestion in

humans. However, the uptake and translocation of microplastics across the mammalian gut has previously been demonstrated (Carr *et al*., 2012). Additionally, Carr *et al*. (2012) demonstrated that PS microspheres (240 nm) could be taken up in the placenta and cross the placental border in rodents, but this has yet to be shown to occur in humans. The transfer of adsorbed toxicants from microplastics to humans is still to be demonstrated in human trials, but has been shown in various marine invertebrates commonly utilized as a food resource (Batel *et al*., 2016; Ziccardi *et al*., 2016). The transfer of absorbed toxicants to various marine invertebrates poses a risk for the transfer of these toxicants from the flesh of the organisms to humans who ingest them.

2.3. Microplastic pollution in South Africa

A large number of recent publications have highlighted the widespread distribution of microplastic pollution (Nel and Froneman, 2015), with reports of microplastics being found in sediment (Stolte *et al*., 2015; Van Cauwenberghe *et al*., 2015; Alomar *et al*., 2016), in both freshwater and marine systems (Wagner *et al*., 2014; Naidoo *et al*., 2015), as well as within organisms (Van Cauwenberghe *et al*., 2015; Naidoo *et al*., 2016) throughout the globe. It has been reported that the highest concentrations of microplastics can be found in ocean gyres (Lebreton *et al*., 2012) as well as anthropogenically impacted estuaries (Browne *et al*., 2011; Luís *et al*., 2015). In comparison with global investigations, there have been limited investigations regarding the distribution of microplastic pollution in South Africa. The first report of microplastic pollution in South Africa was published by Ryan (1988), who reported microplastic concentrations of 3.64 particles.m^{-3} in the sea-surface waters off the coast of the south-western Cape Province. Ryan and Moloney (1990) reported plastic debris as small as 2 mm in beach sediment on the South African southern and western coastline between 1985 and 1989. An investigation by Lamprecht (2013) found an average microplastic concentration of 30.9 ± 17.2 articles.L^{-1} in beach sediment of Milnerton Beach, Cape Town. More recently, Nel and Froneman (2015) investigated microplastic pollution in both sediments and water surfaces along the south-eastern coastline of South Africa, reporting microplastic particle densities ranging from 688.9 – 3308 particles.m^{-2} in beach sediment and 257.9 – 1215 particles.m^{-3} in sea-surface water samples, respectively. Naidoo *et al*. (2015)

reported microplastic concentrations in areas of Durban Bay, located on the eastern coast of South Africa, as high as 70.3 ± 119.3 particles per 10,000 L in surface water and 159.9 ± 271.2 particles per 500 mL in sediment. These reports regarding microplastic pollution in South Africa are concentrated on the south-western coastlines, with little information of microplastic pollution along the eastern coastlines aside from those by Naidoo *et al*. (2015) and Nel *et al*. (2017).

2.4. Factors affecting microplastic abundances in coastal zones near estuaries

2.4.1. Human populations and anthropogenic activities

Land-based microplastic pollution is transported into the marine environment predominantly by freshwater drainage systems and their associated estuaries (Cheung *et al*., 2016). This phenomenon was highlighted by Rech *et al*. (2014), who found a similarity between plastic litter sampled in the upper courses of a Chilean river system and plastic litter sampled in coastal areas located near the associated estuary mouths. As there is a positive relationship between plastic abundance and human population size (Depledge *et al*., 2013), it can be expected that higher abundances of microplastics will be found in estuaries surrounded by larger populations of people than estuaries surrounded by smaller populations, such as Marine Protected Areas (MPA's) (Alomar *et al*., 2016). However, Zhao *et al*. (2015) argued that this apparent correlation may not be the case, as economic structure may also determine quantities of microplastic pollution in estuarine complexes. Nevertheless, an increase in human population will most probably result in an increase in microplastic pollution in the environment (Rocha-Santos and Duarte, 2015). In addition to human population size and economic structure, microplastic abundances in estuaries will depend on the different activities leaching effluent into each estuary. This was shown in a recent study by Naidoo *et al*. (2015) who found high concentrations of microplastics (159.9 ± 271.2 particles per 500 mL) in the densely populated and highly industrialized area of Durban harbour, KZN, but lower microplastic concentrations (13.7 ± 5.6 particles per 500 mL) in ILovu estuary, KZN, which receives no effluents from industrial activities and is surrounded by a relatively less dense population.

2.4.2. Seasonality

Estuaries can be classified as permanently open, temporarily open/closed, estuarine lake systems, estuarine bays and river mouths (Whitfield, 1992). Temporarily open/closed estuaries (TOCEs) are usually closed in the dry winter seasons and open in the wet summer seasons in KZN (Scharler, 2012). The opening and closing of an estuary's mouth is reliant on the freshwater input from the drainage system, the estuary inlet dimensions as well as sand bar width (Scharler, 2012). As TOCEs are only intermittently open to the marine environment, the effluents derived from these estuaries are only transported into the marine environment in seasonal periods of increased rainfall (wet season). Several studies have shown a positive relationship between periods of rainfall and plastic abundance on beaches as well as in estuaries (Ivar du Sol and Costa, 2013; Cheung *et al*., 2016). As TOCEs are subjected to seasonal variations of freshwater riverine inputs, it is important to seasonally monitor microplastic pollution in the near coastal zone originating from these estuaries, as sampling in any particular season may result in inaccurate reports of microplastic abundances. This seasonal variation of microplastics originating from estuaries was investigated by Cheung *et al*. (2016), who noted significantly greater plastic (and microplastic) abundances in wet seasons on beaches located near the Pearl River Estuary on the western shores of Hong Kong than in dry seasons. Seasonal sampling can allow for identification of potential spatial and temporal patterns of microplastic pollution from estuaries, which eventually could be used to identify major point sources of microplastic pollution into individual estuaries.

2.5. Microplastic pollution in South African estuaries

In South Africa, approximately 71 % of estuaries are temporarily open/closed estuaries (TOCEs), with a far smaller proportion being permanently open to the sea (Scharler, 2012). The eastern province of KwaZulu-Natal (KZN), boasts 73 TOCEs (Begg, 1978), making up the predominant type of estuary in this region. Estuaries in KZN, as in most parts of South Africa, are increasingly under stress as a result of rapid urban development, mismanagement of water resources, increasing levels of effluents and habitat destruction (Department of Water Affairs (DWA), 2013). As estuaries transport nutrients into the near inter-tidal environment, the health of the

marine organisms that reside in these areas can potentially be affected by the effluents and microplastics released by these estuaries. A large social sector on the coast extensively harvests marine food resources in KZN inter-tidal zones (Calvo-Ugarteburu *et al*., 2017). Due to the potential impacts of microplastic ingestion by organisms and transfer to humans (as outlined in Section 2.2.3), it is imperative that the state of microplastic pollution in these areas is investigated. To date, only one report has been published regarding microplastic pollution in KZN, focussing on microplastic pollution in five eThekwini estuaries and their surrounding coastlines (Naidoo *et al*., 2015). As data are limited, levels of microplastic pollution in South African estuaries are currently not used in the determination of estuarine health (DWA, 2013). In South Africa, estuaries can be broadly classified in terms of health into categories (excellent, good, fair, or poor) based on their condition in terms of functionality or viability as well as the degree of anthropogenic disturbances (Whitfield and Baliwe, 2013). The South African Department of Water Affairs (DWA) further categorises the health of an estuary into six Ecological Categories (EC) (**Appendix A: Table A 1**) to determine the Present Ecological State (PES) of the estuary, which is the degree to which the current estuarine conditions differ from 'natural' baseline conditions (DWA, 2013). Estuaries near larger human populations may have a lower health status than those estuaries in more pristine areas. As microplastic pollution is purely an anthropogenically produced problem, it may be assumed that higher microplastic pollution loads will be present in estuaries and associated effluents into the marine environment in areas with a higher human population. The health status of an estuary may potentially be used as an indicator of microplastic pollution.

2.6. Microplastic sampling and quantification methodology

2.6.1. The need for microplastic monitoring protocols

As discussed in Sections 2.2.2 and 2.2.3, microplastic pollution poses a suite of physiological and toxicological threats to marine organisms, ecosystems, as well as potentially to humans. This provides good reasoning for microplastic mitigation procedures to be developed and utilized in areas of high microplastic abundance. However, simply removing microplastic debris from the environment is not a viable

option, due to the unmanageably small sizes and large abundances of these particles (Eerkes-Medrano *et al*., 2015). A more appropriate approach would be to reduce microplastic input into the environment (Eerkes-Medrano *et al*., 2015). This has already been implemented by some global corporate cosmetic companies, such as Colgate-Palmolive (Pty. Ltd), which halted the manufacture of microbeads for use in cosmetic scrubbers in 2014 (Rochman *et al*., 2015). There have also been legal cases made, encouraging the adoption of legal policies to ban the use of microplastics in personal care products sold by all corporate cosmetic companies in California, United States of America (Doughty and Eriksen, 2014). However, this will not decrease microplastic abundance in marine environments, as the longevity of plastic polymers will ensure their persistence for many years (Eerkes-Medrano *et al*., 2015). In addition, fragmentation of larger plastic debris into smaller plastic particles within the marine environment will continue to occur (Avio *et al*., 2017). This continual breakdown of plastic debris into microplastics has been described as ‘legacy inputs’ (Eerkes-Medrano *et al*., 2015). Therefore, it is important to monitor the spatial and temporal patterns of microplastic pollution to determine whether input reduction strategies are in place, and if so, whether they are functional.

2.6.2. Lack of standardization of microplastic monitoring protocols

As marine microplastic pollution is a relatively new field of research, methods of sampling, extraction and enumeration of microplastic abundances are relatively limited and unharmonized throughout the literature (Besley *et al*., 2017). Microplastic abundances have traditionally been investigated in water column, water surfaces and sediments (Santana *et al*., 2016). Due to the large differences in methodology and sampling procedures utilized, microplastic reportings cannot successfully be compared and contrasted between studies (Van Cauwenberghe *et al*., 2015). Microplastic pollution monitoring protocols often result in inaccurate representations of microplastic pollution due to the limitations of sampling equipment (Hildago-Ruz *et al*., 2012; Wesch *et al*., 2016). As a result of the rapidly developing number of sampling and extraction techniques for detecting microplastics in natural environments, and a lack of any form of standard operating procedure (SOP), inconsistencies between methodologies used in sediment sampling and extraction of microplastics become glaringly obvious (Hildago-Ruz *et al*., 2012; Besley *et al*.,

2017). These include, but are not limited to, differences in lower and upper size limits used, extraction technique efficiency and sensitivity, as well as overall differences in sampling techniques utilized (Van Cauwenberghe *et al*., 2015). Due to the enormous quantity of available literature of microplastic pollution detection, it was inherently important that the literature was reviewed and the study methodology designed in such a way as to ensure the comparison of the results of this study to global studies. As the lack of standard microplastic pollution detection protocols is of such importance, an entire chapter has been dedicated to the review of the available methods (Chapter 3).

2.6.3. Influence of abiotic factors

Patterns of microplastic pollution in the water column and sediments are erratic as they are influenced by a number of abiotic factors such as wind, tidal actions, and ocean currents (Santana *et al*., 2016). Microplastics may accumulate in sediment in densities far greater than those in the water column and these values cannot be used to extrapolate microplastic abundances for what is available to organisms for ingestion. The limitations and lack of standardisation throughout previously used methodologies highlight the need for a SOP for microplastic sampling, or the development of a new rapid and accurate procedure of microplastic sampling. Many options have been explored, including the biomonitoring of microplastics using filter-feeding marine invertebrates (Santana *et al*., 2016).

2.7. Pollutant biomonitoring

2.7.1. Marine pollutant biomonitoring

Marine pollution biomonitoring involves the use of biological material and organisms to indicate the presence of pollutants in the marine environment (Anandraj *et al*., 2002; Santana *et al*., 2016). Several marine invertebrate species have already been used as bioindicators for a number of pollutants. These include fish and lugworms (Tao *et al*., 2012) as well as polychaetes, barnacles and bivalves (Amoozadeh *et al*., 2014). Invertebrates are commonly used as bioindicators as they are key components of most marine ecosystems and their health and survival are constantly

threatened by increasing levels of marine pollutants (Tosti and Gallo, 2012). Although the use of fish as biomonitors for pollution has yielded some results, this is not considered to be useful since some fish are highly mobile, and as such, cannot be used to monitor spatial differences in pollution concentrations across different areas (Vermeulen and Wepener, 1999).

2.7.2. Mussels as biomonitors of marine pollutants

Bivalve molluscs, including mussels, have been used worldwide as indicators of multiple marine pollutants for more than 40 years (Degger *et al*., 2011). Mussels are sedentary filter-feeders commonly found on rocky shores near estuary mouths where they are susceptible to greater concentrations of pollution carried into the ocean via rivers that lead into estuaries (Chiarelli and Roccheri, 2014; Dahms *et al*., 2014). Mussels are ecologically important as they provide a variety of microhabitats, niches and resources for other organisms, allowing for the co-habitation of a variety of intertidal species, increasing biodiversity in rocky shore populations (Jungerstam *et al*., 2014). Besides their ecological and economic importance, mussels are considered to be one of the best biological indicators of environmental degradation (Kacar *et al*., 2016). This is due to their filter-feeding strategy, as well their sedentary lifestyle which causes them to accumulate pollutants, such as heavy metals, yet remain resilient to natural fluctuations of environmental conditions (Vosloo *et al*., 2012, Kacar *et al*., 2016). An international Mussel Watch Program (MWP) was originally developed by Goldberg (1975) to monitor the scope of coastal zone pollution, which has since led to the widely recognized and accepted use of mussels as marine pollution monitors by many international organisations (Besada *et al*., 2011). Mussels have been used in biomonitoring of marine environmental quality in South Africa since 1974 (Degger *et al*., 2011, Greenfield *et al*., 2014) due to their wide geographic range, size, sessile behaviour, ease of accessibility as well as economic importance to both commercial and subsistence sectors in South Africa (Resgalla *et al*., 2007, Vosloo *et al*., 2012; Martínez-Gómez *et al*., 2017). The South African National Committee for Oceanographic Research (SANCOR) (reconstituted in 1993 as the South African Network for Coastal and Oceanic Research) initiated a Marine Pollution Research Programme (MPRP) for the South African coastal zone in 1985 (SANCOR, 1985) to provide relevant data and scientific input to management

authorities on pollution loads in the coastal environment, successfully utilising a MWP (Wepener and Degger, 2012; Sparks *et al*., 2014). Monitoring of marine pollutants by MWP's in South Africa almost exclusively focuses on heavy metal pollution, as water and sediment sample analysis for heavy metals is unreliable (Greenfield *et al*., 2014).

2.8. Mussels: potential of marine microplastic pollution monitoring

2.8.1. Effects of microplastics on mussels

As a result of their filter-feeding nature, bivalves are susceptible to the ingestion of tiny microplastic particles (Gerber, 2015). Numerous laboratory-based investigations (Browne *et al*., 2008; Von Moos *et al*., 2012) as well as field investigations (Van Cauwenberghe *et al*., 2015) have shown that mussels are capable of microplastic ingestion. This is a cause for concern, as mussels are not only ecologically important, but are an important resource for both subsistence and commercial harvesting (Richir and Gobert, 2016). Previous investigations have found an average of between 0.2 and 0.5 microplastic particles per gram of mollusc tissue, which translates to approximately 1 microplastic particle per individual (De Witte *et al*., 2014; Van Cauwenberghe and Janssen, 2014). A number of studies have documented the effect of microplastic ingestion on mussel physiology. Gerber (2015) determined that *P. perna* decrease filtration rates of microfibre particles (10 – 100 μm) with an increase in microfibre concentrations from 1 $mg.L^{-1}$ to 5 $mg.L^{-1}$. Conversely, the mussels adapted within 24 hours and increased feeding rates of microfibres with an increase in microfibre concentration. This suggests that mussels may be able to filter greater loads of microplastics in the natural environment. A long-term exposure investigation by Rist *et al*. (2016) found that respiration rates and byssus production of *Perna viridis* exposed to PVC microplastics decreased with an increase in microplastic concentration after 44 days of exposure. After 91 days of exposure, median mussel mortality rates increased when exposed to higher concentrations of PVC microplastic particles. Von Moos *et al*. (2012) concluded that *Mytilus edulis* ingested microplastics of size < 80 μm, that these microplastics trans-located into the cells and tissue of the mussels, and that the ingested microplastics produced significant histological changes in the gut of the mussel.

2.8.2. The use of mussels as biomonitors of microplastic pollution

Most land-derived effluent reaches rocky shore habitats, where populations of mussels are concentrated (Greenfield *et al*., 2014) via rivers and estuaries. Marine mussels on rocky shores can therefore be used to monitor microplastic pollution from effluent derived from nearby estuaries. The use of mussels as biomonitors of microplastic pollution enables the identification of the relevant risks of certain microplastics to the mussels (Santana *et al*., 2016), as well as the potential risk these mussels pose to humans who utilize the mussels as a food source. As mussels' lifespans can reach 10 years, as well as being exposed to microplastic pollution throughout their lifetimes (Rist *et al*., 2016), mussels may accumulate high quantities of microplastics and associated toxicants in their tissues. Thus, human exposure to microplastic pollution via the ingestion of mussels is cause for concern. However, this relatively long life span, combined with the sedentary lifestyle, allows for variations in spatial and temporal microplastic pollution patterns to be identified (Degger *et al*., 2011; Greenfield *et al*., 2014).

Numerous studies have investigated microplastic content in several species of wild mussels (De Witte *et al*., 2014; Mathalon and Hill, 2014; Van Cauwenberghe and Jannsen 2014; Van Cauwenberghe *et al*., 2015). However only one study to date has successfully used *P. perna* as an indicator of microplastic pollution in inter-tidal zones (Santana *et al*., 2016). The mussel *P. perna*, belonging to the family Mytilidae, is indigenous to South Africa (Zardi *et al*., 2006) and dominates the KwaZulu-Natal coast line along inter-tidal zones (Zardi *et al.*, 2006). *Perna perna* typically have quicker growth rates than other mussel species (Oliveira *et al*., 2016) and are harvested from natural populations on the KZN coast by subsistence fishermen throughout the year (Yap *et al*., 2004). The use of *P. perna* as biomonitors of microplastic pollution in coastal environments originating from estuaries in KZN seems promising, offering an attractive alternative to 'traditional' microplastic monitoring procedures.

2.9. Conclusion

The growing body of knowledge on microplastic pollution has highlighted the ubiquity of microplastic pollution throughout the globe. Microplastic pollution is mostly as a result of land-based anthropogenic activity, and is transported to marine environments via freshwater systems and their associated estuaries. While microplastic pollution has become a focus for a large body of research, there are still numerous gaps in knowledge regarding microplastic pollution in areas such as South Africa, as well as the potential effects microplastic pollution may have on global ecology and marine seafood resources. To date, microplastic pollution is difficult to compare between studies as a result of the lack of standardization of methodologies and unit reporting. However, this should not limit the research on microplastic pollution, but should stimulate further research in the field to ensure that microplastic pollution can be adequately reported and potential impacts mitigated. The use of mussels, in particular *P. perna*, as biomonitors of microplastic pollution monitoring offers an attractive alternative to more ‘traditional’ methods of microplastic pollution monitoring and should be further investigated.

CHAPTER 3: METHODOLOGY REVIEW

3.1. Introduction

Microplastic pollution is regarded as a relatively new field of research and as a result there are currently no global standard operating procedures (SOP's) regarding data collection, analyses and unit reporting of microplastic pollution in waters, sediments, and in organisms (Eerkes-Medrano *et al*., 2015; Rocha-Santos and Duarte., 2015; Helm, 2017). Despite the rapid growth in the number of published studies regarding global microplastic pollution distributions, there still remains a lack of consistency between sampling methods for the collection, extraction, identification and enumeration of microplastic pollution from field collected samples (Besley *et al*., 2017). Since the methods selected to sample and analyze microplastic samples have a direct interaction with which microplastics are detected in samples (Joint Group of Experts on Scientific Aspects of Marine Environmental Protection (GESAMP), 2015), the differences in research methods limit the potential of resulting data to be compared and contrasted between studies. The limited comparability potentially prevents any meaningful comparison of microplastic abundances between studies and limits the identification of spatial and temporal microplastic pollution distributions in the marine environment (Eerkes-Medrano *et al*., 2015).

Although some efforts have been made to standardize research methods suitable for microplastic pollution monitoring (Marine Strategy Framework Directive (MSFD), 2013; GESAMP, 2015; United Nations Environment Programme (UNEP), 2016), it has been argued that standardized protocols for microplastic pollution monitoring may not be applicable in every situation (Rochman *et al*., 2017). Each study investigating microplastic pollution may have specific circumstances in which harmonized approaches may not be appropriate. Regional differences in terms of weather, accumulation of plastic and availability of resources may limit the extent to which local study methodologies of microplastic pollution monitoring may mirror global approaches (Lusher *et al*., 2017). However, these differences should not limit the comparisons between studies, provided that the methods used are similar to previously used methods and any differences between methods are described

(UNEP, 2016). For the purposes of the study, an extensive literature review was conducted to investigate and compare recent microplastic sampling procedures for estuarine waters, beach sediment and field collected mussels. The methodology used for this study was derived from the methodology in this chapter to allow the findings of this study to be comparable within the broader literature.

3.2. Research techniques and instruments

3.2.1. Environmental variables

Environmental variables such as wind, tide, and air temperature all play a role in the distribution of microplastics within marine and estuarine environments (Rochman *et al*., 2017). In addition, factors such as water density may affect the microplastics sampled in surface-water sample collection (Carr *et al*., 2016). Season, and associated rainfall, may also influence microplastic abundances within a particular area (Kuo and Huang, 2014). Kuo and Huang (2014) determined that microplastic abundances in a particular area differed between neap and spring tides. Therefore, any cross-sectional study of microplastic pollution should ensure limited variances between these environmental factors by simultaneous sampling of each site. When simultaneous data collection cannot be achieved, the samples should be collected within a short time frame. In any case, these environmental variables should be recorded and reported along with microplastic abundance values to ensure accurate interpretation of microplastic abundances and comparability of information between studies (Qiu *et al*., 2016). In this particular study, sampling was done within a three day period during the summer season, when all estuary mouths were open. All estuaries were sampled at low tide.

3.2.2. Estuarine water surfaces

Rivers and their associate estuaries are considered as major sources of plastic and microplastic pollution into the marine environment (UNEP, 2016). Therefore, an important aspect of microplastic pollution monitoring is the identification of microplastic pollution levels in these entryways. Estuarine water surfaces are often sampled to determine microplastic abundances in a particular estuary due to the

tendency of buoyant microplastics to accumulate at the water's surface (Gago *et al*., 2016). Large volumes of water, in relation to water samples used for standard chemical analysis (1 - 100 L), are often sampled due to the relatively low concentrations of microplastics in water bodies in comparison to microplastic concentrations in sediment (GESAMP, 2015; Löder and Gerdts, 2015). Manta trawl tows are the most commonly used methods for sampling microplastics on water surfaces (Hildago-Ruz *et al*., 2012). The use of manta trawls allow for large volumes of water to be rapidly sampled while retaining a volume-reduced sample (Gago *et al*., 2016). The most commonly used net mesh size is 333 - 335 μm (GESAMP, 2015; Rocha-Santos and Duarte, 2015) and net aperture sizes (mouth opening of net) range between 0.03 - 2 m^2 (Gago *et al*., 2016; UNEP, 2016). The use of a net for microplastic sampling is limited by the net's mesh size and often results in underestimations of microplastic particles smaller than the mesh size. Conversely, the use of smaller mesh sizes may result in net resistance, clogging and potential ripping of the mesh, leading to underestimates of microplastic abundance (MSFD, 2013). In light of the underestimations of microplastic abundances by trawl sample collection, surface grab samples have been used to collect microplastic abundance data in particular water bodies (Barrows *et al*., 2017). Grab sampling involves collection of a sample of water in a vessel and subsequent filtration under vacuum filter. This method allows for the identification of smaller microplastics that are possibly not sampled using manta trawl tows. Barrows *et al*. (2017), comparing the effectiveness of a 0.335 mm neuston net tow and a 1 L surface grab, found that grab samples collected three orders of magnitude more microplastics per volume of water than a neuston net tow. However, the large variances of microplastic abundances between grab samples does not allow for the environmentally relevant microplastic abundances to be reported (Barrows *et al*., 2017). Therefore, manta trawls remain the standard data collection techniques for surface water microplastic pollution sampling. For the purposes of this study, the use of a manta trawl to collect estuarine surface water samples for microplastic analysis was considered to be the most appropriate and repeatable sample collection strategy.

Standard manta trawls are often constructed from aluminium or stainless steel, allowing the manta trawl to be towed for long distances and to be used almost indefinitely (Coyle *et al*., 2016). However, the construction of a standard aluminium or

stainless steel manta trawl is limited by the cost and technical difficulty in construction, and is therefore almost exclusively available to researchers with appropriate funding. Standard manta trawls have also been cited as heavy and difficult to transport (Coyle *et al*., 2016). For the purposes of this study, a manta trawl was constructed largely based on the open source design for the Low-tech Aquatic Debris Instrument (LADI) (Coyle *et al*., 2016).

Figure 3.1: The completed manta trawl, the "Manta-Reg"

The manta trawl (**Figure 3.1**), was constructed with low cost and readily available hardware supplies. The mouth of the manta trawl (mouth size 0.28 m length x 0.32 m width) was constructed using marine plywood, coated with a marine-grade wood sealant. The ‘wings’ were constructed with equal lengths of PVC pipe and sealed with cap ends and PVC weld. The wings allowed the manta trawl mouth to float on the water surface with half of the mouth submerged. The net of the manta trawl was constructed from 300 *µ*m mesh (Dawning Filters) and nylon ripstop fabric. The manta trawl was designed such that the net may be changed with another net. The cod end of the manta trawl is clamped on using standard hose clamps, which can be removed to obtain samples collected by the manta trawl whenever needed.

Once data is collected, microplastic pollution in surface water samples is preferred to be reported as abundance when determining ecological significance (GESAMP, 2015). Microplastic abundance in surface water samples are most commonly reported in number of items per area (m^2) or volume (m^3) sampled (MSFD, 2013; Löder and Gerdts, 2015). However, reporting surface water microplastic abundance as number of items per m^3 may be an inaccurate measurement, as surface water sample collection does not allow for the exact volume of water sampled to be recorded. As such, water surface microplastic pollution may only be accurately reported as number of items per m^2. The reporting of microplastics per unit area of surface water was used in this study to ensure the accurate representation of data collected as the extrapolation of microplastics per unit volume may result in underestimations or overestimations of volume filtered.

3.2.3. Microplastic sampling and extraction from sediment

Techniques of microplastic pollution monitoring have often involved the sampling and analysis of microplastics deposited in benthic sediments which has been successfully applied to beach, estuarine and sea-floor sediments (Solomon and Palanisami, 2016). Microplastic deposition on sandy beaches has previously been used to extrapolate microplastic pollution levels in a particular coastal area (Nel and Froneman, 2015). Microplastic abundances are commonly investigated in sandy beaches due to the ease of accessibility (Van Cauwenberghe *et al*., 2015). Floating microplastics from the ocean are primarily deposited in inter-tidal zones of beaches, most commonly on strand or drift lines (Moreira *et al*., 2016). However, the deposition and distribution of microplastics along beach profiles is not considered to be uniform (Turra *et al.,* 2014). Turra *et al*. (2014) determined that the majority of microplastics in the upper inter-tidal zone are limited to the sediment surface, while microplastics were concentrated in the backshore up to a 2 m depth. However, Besley *et al*. (2017) found no significant distributional patterns of microplastics between inter-tidal zones, high-tide marks, and supralittoral zones. Nonetheless, patterns of microplastic distributions in beach sediment are subject to numerous influencing factors and are therefore considered highly dynamic (Besley *et al*., 2017). The evaluation of microplastics found in the inter-tidal zone would therefore be appropriate to determine the amount of microplastic input from the ocean (Moreira *et al*., 2016) and

not necessarily the amount of accumulated microplastics over time. When an estuary mouth is open, the water flowing from the estuary will mix with the ocean water, often resulting in the pollution of the nearby coastal environments. For this reason, the analysis of sediment samples collected from the inter-tidal zone of sandy beaches near estuary mouths may be useful to determine the input of microplastic pollution from estuaries into the nearby coastal environments.

While sediment has often been sampled for microplastics, techniques of sediment collection are often varied and specific to each study (Hildago-Ruz *et al*., 2012; Besley *et al*., 2017). Beach sediment samples for microplastic analysis have previously been collected using grab samplers (Löder and Gerdts, 2015), such as an Ekman grab. During sample collection, grab-samplers mix sediment layers, thereby preventing the identification of microplastic depositions at specific sediment depths (Löder and Gerdts, 2015). In addition, the mixing of sediment layers collected does not allow for consistency of the quantity of sediment sampled, potentially leading to inaccurate reporting of microplastics within those sediment samples. The problem is easily solved with the use of sediment corers. Sediment corers allow the collection of sediment at specific depths and prevent the mixing of sediment layers (Löder and Gerdts, 2015). Sediment sample depths range from 20 – 100 mm, forming the basis of the recommendations put forward by MSFD (2013) to sample the upper 50 mm of beach sediment, ensuring comparability of results between studies (MSFD, 2013; Besley *et al*., 2017). For the purposes of this study, beach sediment samples for microplastic analysis were collected to a depth of 5 cm from the high tide mark at each site using sediment corers to ensure comparability among previous and future studies.

In order to quantify microplastics in sediment samples microplastics need to be separated from sediment and other biotic particles. Techniques previously used involved floatation, filtration and sieving (Rocha-Santos and Duarte, 2015). Density separation procedures have been shown as an effective method for microplastic quantification in sediments (Claessens *et al*., 2013; Van Cauwenberghe *et al*., 2015). This is based on the inherently different density properties of sediments and microplastics. Sediment density has been approximated at 2.65 $g.cm^{-3}$ (Rocha-Santos and Duarte, 2015) and this density difference can be utilized to separate the

microplastics from sediment. Microplastic extraction from sediments has commonly involved the use of a hypersaturated sodium chloride (NaCl) solution (density 1.2 $g.cm^{-3}$) which allows the lower density microplastics to float out of the higher density sediment. The use of NaCl solution has been highly recommended because of the low cost and eco-friendly nature (MSFD, 2013; Solomon and Palanisami, 2016). The use of NaCl solution in density separation of microplastics from sediments has been successfully demonstrated in a number of investigations (Naidoo *et al*., 2015; Kedzierski *et al*., 2016). However, microplastics with a higher density such as polyvinylchloride (PVC, density 1.14 - 1.56 $g.cm^{-3}$) and polyethylene terephthalate (PET, density 1.32 – 1.41 $g.cm^{-3}$) possibly may not be extracted using this methodology, resulting in underestimations of microplastics present in the sample (Van Cauwenberghe *et al*., 2015). To overcome this potential shortfall Claessens *et al*., (2013) suggested the use of a hypersaturated sodium iodide (NaI) solution (density 1.6 - 1.8 $g.cm^{-3}$). This was demonstrated by Nuelle *et al*. (2014) who obtained higher extraction efficiencies of PVC and PET than the more frequently used NaCl solution. However, the relative cost of NaI is much greater than NaCl, and the higher density of NaI solution may also float interfering substances such as small sediment particles (Qiu *et al*., 2016). Taking the previous recommendations for microplastic extraction from sediment into consideration, this study used a pre-filtered (to remove potential microplastic contaminants) saturated NaCl solution to separate microplastics from sediment to ensure comparability of the study results with previous studies, as well as to minimize financial resources required to carry out this study and any future investigations. Microplastic abundances in sediment have previously been reported as number of items per area (m^2), per volume (L or m^3), or per dry sediment weight (g) (Löder and Gerdts, 2015). In this study, the data collection of microplastic abundances in sediment was reported as number of items per area (m^2) for increased comparability with previous studies (Besley *et al*., 2017).

3.2.4. Microplastic sampling and extraction from mussels

As mussels are ecologically important and a major seafood resource (Li *et al*., 2015), it is important to classify microplastics in mussels in the interest of ecosystem and human health (Vandermeersch *et al*., 2015; Phuong *et al*., 2017). Mussels may also be useful to monitor smaller microplastics which recommended procedures may

underestimate (Lusher *et al*., 2017). Procedures of microplastic extraction and quantification from field collected mussel samples previously described in literature are limited and not harmonized (Claessens *et al*., 2013; Vandermeersch *et al*., 2015) and as such, the comparison between studies are often unsuccessful.

Collection of bivalve molluscs, such as *P. perna,* from study sites is often simply achieved by hand collection (Li *et al*., 2016). Once collected, the individual samples are normally preserved until further analysis (Lusher *et al*., 2017). The fixative used to preserve animals prior to microplastic analysis has always been dependent upon the research questions asked (Lusher *et al*., 2017). Common fixatives, such as 4 % formalin, have been used to preserve animal tissue, however, chemical fixatives have the potential to degrade microplastic particles within biotic samples (Catarino *et al*., 2016). Cyro-preservation (i.e. cold storage) is frequently used to minimize the potential of microplastic degradation within samples (Lusher *et al*., 2017; Phuong *et al*., 2017).

Previously, the presence of microplastics in marine invertebrate tissue was determined by dissection of the animal gut and visual identification (Lusher *et al*., 2017). However, microplastics have been shown to translocate from the gut of mussels to the circulatory system (Browne *et al*., 2008), therefore the extraction analysis of microplastics throughout the organism tissue is required (Phuong *et al*., 2017). To minimize time of visual analysis and potential of misidentification of microplastics, digestion techniques have been developed to eliminate or reduce the quantity of organic matter, whilst ensuring the preservation of microplastics in the samples (Karami *et al*., 2016; Phuong *et al*., 2017). Extraction of microplastics from field collected organisms has previously been achieved by acid/alkaline or enzymatic digestion procedures (Lusher *et al*., 2017), which removes organic tissues, leaving the microplastics behind. Enzymatic digestion of mussel tissues has only been demonstrated in a handful of studies (Cole *et al*., 2014; Catarino *et al*., 2016), with the vast majority of studies relying on chemical digestion procedures (Vandermeersch *et al*., 2015). The extraction of microplastics from field collected samples using chemical digestion often involved strong acids or bases to remove organic matter (Claessens *et al*., 2013). However some plastic particles may be partially degraded or destroyed as a result of the strong acids/bases and high

temperatures used (Catarino *et al*., 2016). This may lead to underestimated microplastic levels within biota (Dehaut *et al*., 2016). Claessens *et al*. (2013) compared the extraction efficiencies as well as polymer degradation of microplastics using different digestion techniques. The most efficient acid digestion technique was using nitric acid (HNO_3) (22.5 M) followed by boiling (± 100 °C) for 2 hours (Claessens *et al*., 2013). However, the use of HNO_3 to digest biological sample tissue has been shown to degrade a number of polymer types (Dehaut *et al*., 2016). More recently, the International Council of the Exploration of the Sea (ICES) released a preliminary protocol for the extraction and identification of microplastic particles in fish guts (Vandermeersch *et al*., 2015). This includes a HNO_3:$HClO_4$ 4:1 v:v mixture as it digests both tissues and other organic matter, such as detritus. A more recent and comprehensive report by Dehaut *et al*. (2016) compared the extraction efficiencies and microplastic degradation using a number of previously reported acidic, alkaline and enzymatic digestion procedures. The use of a 10 % potassium hydroxide (KOH) solution at 60 ºC for 24 hours was recommended to digest mussel tissue due to the high tissue digestion efficiency with no resulting degradation of microplastic particles (Dehaut *et al*., 2016). Similar results were found by Phuong *et al*. (2017), who demonstrated that the use of a 10 % w/w KOH solution resulted in the greatest reduction of mussel tissue with the least microplastic damage. For the purposes of this study, the digestions of mussel tissue with a 10 % w/w KOH solution was chosen to minimize the loss of microplastics and maximize the digestion efficiency of mussel tissue. Once samples have been digested, the resulting liquid is filtered and analysed (Phuong *et al*., 2017). Microplastic abundances within biota is recommended to be reported in reference with wet weight of biota tissue (microplastics.g^{-1} w/w) to avoid invalid extrapolations of this value to relative quantities in the environment (GESAMP, 2015; Phuong *et al*., 2017).

3.2.5. Microplastic identification, enumeration and analysis

To identify quantities and trends of microplastic pollution within the environment, microplastic particles need to be counted and analysed to determine relative abundances, potential sources and potential threats to exposed organisms (Rodríguez-Seijo and Pereira, 2017). Perhaps the most obvious and important step in determining microplastic abundances within samples is to distinguish between

plastic and non-plastic particles. To date, there is no standard procedure for microplastic polymer characterization (GESAMP, 2015). Multiple methods of microplastic characterization have been previously described (Eerkes-Madrano *et al*., 2015) which can be broadly divided into visual identification and chemical analysis techniques. Visual identification of microplastics is considered to be one of the most rapid and technically simple methods (Lusher *et al*., 2017). Visual identification of microplastics is most commonly used to sort microplastics from non-plastics and are thereafter categorized into groups based on morphological differences (**Table 3.1**) which most commonly include size (longest dimension) (Lusher *et al*., 2017), colour, degree of erosion, and type (**Table 3.2**) (Gallagher *et al*., 2016; Gago *et al*., 2016).

Table 3.1: Morphological characterization of microplastics (Hildago-Ruz *et al*., 2012; Naidoo *et al*., 2015; Coyle *et al*., 2016; Gallagher *et al*., 2016; Helm, 2017, Rodríguez-Seijo and Pereira, 2017)

Category	Description
Source	Primary microplastics: raw resin pellets, cosmetic scrubbers
	Secondary microplastics: degradation of larger plastic items
Type/Shape	Fragment (angular, subangular, subrounded, rounded), pellet (cylindrical, ovoid, disk, flat), microbead, fibre, thread, foam, film, other (e.g. cigarette butts, rubber)
Size	< 5 mm in longest dimension
Erosion	Weathering, biofilms, cracking, grooves, ridges
Colour	Wide range, subjective to researcher
General	Irregular, elongated, rough, broken edges

Table 3.2: Description of common microplastic types (Hildago-Ruz *et al*., 2012; Coyle *et al*., 2016; Li *et al*., 2016; Helm, 2017; Rodríguez-Seijo and Pereira, 2017)

Type	Shape description	Potential Source
Fragment	Isolated, often fragmented particle. No definitive shape	Broken off from a larger plastic particle
Pellet	± 4 mm in diameter. Cylindrical, disc or rectangular in shape	Raw materials for plastic injection moulding
Microbead	Very small, almost perfectly spherical. Often brightly coloured	Cosmetic scrubbers, industrial airblasting
Fibre	Slender, elongated	Synthetic fabrics
Thread	Thread-like user plastics	Nylon line, fishing line, packaging straps
Foam	Foamed user plastics	Polystyrene packaging, or polyurethane from construction foam
Film	Thin, sheet-like user plastic	Plastic bags, foils, candy wrappers
Other	Plastic-like, but do not fit into any other category	Cigarette butts, rubber, elastics

The reporting of microplastic sizes and shapes within environments enables the identification of potential microplastic sources and the potential physical and/or chemical harm the microplastics pose to organisms (Rodríguez-Seijo and Pereira, 2017). Within each microplastic shape category, it has been recommended that microplastics be further categorized based on degree of weathering, erosion, shape of fragments as well as colour (Helm, 2017). The more specific categorization of microplastics allows for the identification of the relative abundance of particular microplastics in the environment, as well as the understanding of potential sources of the microplastics (Helm, 2017; Lusher *et al*., 2017). Microplastic colour has previously been used for preliminary identification of microplastic composition

(Rodríguez-Seijo and Pereira, 2017). Visual identification of microplastics, on its own, is open to multiple sources of bias such as the person counting the microplastics, microscope magnification and quality, the sample matrix being examined, and is limited to larger microplastics (Löder and Gerdts, 2015). The MSFD (2013) recommended that visual identification of microplastics be combined with further analytical procedures for microplastic polymer analysis to negate the shortcomings of visual identification alone. Visual identification of microplastics > 500 mm is considered appropriate (Dehaut *et al*., 2016), but subsamples of microplastics smaller than 500 mm are recommended to be further analyzed (Lusher *et al*., 2017).

To further determine the polymer composition of microplastics, a number of chemical analyses have been used to confirm polymer identity (Helm, 2017). Fourier Transform Infrared spectroscopy (FTIR) has commonly been used for the chemical analysis of microplastic particles and is considered the most reliable method of plastic polymer identification to date (Hildago-Ruz *et al*., 2012). The use of FTIR to determine polymer types of microplastics has been widely recommended (MSFD, 2013; GESAMP, 2015; UNEP, 2016). Using FTIR, polymer composition can rapidly be identified by comparing the unique spectral signal of each polymer type to a library of known polymer spectral signals. FTIR has been successfully utilized in a number of investigations (Naidoo *et al*., 2015; Song *et al*., 2015; Vandermeersch *et al*., 2015; Carr *et al*., 2016) as a means for polymer identification in addition to visual identification. This method allows for the composition analysis of particles which visibly identify as microplastic but may in fact not be microplastics. For example, Eriksen *et al*. (2013) found that many particles initially identified as microplastics were actually aluminum silicates which made up approximately 20 % of the 0.355 – 1 mm particle size fraction in samples. However, due to the high cost and level of technicality of FTIR analysis, it is recommended that FTIR only be used when a few samples are to be analysed (Rodríguez-Seijo and Pereira, 2017). An alternative and perhaps the simplest method of microplastic identification is the ‘hot needle test’ or the ‘hot point test’ (Lusher *et al*., 2017). The hot needle test involves a hot needle being placed near a microparticle and observed under a microscope (De Witte *et al*., 2014). If the particle reacts by bending or melting, the particle is classified as a plastic. If the particle does not react, it is considered to be of a non-plastic origin and hence omitted from the results (De Witte *et al*., 2014). While the hot needle test is an

effective and low-tech method to identify microparticles as plastic, the test does not allow for the specific polymer composition of the particle to be identified (Lusher *et al*., 2017). In the context of this study, the 'hot needle test' was determined as the most efficient in terms of time and available resources.

To aid in the identification of microplastics from non-plastic particles in this study, a simplified dichotomous key was developed using previously mentioned recommendations of microplastic visual analysis (**Table 3.3**). Microplastics were distinguished from non-plastics by following guidelines outlined in **Table 3.3.** Once a particle had been identified as plastic, the maximum size dimension of each particle was recorded in *µ*m (Lusher *et al*., 2017), as well as microplastic type, colour, and state of degradation (Coyle *et al*., 2016).

Table 3.3: Guideline for visual identification of microplastics under magnification.

	Visual property	Present?	Reference
1.	Particle < 5 mm	Yes (See 2.) No (Not a microplastic)	Arthur *et al*., (2009)
2.	Cellular or organic structures present (excluding surface biofouling)	Yes (Not a microplastic) No (Fibre – See 3. Other – see 6.)	Norén (2008) Coyle *et al*., (2016) Qiu *et al*., (2016)
3.	Fibres equally thick throughout length	Yes (See 4.) No (Not a microplastic)	Norén (2008) Coyle *et al*., (2016) Qiu *et al*., (2016)
4.	Fibre split/frayed	Yes (See 5.) No (Not a microplastic)	
5.	Particle homogenous in colour	Yes (Microplastic) No (See 6.)	Norén (2008) Qiu *et al*., (2016)
6.	Particle positively reacts to hot needle test	Yes (Microplastic) No (Not a microplastic)	De Witte *et al*., (2014)

3.2.6. Precautions and quality control

In modern times, a multitude of plastic products are used on a daily basis, therefore the use of plastic products is often unavoidable. In microplastic sampling, this translates to the omnipresent possibility of sample contamination with airborne microplastics and clothing (Löder and Gerdts, 2015). As with any investigation, precautions need to be taken to minimize sample contamination. Post-sample contamination of samples with microplastics has previously been shown to be

minimized by using non-plastic equipment such as glass (Nel and Froneman, 2015). Contamination of microplastic samples by airborne microplastics in another recurring issue in microplastic research (Van Cauwenberghe and Janssen, 2014). To avoid airborne microplastic contamination, it has been recommended that laboratory procedures take place in a fume cupboard and all samples covered when not in use (Nel and Froneman, 2015). Clothing made from synthetic materials such as nylon and polyester are commonly worn and as such, may provide a source of microfiber contamination in samples (Woodall *et al*., 2015). As such, it is recommended that personnel involved in laboratory procedures of microplastic investigations wear protective clothing made from 100 % natural materials, such as cotton, to avoid any microfibre contamination of samples. Procedural blanks should be included in investigations to account for contamination by airborne microplastics (Catarino *et al*., 2016).

3.3. Conclusion

There are numerous methodologies available for microplastic pollution monitoring, however, this limits the extent to which microplastic pollution levels can be compared and contrasted among global studies. A number of low cost sample collection and identification methods have been identified in the relevant literature for the use in estuarine, beach and mussel microplastic monitoring in this study. The low cost of the data collection and analysis enables the replication of the methodologies outlined in areas where resources may be limited, allowing microplastic pollution monitoring to be expanded to areas where research may otherwise be hindered. In addition, the methodologies outlined for use in this study enable easy replication for further research of both spatial and temporal trends of microplastic pollution.

CHAPTER 4: A NOVEL MACRO-BASED METHODOLOGY FOR ACCURATE ESTIMATION OF MICROPLASTIC FIBRE UPTAKE IN MUSSELS

4.1. Abstract

Microplastic (< 5 mm) pollution has recently become the focus of a large area of research due to the ubiquity in marine environments and ingestion by marine invertebrates such as mussels. However, the microscopy methods used to count and measure microplastics in samples are time-consuming. The counting and measurement of microplastics within samples is an important aspect of marine microplastic biomonitoring but there is a need to develop a more rapid and repeatable method of visual analysis. This study presents the first step in developing an automated computer macro to count and measure microplastic particles within mussel samples. The aim of this investigation was to determine if the developed automated counting feature could be used as a viable time-saving alternative to manual counting of microplastic fibres (microfibres) ingested by the rocky shore bivalve *Perna perna* (L.) under laboratory conditions. Results showed that mean microfibre counts, lengths, and widths were not statistically different between manual and automated methodologies. The time taken to count microfibres in images was significantly reduced using the automated counting and measurement method (1.00 ± 0.14 minutes) as opposed to the manual counting and measuring method (23.91 ± 7.68 minutes). The findings showed that this novel counting methodology for microfibre uptake in mussels under laboratory conditions is as effective and reliable as manual microscopy, but resulted in significant reductions in microscopy time analysis. Further research is required for the rapid polymer identification of microplastic particles. As research of microplastic pollution is a relatively new area of interest, the application of this novel methodology will reduce the microscopy time necessary for sample analysis, benefitting future assessments of microplastics in environmental samples.

Keywords: microplastics, microscopy, automation, novel methodology, *Perna perna*

4.2. Introduction

Microplastics are any plastic particles smaller than five mm in size in their maximum dimension (Lusher *et al*., 2017) and are primarily manufactured as raw plastic virgin pellets for injection moulding and as scrubbers in cosmetic exfoliants (Ivar do Sul and Costa, 2014; Luís *et al*., 2015). Microplastics are also formed from the breakdown of larger plastic items in the environment via mechanisms such as wave, tidal, chemical, and photo-degradation (Carr *et al*., 2016). One of the most common sources of secondary microplastic particles is the plastic textile fibres from clothes washed in washing machines (Cole *et al*., 2011; Lusher *et al*., 2013). As a result of their small size and ubiquity throughout the marine environment (Van Cauwenberghe *et al*., 2015), microplastics are of ecological concern as they are available for ingestion to a wide variety of marine organisms (Van Cauwenberghe and Janssen, 2014). These ingested microplastics may not only affect the organisms which ingest them, but can potentially be passed along the food web (Farrell and Nelson, 2013; Setälä *et al*., 2014), further affecting a wide variety of organisms.

As a result of their filter-feeding nature, mussels are susceptible to the ingestion of small microplastic particles (Von Moos *et al*., 2012). Numerous laboratory-based investigations have shown that the ingestion of microplastics by mussels has a negative effect on mussel physiology (Browne *et al*., 2008; Gerber, 2015). Organisms in laboratory experiments are usually exposed to microplastic quantities far greater than those found in natural environments (Phuong *et al*., 2016). The higher exposure levels can be used to determine potential uptake rates of microplastics and the associated adsorbed toxicants to the organisms should microplastic pollution levels continue to increase in the natural environment. Studies of wild mussel populations have shown that microplastics are being ingested by mussels in the marine environment (Van Cauwenberghe *et al*., 2015), indicating that microplastics are entering the food web. The ingestion of microplastics by wild mussels is both an ecological and economic concern, as mussels are an important resource for subsistence and commercial sectors (Richir and Gobert, 2016). However, the presence of microplastics in mussels allows for the biomonitoring of microplastic pollution within marine environments (Santana *et al*., 2016). Mussels are regarded as an important biological indicator of marine pollution (Kacar *et al*., 2016) due to their

sedentary lifestyle and filter-feeding strategy, which allows for spatial and temporal patterns of marine pollutants to be identified (Martínez-Gómez *et al*., 2017). Mussels have been used to monitor levels of heavy metal pollution in the South African marine environment for a number of years (Degger *et al*., 2011, Greenfield *et al*., 2014). However, it may be in the interest of ecosystem and human health to classify and monitor microplastics within mussels in addition to heavy metals (Phuong *et al*., 2017). The brown mussel, *Perna perna* (L.), is indigenous to South Africa (Zardi *et al*., 2006) and is often harvested from natural populations along the KwaZulu-Natal coastline by subsistence fishermen (Yap *et al*., 2004). The use of *P. perna* as a biomonitor of microplastic pollution has previously been successful (Santana *et al*., 2016), and offers an attractive alternative to abiotic monitoring of microplastic pollution in the marine environment.

To identify quantities of ingested microplastics by organisms in laboratory and field studies, microplastic particles need to be counted and analysed to determine relative abundances, and potential threats to exposed organisms (Rodríguez-Seijo and Pereira, 2017). Due to a lack of standardised procedures, multiple methods of microplastic characterization in laboratory and field studies have been developed (GESAMP, 2015). Visual characterisation has frequently been used as a rapid and simple method to determine microplastic quantities, sizes, and shapes within samples (Gallagher *et al*., 2016; Lusher *et al*., 2017). The quantification of microscopic particles, such as microplastics, usually involves the use of light, fluorescence or electron microscopy (Lusher *et al*., 2017) to manually count and size each particle by photo enlargement or by counting and assigning particles to a size class on the display (Rodríguez-Seijo and Pereira, 2017). Visual identification of microplastics, on its own, is open to multiple sources of bias, such as the sample matrix being examined, human error, and is limited to larger microplastics (Löder and Gerdts, 2015). Visual counting is not only time-consuming, but researchers may be prone to fatigue (Qiu *et al*., 2016). There is therefore a great need to introduce time-saving, yet cost-effective alternatives to manual counting and sizing of microplastics.

Many freeware image analysis software applications, such as Image J and, DeconvolutionLab, are available allowing for either the automated counting, measurement or sizing of items of interest, but not all at once. The closest freeware

that offers all options of the above is CellC, which was developed by Selinummi *et al*. (2005) and has been used extensively for particle counting (Lehmussola *et al*., 2008; De Vylder *et al*., 2013; Freimann *et al*., 2013). A limitation of CellC software is that it can not be used for calibrating images for accurate dimensional estimates. It should be noted that numerous other freeware image analysis software do exist (Heintzman, 2009) with each program structured around its intended purpose such as axon length mapping, and three dimensional (3D) morphometries, with a few being solely developed for bacterial enumeration and morphological characterization by white light microscopy. In order for the accurate and rapid counting of microplastic particles, the use of commercially available image analysis software Image Pro Plus (IPP) was utilized as it offered the potential for automated counting and measurements of microplastic particles, as well as providing continued technical support. Although this software offers no repeatable automated counting feature, simple macro steps can be programmed in order to make such repetitious functions automated with minimal user input. Originally the macro herein was written specifically to count and size DAPI (4’6-diamidino-2-phenylindole) stained bacterial cells. The use of this macro for counting bacterial cells was therefore adapted for the use in counting and sizing microplastic fibres. The counting and measurement of microplastics within mussels is an important aspect of marine microplastic biomonitoring, however, there is a need to develop a more rapid and repeatable method of visual analysis. This study presents the first step in developing an automated computer macro to count and measure microplastic particles within mussel samples. The aim of this investigation was to determine if the developed automated counting feature could be used as a viable time-saving alternative to manual counting of microplastic fibres ingested by the rocky shore bivalve, *P. perna* under laboratory conditions. Objectives included 1) data comparison between volunteers manually counting and measuring microfibres ingested by mussels using IPP to compare with data collected by using the developed automated counting feature and 2) account for potential time savings between the two methodologies. It was hypothesized that 1) there would not be a significant difference in microfibre counts and measurements between manual and automated methodologies and 2) there would be a significant difference in data capture time between the methodologies.

4.3. Methods and Materials

4.3.1. Microfibre manufacture

To ensure homogeneity of size, shape and polymer composition of microplastics particles to be counted, microplastic fibres were manufactured for the purposes of this investigation. Ultra-violet (UV) fluorescent polyethylene-terephthalate (PET) textile (395 nm) was manually sheared into fine fibres with scissors that had been previously rinsed with deionised water. The fibres were placed in 500 mL of 100 % ethanol and manually agitated until the fibres had separated. The fibre-ethanol suspension was filtered through a 100 μm nylon mesh, and the filtrate was then filtered through a 10 μm nylon mesh using a vacuum manifold. The plastics retained on the 10 μm nylon mesh were collected and dried at 60 °C until constant mass. The plastics (size range approximately 10 – 100 μm) were then weighed according to the quantities needed for each experiment.

4.3.2. Microplastic ingestion experiment

Perna perna specimens (size class 50 – 60 mm) were sourced from the rocky shore habitat at Park Rynie Beach, KwaZulu-Natal, South Africa (30°18'31.4"S 30°44'46.2"E) in October 2015 at low tide (average water temperature ± 24 °C). Mussels were removed from the rocks by shearing the byssal threads with a 30 mm titanium blade. Fouling organisms and debris were removed from the outer shell of the mussels using a similar titanium blade, scrubbing brush and distilled water. Mussels were contained in 50 L recirculating artificial saltwater (Red Sea Salt® with distilled water) at a salinity of 30 psu and constant temperature (24 °C) (Srisunont and Babel, 2015) for 48 hours to allow for depuration. During the 48 hour period, mussels were initially fed ¼ teaspoon PhytoPlan® Advanced Plankton Diet and then again after 24 hours to prevent starvation and to ensure that the guts were clear of any microplastic particles prior to the experiment (Van Cauwenberghe and Janssen 2014). Ten mussels were individually distributed among ten buckets each containing five L of recirculating and aerated artificial seawater (24 °C and 30 psu). Five mg [1 $mg.L^{-1}$] of previously manufactured UV fluorescent microplastic fibres were dispensed into each bucket and the mussels were allowed to feed for 24 hours. It has been

noted that laboratory procedures often use microplastic concentrations several thousand times greater than concentrations reported in field studies (Claessens *et al*., 2013; Phuong *et al*., 2016). This is partly due to the difficulty of accurately replicating the minute concentrations of microplastics found in natural environments, and allows for the control of variables that cannot be controlled for in field investigations. Mussels were removed and thoroughly rinsed with distilled water. Each mussel was then placed into 50 mL of distilled water and boiled for 15 minutes at 100 °C to allow for easier dissection of the gut tissue. The gut of each mussel was carefully removed and alkaline digested according to methodology adapted from Dehaut *et al*., (2016). Each gut was placed in jars containing 250 mL of 10 % (w/w) potassium hydroxide (KOH). The jars were sealed and placed in an oven at 60 °C for 24 hours. Thereafter, samples were diluted to one L using distilled water and vacuum filtered through a 5.0 *µ*m polycarbonate track-etched ISOPORE™ membrane filter (47 mm ⌀) (Merck Millipore Ltd.). The filters were dried at a constant temperature of 40 °C for 24 hours.

4.3.3. Microscopy and image analysis

The filters were viewed under UV illumination with a Nikon Eclipse 80i epifluorescent microscope with external UV lamp using a 2X objective lens with the neutral density filter (NDF) set at 4. The filter set comprised a UV – 2B filter with an excitation wavelength of 330 – 380 nm, dichromic mirror of 400 nm and barrier filter of 420 nm. The software program NIS Elements D (NIKON) was used for image capture, using a Nikon Digital Sight DS–F1i digital camera for each sample. Image acquisition parameters within NIS Elements D were adjusted to give the best image quality due to the extreme distance between the sample and the objective lens when using fluorescence. Ten fields of view were digitally captured within the working filtered area with each captured field being 2.90 x 10^7 μm^2 at 20X magnification. Ten captured images covered approximately 94.77 % of the filter, allowing for a high degree of accuracy while avoiding the potential of overlapping images. When required, due to an inconsistent X – plane, multiple images of one field of view were captured to be digitally Z stacked for improved quality and focus. Images to be stacked were run through Image Pro Plus v.6.2 (IPP) with a specifically scripted macro enabled Extended Depth of Field (EDF) for this study.

Stacked images were then run through another specifically scripted macro in IPP for data acquisition. Briefly, each sequential image was low level gray-scaled then calibrated to a pre–set calibrated scale bar. Following this the image was automatically assessed by a pre–selected histogram segmentation threshold that was set to 27:255 for optimal data collection. This setting was previously determined to give the best overall data acquisition based on image brightness and pixilation around the microfibres. Data were then exported to a Microsoft Excel® data sheet for further analysis. Data gathered included, but was not limited to, area, diameter, length, width, ferret min, ferret max and the perimeter length of each microfibre. Microfibres that were touching any edge of the captured image were not counted as it could not be determined what proportion of the microfibre was outside of the image. Macros were written using MS Excel® VBA (Visual Basic for Applications) to automatically analyze and calculate the data within the Excel file captured from IPP. To test the efficiency, accuracy and reproducibility of the above automated counting feature, 50 microfibre images were given to five volunteers to count and size. For ease of manual data gathering, only the width and best arc length were counted. This data was compared to data collected from the automated counting feature for accuracy of counts, measurements and time taken for analysis.

4.3.4. Calculations and statistical analyses

Calculation and extrapolation of microfibre counts per mussel (microfibres.mussel^{-1}) for both manual and automated counting methodologies was calculated using the following equation:

$$N = A_F \left(\frac{n}{A_I \times I} \right)$$

Where N = final estimated microfibre counts per mussel (microfibres.mussel^{-1}), A_F = working surface area of the filter used (3.06 x 10^8 μm^2), n = total microfibres counted in I number of images, I = number of images per filter (10), A_I = total area of field of view (FOV) for one image (2.90 x 10^7 μm^2).

All statistical analyses were done using IBM SPPS Statistics (version 23 for Microsoft® Windows® 10). Statistical significance was set at α = 0.05 for all statistical tests. Mean microfibre counts per mussel (microfibres.mussel^{-1}) between manual counting and automated counting were compared using a Paired samples *t* test after assumptions of normally distributed data were met (One-sample Kolmogorov-Smirnov test = 0.224, p = 0.20). Mean microfibre length (*μ*m) was compared between manual and automated counting methodologies using a Paired samples *t* test after assumptions of normally distributed data were met (One-sample Kolmogorov-Smirnov test = 0.233, p = 0.20). Mean microfibre width (*μ*m) was compared between manual and automated counting methodologies using a Paired Samples *t* test after assumptions of normally distributed data (One-sample Kolmogorov-Smirnov test = 0.23, *p* = 0.20) was met. Mean time (minutes) taken to count and measure microfibres in images was compared between manual and automated counting methodologies using a Paired samples *t* test after assumptions of normally distributed data were met (One-sample Kolmogorov-Smirnov test = 0.320, p = 0.103)

4.4. Results

There was no significant difference in the number of microfibres.mussel^{-1} between manual (85.32 ± 42.61 microfibres.mussel^{-1}) and automated (83.36 ± 43.33 microfibres.mussel^{-1}) counting methodologies (Paired samples *t* test: *t* = 0.71, *p* = 0.52) (**Figure 4.1a**). There was no significant difference in counted microfibre length (*μ*m) within each mussel between manual (338.61 ± 81.48 *μ*m) and automated (370.56 ± 79.51 *μ*m) counting methodologies (Paired samples *t* test: *t* = -2.48, *p* = 0.069) (n = 5) (**Figure 4.1b**). There was no significant difference in counted microfibre widths (*μ*m) within each mussel between manual (23.23 ± 1.45 *μ*m) and automated (23.03 ± 1.82 *μ*m) counting methodologies (Paired samples *t* test: *t* = 1.16, *p* = 0.31) (**Figure 4.1c**). Mean time taken (minutes) to count and measure the number of microfibres.mussel^{-1} was significantly different between manual and automated counting methodologies (Paired samples *t* test: *t* = 6.66, *p* = 0.003). Mean time taken (minutes) to count and measure the number of microfibres.mussel^{-1} was significantly less using the automated methodology (1.00 ± 0.14 minutes) than the manual methodology (23.91 ± 7.68 minutes) (**Figure 4.1d**).

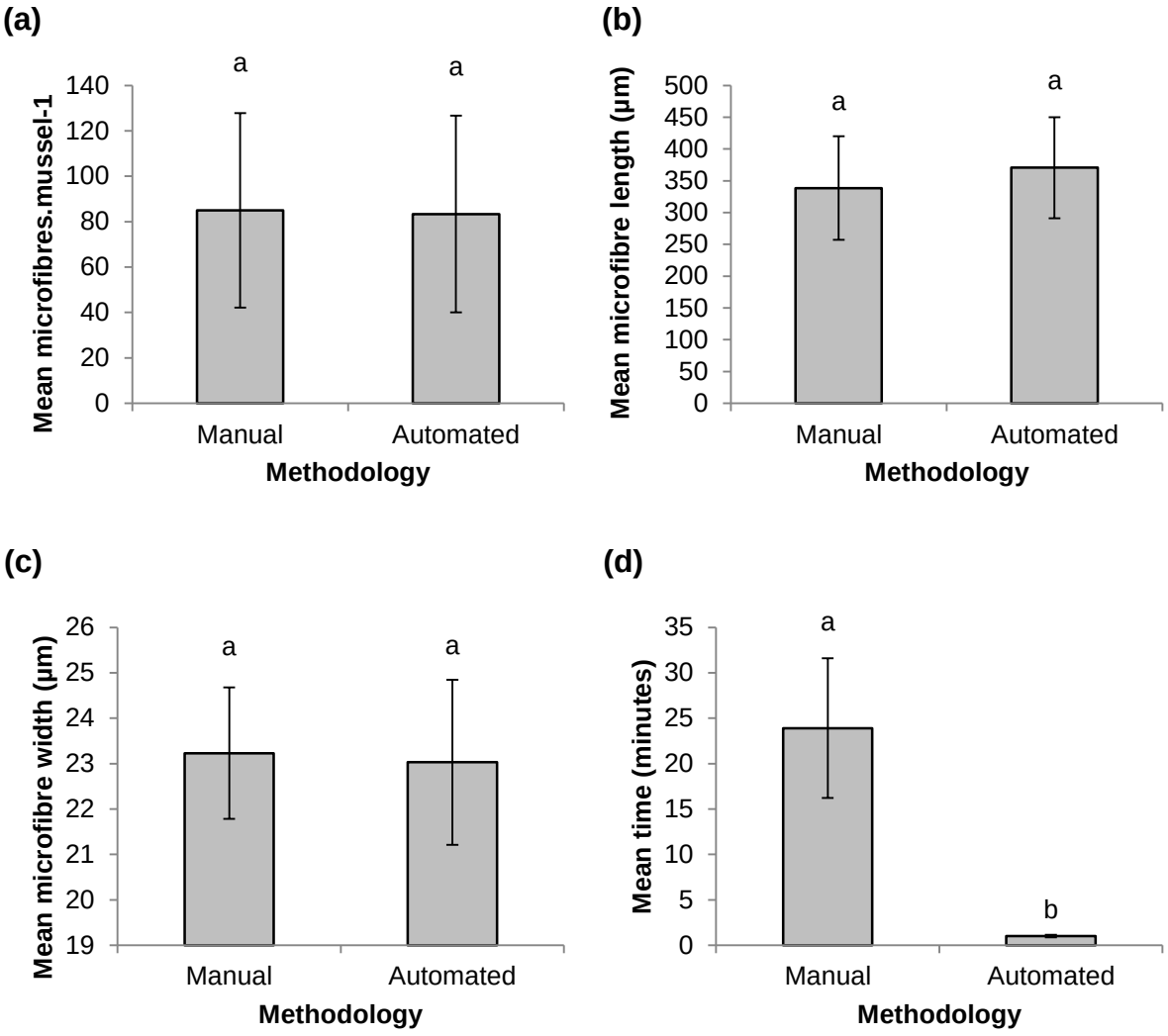

Figure 4.1: Comparisons of mean microfibre counts (microfibres.mussel^{-1}) (a), mean microfibre length (*µ*m) (b), mean microfibre width (*µ*m) (c), and mean time taken (seconds) to count images for one mussel sample (d) between manual and automated counting methodologies (n = 5 volunteers). Lowercase letters indicate significant differences between data ($p < 0.05$). Error bars represent ± 1 standard deviation (SD).

4.5. Discussion

The results show that there was no overall difference in microfibre counts and measurements between manual and automated counting methodologies (**Figure 4.1**), therefore the hypothesis stating there will be no significant difference in microfibre counts and measurements between manual and automated counting methodologies was accepted. However the average time taken time taken (minutes) to count and measure the number of microfibres.mussel^{-1} was significantly quicker using the automated methodology than the manual methodology (**Figure 4.1d**). As a result, the hypothesis stating there would be a significant difference in data capture time between the methodologies was accepted. This data shows that the innovative automatic counting feature developed can be used as a time-saving alternative to manual counts of microfibres. There was also significantly less variation in time taken to count and measure microfibres using the automated counting method than the manual counting (**Figure 4.1d**), which indicates that the automated counting methodology is far more reliable in data acquisition across all samples as compared to manual counting. In addition to significantly reducing data capturing times, automated counting allows for the removal of bias due to human error and fatigue (Qiu *et al*., 2016).

Although there was no significant difference in microplastic counts between automated counting and manual counting by human volunteers (**Figure 4.1a**), the automated counting could not distinguish between particles of interest (in this case, UV-fluorescent microfibres) and any other potentially interfering material (**Figure 4.2**). **Figure 4.2** shows microfibre particles of interest in this study (A), and cotton fibres (B) that had been intentionally placed in samples, all of which fluoresce under UV illumination. The lack of distinction between particles of interest and debris by the automated counting method may be attributed to the pre-grayscaling of the images, whereas with the manual counting all volunteers were supplied colour images. However, this lack of distinction may also be reflected in manual counting, where volunteers with pre-existing physiological conditions (i.e. colour-blindness) may not be able to differentiate between microplastic particles and other particles that may fluoresce under UV illumination. These results indicate that regardless of manual or automated methods, visual identification of microplastics and other debris may need

to be supplemented with chemical analysis (Helm, 2017) to confirm particle composition. The use of chemical analysis to identify microplastic polymer type has been widely successful (Helm, 2017), with many studies using Fourier Transform Infrared (FTIR) spectroscopy and Raman microspectroscopy (Hildago-Ruz *et al*., 2012; MSFD, 2013; GESAMP, 2015; Naidoo *et al*., 2015; Carr *et al*., 2016; UNEP, 2016). However, FTIR and Raman microspectroscopy demand a relatively high level of technical skill and financial resources (Helm, 2017; Maes *et al*., 2017). It has been recommended that these analyses be used only for small sample sizes or for sub-samples of data (Rodríguez-Seijo and Pereira, 2017). Automation procedures of infrared (IR) microscopy to identify microplastics have been developed (Tagg *et al*., 2015; Löder and Gerdts, 2015), but these methods are not recommended for monitoring due to slow speed, high financial cost and poor spectral signals (Maes *et al*., 2017).

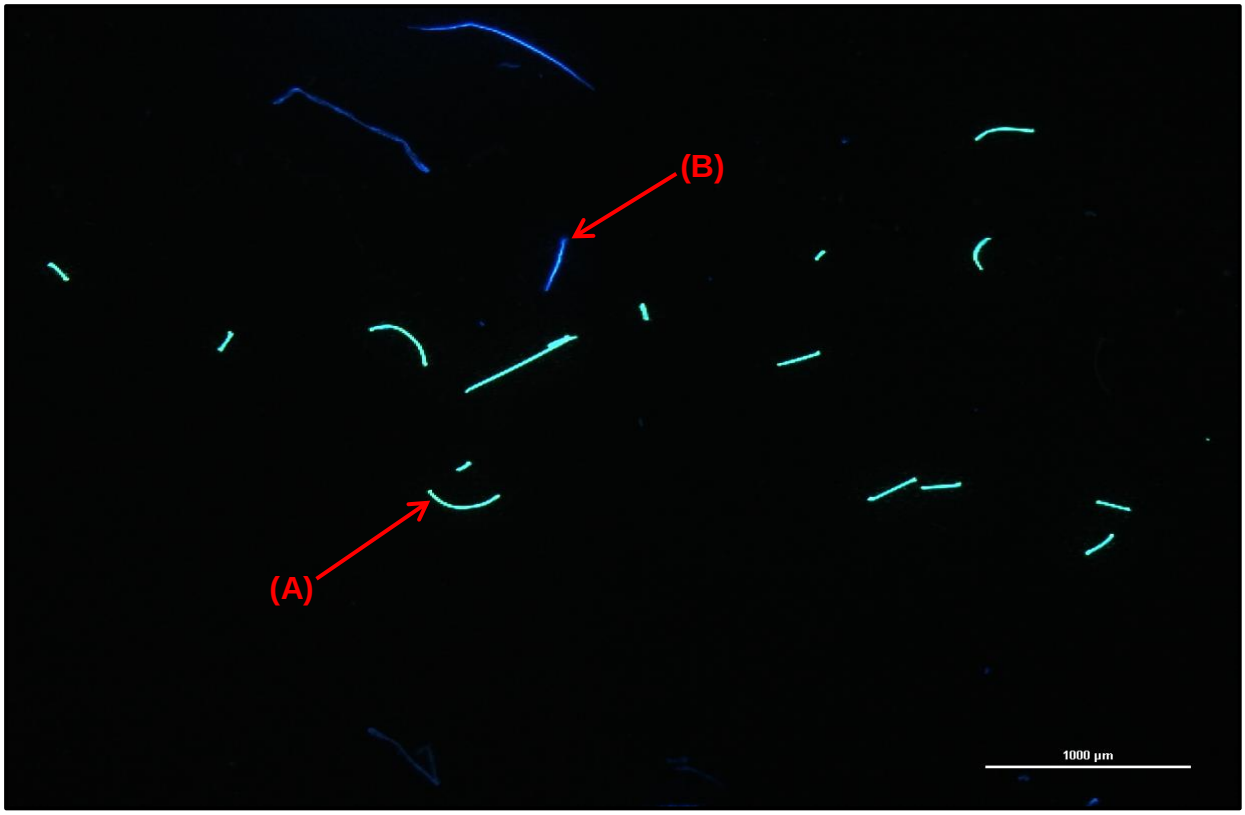

Figure 4.2: Micrograph showing plastic microfibre (A) and non-plastic cotton fibre (B) under ultra-violet (UV) illumination at 20X magnification. Scale bar represents 1000 *µ*m.

A simple and effective procedure for distinguishing between microplastic particles and other particles of non-plastic origin was developed by Maes *et al*. (2017), using *in situ* fluorescent tagging of microplastics with the lipophilic dye Nile Red (NR). The fluorescent tagging of microplastics with NR allows microplastic particles to be distinguished from non-plastic particles under blue light (450 – 510 nm) and orange filter (529 nm) (**Figure 4.3**). Although the automated macro used in **Chapter 4** is currently not useful for the characterization of plastic particles from non-plastic debris, the adoption of fluorescent tagging of samples with NR, together with the automated counting methodology, may aid in the rapid assessment of microplastics from both laboratory and field studies. Microfibres used in this investigation were all of consistent shape and colour, however the automated counting feature could be capable of counting and measuring many varieties of microplastics.

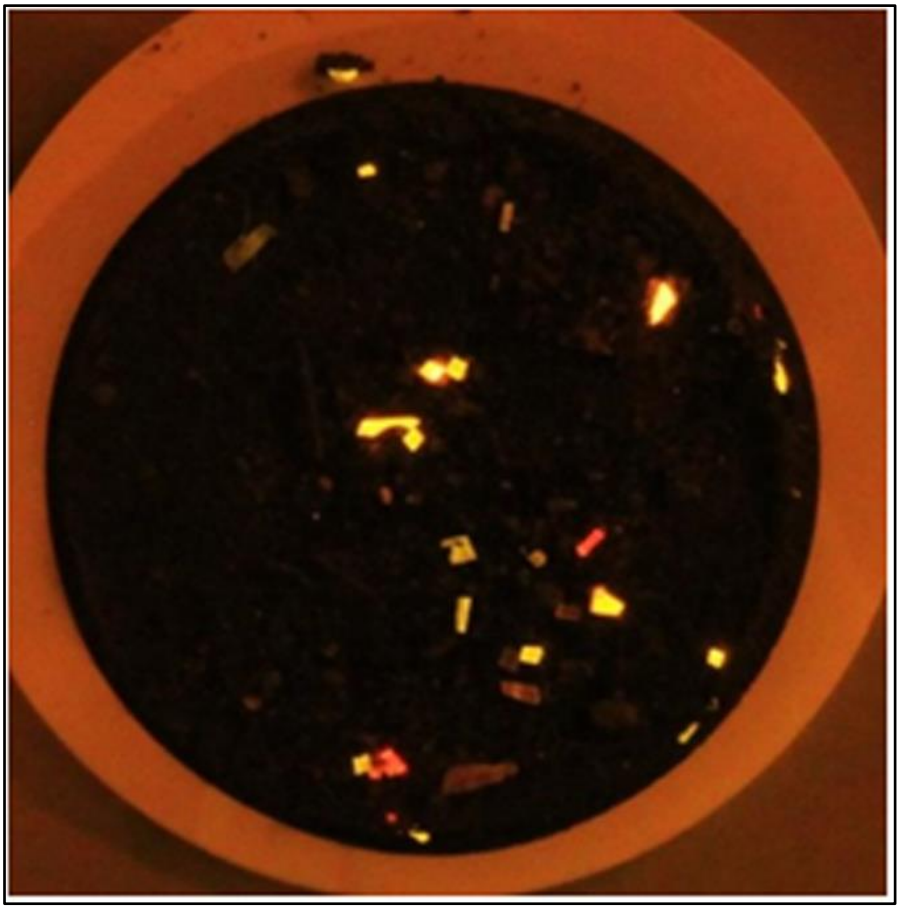

Figure 4.3: Marine sediment spiked with microplastics of six different polymer types, dyed with Nile Red, and filtered on to a Whatman GF/F filter. Photo taken with blue light (450 – 510 nm) and orange filter (529 nm) (Maes *et al*., 2017).

4.6. Conclusion

The novel automated methodology developed for the purposes of this investigation resulted in significant saving of time with regard to microplastic counting. However, further research is required regarding the identification of plastic and non-plastic particles within samples. Automated features for the counting and measurement of microfibres may be used in a wide variety of applications, including the use of rapid bioassessement of microplastics ingested by mussels. The use of the automated counting feature not only saves time, but reduces human error and fatigue and allows for minimal training on the software thus enabling more samples to be processed within a shorter period of time. In combination with fluorescent tagging of microplastics, the automated method of counting will produce reliable estimates of microplastic abundances within samples in future assessments of microplastic pollution.

CHAPTER 5: MICROPLASTIC POLLUTION DISTRIBUTION IN SELECTED KWAZULU-NATAL TEMPORARILY OPEN/CLOSED ESTUARIES DURING AN OPEN MOUTH PHASE.

5.1. Abstract

Microplastic (< 5 mm) pollution has recently become the focus of a large body of research due to its ubiquity and negative effects on organisms that ingest microplastics. Global research trends are comparatively more focused on marine microplastic pollution as opposed to freshwater microplastic pollution. As rivers and their associated estuaries are considered to be major conduits of microplastic pollution to the marine environment, it is important to identify microplastic pollution levels in these estuarine entryways. To date, there are very few studies investigating microplastic pollution in KwaZulu-Natal estuaries. This investigation aimed to identify and compare microplastic pollution in three KwaZulu-Natal estuaries during an open mouth phase. Results showed that Bilanhlolo Estuary had significantly greater levels of microplastic pollution in sediment ($4.22 \times 10^4 \pm 2.17 \times 10^3$ microplastics.m^{-2}) and surface water (5.98 ± 0.46 microplastics.m^{-2}) as compared to Mhlangeni Estuary (sediment: $1.33 \times 10^4 \pm 1.52 \times 10^3$ microplastics.m^{-2}; surface water: 4.50 ± 0.59 microplastics.m^{-2}) and Kongweni Estuary (sediment: $1.89 \times 10^4 \pm 2.31 \times 10^3$ microplastics.m^{-2}; surface water: 2.34 ± 0.23 microplastics.m^{-2}). Microplastic fibres were the most dominant microplastic type in all studied systems (60.07 %) and smaller microplastics were more abundant than larger microplastics in all studied systems. This investigation is the first of its kind to investigate microplastic pollution in these three estuaries and may be used as a baseline survey for future research of South African microplastic pollution.

Keywords: microplastics, temporarily open/closed estuaries, open mouth phase, South Africa, baseline

5.2. Introduction

Microplastics are plastic particles smaller than 5 mm in their longest dimension (Lusher *et al*., 2017) and are classified as primary or secondary based on their origin (Cole *et al*., 2011). Primary microplastics are manufactured to be of a small size, such as cosmetic scrubbers (Thompson *et al*., 2004), and secondary microplastics result from the disintegration of larger plastic items via physical, chemical, and biological degradation (Barnes *et al*., 2009). Another common source of secondary microplastics is the microfibres which are produced as a result of washing synthetic garments (Cole *et al.*, 2011; Lusher *et al.*, 2013). The inner drums of washing machines act as a 'grater' and sheer off minute synthetic fibres (Lusher *et al.*, 2013). These microplastics, along with primary microplastics, go directly from domestic sources to municipal wastewater treatment works (WWTW). Microplastics are a cause for concern as they may become available for ingestion by a number of organisms (Van Cauwenberghe and Janssen, 2014). The ingestion of microplastics have been shown to have numerous negative physiological effects on organism (Luís *et al*., 2015), in addition to acting as vectors of toxicants to organisms (Chua *et al*., 2014).

Recent publications have highlighted the widespread distribution of microplastics in sediment (Alomar *et al*., 2016), freshwater and marine environments (Wagner *et al*., 2014; Naidoo *et al*., 2015), as well as within organisms (Van Cauwenberghe *et al*., 2015; Naidoo *et al*., 2016). Global research trends of microplastic pollution are noticeably more focused on marine environments (Eerkes-Medrano *et al*., 2015) and this has led to numerous clean-up projects and plastic-collection devices within marine environments (Mahon *et al*., 2017). Despite approximately 80 % of plastic and microplastic pollution in the marine environment being derived from terrestrial sources (Andrady, 2011), there are still enormous knowledge gaps regarding the impacts on ecological and human health of freshwater microplastic pollution and consequential transport to marine environments (Eerkes-Medrano *et al*., 2015; Cheung *et al*., 2016). Rivers and their associated estuaries are considered as major sources of plastic and microplastic pollution into the marine environment (United Nations Environmental Program) (UNEP), 2016). This phenomenon was highlighted by Rech *et al*. (2014), who found a similarity between plastic litter sampled in the

upper courses of a Chilean river system and plastic litter sampled in coastal areas located near the associated estuary mouths. Therefore, an important aspect of microplastic pollution monitoring is the identification of microplastic pollution levels in these estuarine entryways. The lack of microplastic pollution research in freshwater systems is mirrored within the South African context, as the majority of investigations of microplastic pollution in South Africa focus on ocean and coastal microplastic pollution (Ryan, 1988; Ryan and Moloney, 1990; Lamprecht, 2013; Nel and Froneman, 2015). Only two South African studies to date have investigated microplastic pollution in freshwater systems (Naidoo *et al*., 2015; Nel *et al*., 2018).

Temporarily open/closed estuaries (TOCEs) are a type of estuary characterized by the opening of the estuary mouth during periods of increased rainfall, and the closure of the estuary mouth during periods of decreased rainfall (Scharler, 2012). As estuaries are intermittently open to the marine environment, effluents derived from these estuaries are only transported in to the marine environment during periods of mouth opening. 71 % of South African estuaries are classified as TOCEs (Scharler, 2012). In KwaZulu-Natal (KZN), there are 73 TOCEs, which makes up the predominant type of estuary in this region (Begg, 1978). To date, there is only one published study regarding microplastic pollution in KZN estuaries (Naidoo *et al*., 2015), focussing on microplastic pollution in selected eThekwini estuaries and their surrounding coastlines (Naidoo *et al*., 2015). Due to the relatively new understanding of microplastic pollution and a lack of data, levels of microplastic pollution are not used in the determination of estuarine health status in South Africa (DWA, 2013). The South African Department of Water Affairs categorises estuaries based on their health status in to six Ecological Categories (EC). The EC is then used to determine the Present Ecological State (PES) of the estuary, which is the degree of which the current estuarine conditions differ from ‘natural’ or baseline status (DWA, 2013) (**Appendix A: Table A 1**). As microplastic pollution is purely an anthropogenically produced problem, it can be assumed that more microplastic pollution will be present in estuaries and the associated effluent into the marine environment in area with a higher human population (Rocha-Santos and Duarte, 2015). However, Zhao *et al*. (2015) argued that population demographics surrounding an estuary are not the primary causation of microplastic pollution within the estuaries, as economic structure may also determine quantities of microplastic pollution in estuarine complexes. In

addition to human population size and economic structure, microplastic abundances in estuaries will depend on the different activities leaching effluent into each estuary. Therefore, the PES of an estuary may be a more accurate predictor of microplastic pollution levels in estuaries than surrounding population sizes. Due to the potential impacts of microplastic ingestion by organisms (Chua *et al*., 2014) and transfer to humans (Ziccardi *et al*., 2016), it is imperative that that state of microplastic pollution in these areas is investigated.

The aim of this investigation was to determine microplastic pollution status in three temporarily open/closed estuaries in KwaZulu-Natal during an open mouth phase. The three estuaries, Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary, all fall within the Ugu District Municipality, KwaZulu-Natal and experience similar climate and weather due to their close geographic proximity. As microplastic pollution in these estuaries has not been previously investigated, this investigation serves as a baseline survey of microplastic pollution in these areas. Objectives of the study were to compare 1) microplastic abundance in surface water and sediment among estuaries, 2) compare microplastic type composition in surface water and sediment among estuaries, and 3) compare microplastic size class distribution in surface water and sediment among estuaries. As a descriptive analysis, the quantity of microplastic pollution in each estuary was compared to the PES of each estuary It was hypothesized that there would be a difference in microplastic abundance, type and sizes in surface water and sediment between the three estuaries.

5.3. Methods and Materials

5.3.1. Data collection and processing

All data was collected from the selected estuaries (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) (**Chapter 1: Figure 1.1**) over a three day period in January 2017. Samples were collected at low tide during estuarine open mouth phases. Estuarine surface water samples from each estuary mouth were collected using a manta trawl (mouth size 0.28 m length x 0.32 m width; mesh size 300 μm). The mouth of the manta trawl floated on the water surface so that approximately half of the mouth was submerged. The manta trawl was towed for 50 m perpendicularly

across each estuary mouth at ebb tide (Frère *et al*., 2017) for five replicate trawls per estuary. Samples were taken only from the mouth of each estuary because this water is most likely to enter the ocean at the time. An analysis of the upper reaches of the estuaries would be redundant for the purposes of this study. Samples were washed into the cod-end of the manta trawl. The cod-end was removed and contents washed through a 5 mm sieve and then into a 300 *µ*m sieve. The sample contents retained on the 300 *µ*m sieve were carefully transferred into previously acid-rinsed polyethylene jars and sealed. Sample bottles were stored in a freezer at -20 ºC until laboratory processing. Prior to laboratory processing, surface water samples were removed from the freezer and completely thawed at room temperature (± 25 °C). Surface water samples were vacuum-filtered on to 5.0 *µ*m polycarbonate track-etched ISOPORE™ membrane filters (47 mm ⌀) (Merck Millipore Ltd.). Each filter was placed in to a new, clean plastic petri dish, loosely covered with aluminium foil and dried in the oven at 60 ºC for 24 hours. Petri dishes were removed from the oven and covered with aluminium foil until further microscope analysis.

Sediment samples for microplastic analysis were collected at two stations (North and South) within each estuary mouth. At each station, using a plastic corer (45 mm internal ⌀), five replicate sediment core samples of the top 5 cm of sediment in were taken at the hightide mark at a minimum of 1 metre apart as per recommendations (see **Chapter 3**, Hildago-Ruz *et al*., 2012; MSFD, 2013; Naidoo *et al*., 2015; Besley *et al*., 2017). Each sample was collected and sieved through a 5 mm stainless steel sieve and placed into previously acid-washed 300 mL bottles and sealed. Personnel collected samples standing downwind of each sample site, to minimize airborne microplastic contamination of collected samples (MFSD, 2013). Sediment samples were stored in a freezer at -20 °C until laboratory processing. Prior to laboratory processing, sediment samples were removed from the freezer and completely thawed at room temperature (± 25 °C). Sediment samples were decanted into aluminium ‘boats’ and covered with additional aluminium foil. Samples were placed into an oven and dried at 60 ºC for 48 hours until constant mass (Naidoo *et al*., 2015). Density separation was used to separate microplastics from sediment. A fully saturated sodium chloride (NaCl) solution was prepared by adding 360 g of commercially available iodated table salt (First Value®) to a beaker containing 1 L of distilled water and a magnetic stirrer bead. The beaker opening was covered with

aluminium foil and placed on a magnetic stirrer. The mixture was mixed using a magnetic stirrer at high speed at room temperature (25 ºC) for ten minutes. Thereafter, the mixture was allowed to stand for a further 10 minutes. As microplastics have been found in commercially available table salt (Yang *et al*., 2015; Karami *et al*., 2017), the NaCl solution was vacuum-filtered on to a Whatman® borosilicate glass microfibre filter (47 mm ⌀, 0.7 *µ*m pore size) (Sigma-Aldrich©). The supernatant was collected and the procedure was repeated until the appropriate quantity of saturated NaCl solution had been obtained. Each dried sediment sample was weighed to the nearest 0.001 g and thereafter mixed with 200 mL of saturated NaCl solution with a glass rod in a 250 mL beaker (both previously rinsed with deionized water) for approximately 2 minutes. The sediment-salt mixture was allowed to stand for a minimum of one hour until the sediment had visibly settled, thereafter the supernatant was carefully poured into the vacuum filtration receiver as to exclude larger sediment particles. The supernatant was filtered through a 5.0 *µ*m polycarbonate track-etched ISOPORE™ membrane filter (47 mm ⌀) (Merck Millipore Ltd.). The sediment sample was replenished with a further 200 mL of filtered NaCl solution and the extraction procedure was repeated. As per recommendations by MSFD (2013) and Besley *et al*., (2017), the extraction procedure was repeated three times per sediment sample. Each filter was placed in a clean plastic petri dish and covered with aluminium foil. The samples were dried in an oven at 60 ºC for 24 hours and thereafter stored (still covered) at room temperature until microscope analysis.

To reduce the possibility of airborne microplastic contamination during laboratory processing, all samples were covered with aluminium foil when not in use (Nel and Froneman, 2015; Catarino *et al*., 2016). Where possible, non-plastic equipment was used instead of plastic to reduce the possibility of sample contamination (MSFD, 2013; Nel and Froneman, 2015; Lusher *et al*., 2017). To reduce cross-contamination of samples with synthetic fibres from clothes, personnel wore clean laboratory coats at all times (MFSD, 2013; Catarino *et al*., 2016; Frère *et al*., 2017). Five blank samples for both surface water and sediment samples were included to quantify for any microplastic contamination during laboratory processing and analyses.

5.3.2. Microplastic analyses

Filtered samples were viewed up to 40 X magnification using a Nikon© AZ100 stereomicroscope. Microplastics were distinguished from non-plastics by following guidelines outlined in **Table 3.3** (**Chapter 3**). Once a particle had been identified as plastic, the maximum size dimension of each particle was recorded in *µ*m (Lusher *et al*., 2017), as well as microplastic type, colour, and state of degradation (Coyle *et al*., 2016). Microplastic abundances (microplastics.m^{-2}) were calculated for surface water as follows:

$$(particles.m^{-2}) = \frac{Number\ of\ particles}{Width\ of\ trawl\ mouth\ (m) * distance\ towed\ (m)}$$

Microplastic abundances (microplastics.m^{-2}) were calculated for sediment as follows:

$$(particles.m^{-2}) = \frac{Number\ of\ particles}{area\ of\ core\ (m^2)}$$

5.3.3. Statistical analysis

Statistical significance was set at α = 0.05. Absolute *p* values were reported for all values > 0.001. Where *p* values were less than 0.001, the significance was reported as $p < 0.001$. Univariate statistics were conducted using IBM SPSS Statistics® (version 23 for Microsoft® Windows® 10). Mean surface water microplastic abundance (microplastics.m^{-2}) was compared between the three estuaries using a one-way analysis of variance (ANOVA) after data was $\log_{10}(x)$-transformed to meet assumptions of normally distributed residuals (Shapiro-Wilk statistic = 0.91, $p > 0.05$), and homogeneity of variances (Levene's Test statistic = 0.78, $p > 0.05$). A Tukey HSD post hoc test was used to determine statistically significant differences in surface water microplastic abundance (microplastics.m^{-2}) among the three estuaries. Mean sediment microplastic abundance (microplastics.m^{-2}) was compared between the three estuaries using a one-way ANOVA after $\log_{10}(x)$ data transformation to meet ANOVA assumptions (Shapiro-Wilk statistic = 0.94, $p > 0.05$; Levene's Test

statistic = 0.06, $p > 0.05$). A post hoc Tukey HSD test was used to determine statistically significant differences in sediment microplastic abundance (microplastics.m^{-2}) among the three estuaries.

Multivariate statistics were conducted using Plymouth Routines in Multivariate Ecological Research (PRIMER) version 6 and permutational multivariate analysis of variance (PERMANOVA) package to determine significant differences in microplastic type composition (%) in estuarine surface waters of each estuary and estuarine sediment of each estuary. Data was square root transformed to weight the contributions of common and 'rare' microplastic types. A Bray Curtis matrix of similarity was constructed with the square root transformed data. A nested PERMANOVA and post hoc PERMANOVA pairwise comparisons were conducted on the Bray Curtis matrix of square root transformed data to determine if microplastic types in estuarine waters were significantly different among the three estuaries and if microplastic type in sediment were significantly different among the three estuaries. A similarity percentage analysis (SIMPER) was used to determine percentage similarities and dissimilarities between microplastic types in the different estuaries. The multivariate analysis was repeated for microplastic size distributions in estuarine surface water and sediment among the three estuaries. Microplastic size class ranges were selected from 20 µm (smallest detectable particle), 300 µm (mesh size), 1000 µm upper size limit for small microplastics (MSFD, 2013), and 5000 µm upper size limit for microplastics (MSFD, 2013). The range values in between these values (150 µm, 2500 µm) were chosen to assist detection of patterns in microplastic size classes within these larger categories.

5.4. Results

5.4.1. Estuarine surface water and sediment microplastic abundance

No microplastics were found in blank samples. Microplastics were recorded in all estuarine surface water samples and estuarine sediment samples. A total of 2209 microplastics were found in surface water samples and sediment samples combined between the three estuary mouths. Overall, most microplastics were found in sediment samples (53.60 %) among estuary study sites, with surface water samples

accounting for relatively less (46.40 %) of the total microplastic abundance. Most microplastics were found in the combined surface water and sediment samples from Bilanhlolo Estuary (52.00 %), with relatively fewer from Kongweni Estuary (22.10 %) and Mhlangeni Estuary (25.90 %).

There was a significant difference in $\log_{10}(x)$-transformed mean surface water microplastic abundance (microplastics.m^{-2}) (One-way ANOVA: $F_{(2,12)} = 109.62$, $p < 0.001$), and sediment samples (One-way ANOVA: $F_{(2,27)} = 357.40$, $p < 0.001$). Mean surface water microplastic abundance (microplastics.m^{-2}) was significantly different among Mhlangeni Estuary (4.50 ± 0.59 microplastics.m^{-2}), Kongweni Estuary (2.34 ± 0.23 microplastics.m^{-2}), and Bilanhlolo Estuary (5.98 ± 0.46 microplastics.m^{-2}) (Tukey HSD post hoc comparison test: $p < 0.001$ for all interactions) (**Figure 5.1a**). Mean sediment microplastic abundance (microplastics.m^{-2}) was significantly different among Mhlangeni Estuary ($1.33 \times 10^4 \pm 1.52 \times 10^3$ microplastics.m^{-2}), Kongweni Estuary ($1.89 \times 10^4 \pm 2.31 \times 10^3$ microplastics.m^{-2}), and Bilanhlolo Estuary ($4.22 \times 10^4 \pm 2.17 \times 10^3$ microplastics.m^{-2}) (Tukey HSD post hoc comparison test: $p < 0.001$ for all interactions) (**Figure 5.1b**). Microplastics (microplastics.m^{-2}) were most abundant in both surface water and sediment of Bilanhlolo Estuary as compared to Mhlangeni Estuary and Kongweni Estuary (**Figure 5.1**). Although Kongweni Estuary showed the lowest microplastic abundance in surface water samples, this pattern was not reflected within the sediment samples.

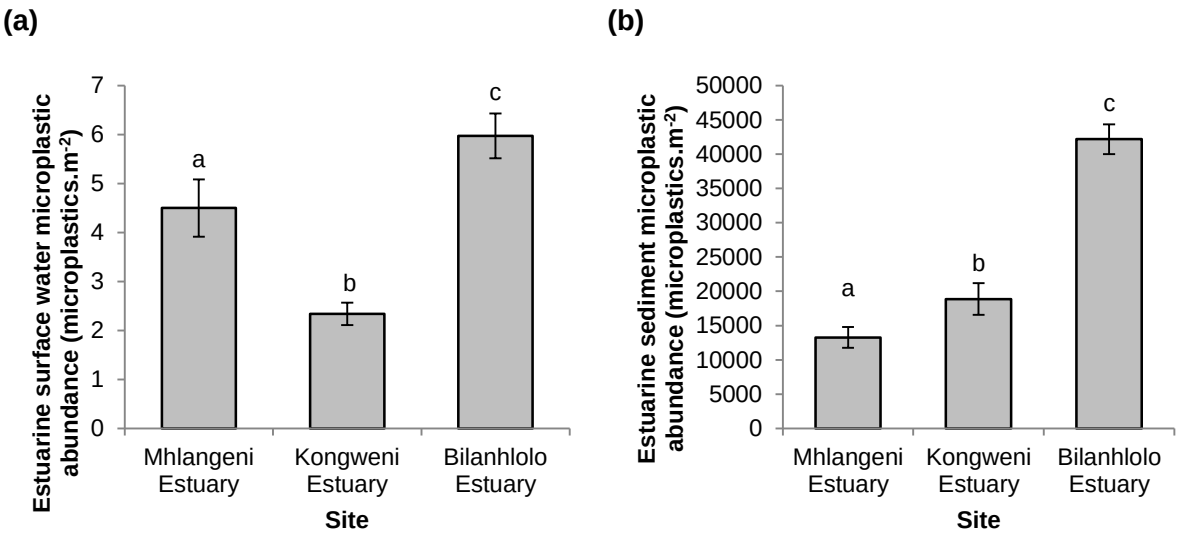

Figure 5.1: Mean microplastic abundance (microplastics.m^{-2}) in estuarine surface water (n = 5) (a) and sediment (n = 10) (b) of Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary. Lowercase letters indicate Tukey HSD post hoc significant differences among estuaries. Error bars indicate ± 1 standard deviation (SD).

5.4.2. Estuarine microplastic type composition

There was no significant difference in overall square-root transformed microplastic type composition (%) across all estuaries (surface water and sediment combined) (PERMANOVA: Pseudo-$F_{(2, 39)}$ = 1.86, p = 0.383) (**Figure 5.2**). Microplastic fibres were the most abundant microplastic type (60. 07 %) in all estuaries with surface water and sediment combined (Mhlangeni Estuary: 60.40 %, Kongweni Estuary: 63.70 %, Bilanhlolo Estuary: 57.90 %) (**Figure 5.2**). Fragments were the second most abundant microplastic type (22.95 %) in combined surface water and sediment samples in all estuaries (Mhlangeni Estuary: 23.40 %, Kongweni Estuary: 22.20 %, Bilanhlolo Estuary: 23.10 %) (**Figure 5.2**). **Figure 5.3** shows micrographs of selected microplastics found within samples.

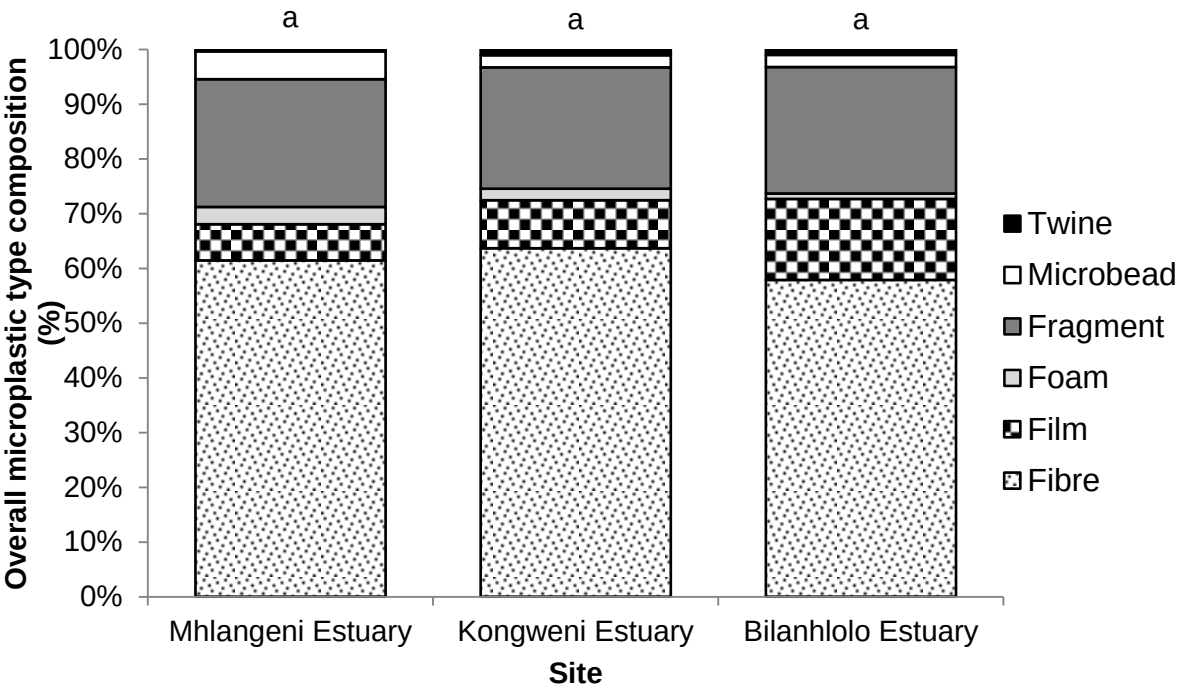

Figure 5.2: Overall microplastic type composition (%) (combined surface water and sediment) between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary. Lowercase letters indicate PERMANOVA pairwise comparison significant differences in microplastic composition (%) among sites.

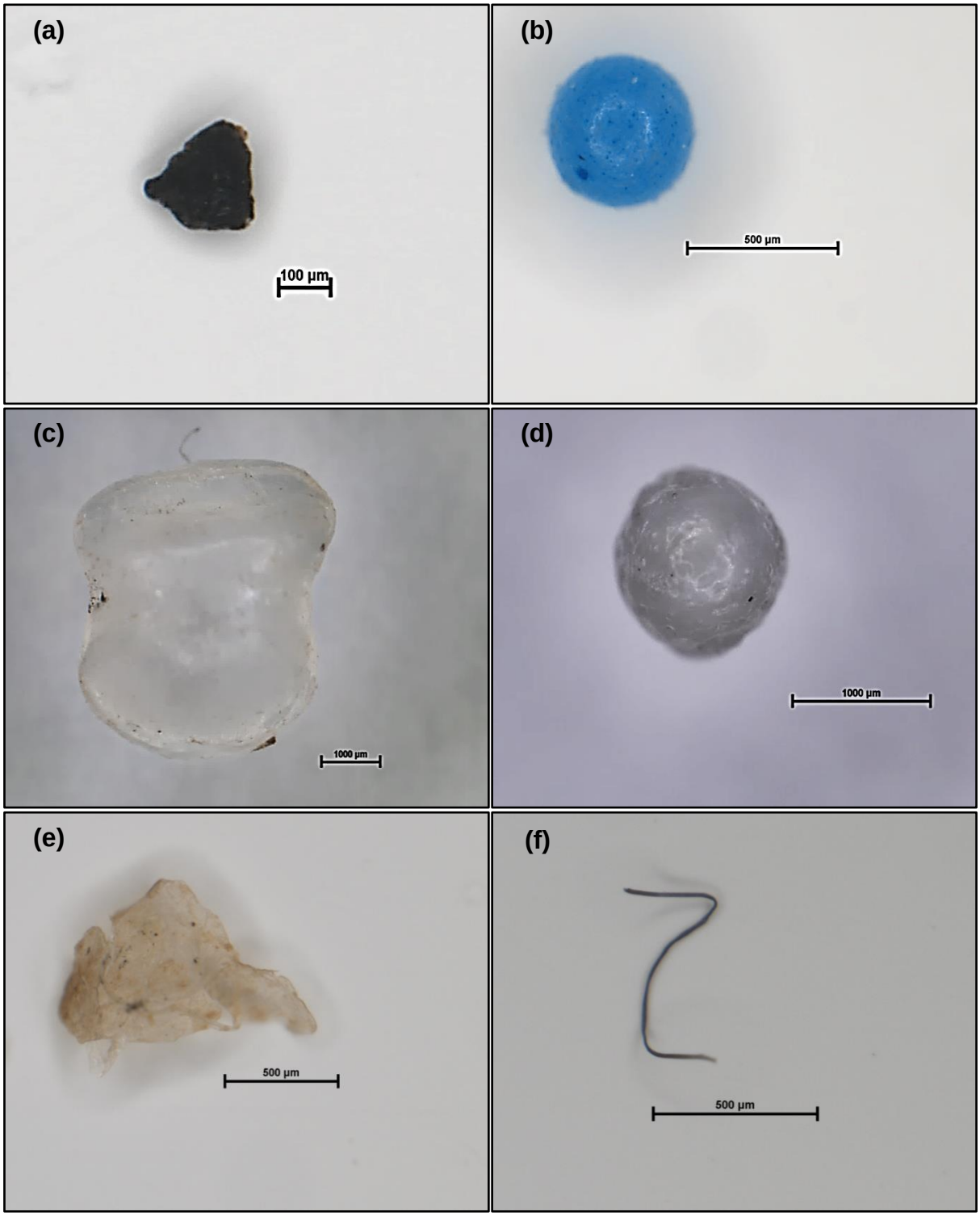

Figure 5.3: Micrographs of microplastic types found in samples, an example of a microplastic fragment (a), microplastic bead (b), microplastic pellet (c), white microbead, (d), microplastic film (e), microplastic fibre (f).

There was a significant difference between square-root transformed microplastic type composition (%) between surface water and sediment samples across all estuaries (PERMANOVA: Pseudo-F= 34.18, df = 1, $p < 0.001$) (PERMANOVA pairwise comparison: Bilanhlolo Estuary: t = 4.28, $p = 0.002$; Kongweni Estuary: t = 3.15, $p < 0.001$; Mhlangeni Estuary: t = 4.38, $p = 0.002$). Square-root microplastic type composition (%) was significantly different between surface water samples of each estuary (PERMANOVA: Pseudo-F = 6.53, df = 2, $p < 0.001$). Square-root microplastic type composition (%) in surface water was significantly different between Bilanhlolo Estuary and Kongweni Estuary (PERMANOVA pairwise comparison: t = 3.62, $p = 0.006$), and between Kongweni Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 2.30, $p = 0.007$) (**Figure 5.4a and 5.4b**). There was no significant difference in surface water microplastic type composition between Bilanhlolo Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 1.48, $p = 0.101$) (**Figure 5.4a and 5.4b**). SIMPER similarity percentage analysis indicated an average dissimilarity in surface water microplastic type composition of 27.50 % between Bilanhlolo Estuary and Kongweni Estuary. The majority of the dissimilarity was largely due to fibres (31.64 %), fragments (25.52 %), and microbeads (15.44 %). SIMPER analysis determined the average dissimilarity in surface water microplastic type composition between Kongweni Estuary and Mhlangeni Estuary (25.36 %) was largely attributed to fibres (30.96 %), microbeads (22.82 %), and foam (16.77 %). Fibres were the most dominant microplastic type within surface water samples in all estuaries (46.10 %), contributing 51.54 % to the total microplastic composition in Mhlangeni Estuary, 40.10 % in Kongweni Estuary and 44.60 % in Bilanhlolo Estuary (**Figure 5.4a**). Fragments were the second dominant microplastic type within surface water samples (36.50 %), contributing 30.30 % to the total microplastic composition in Mhlangeni Estuary, 42.20 % in Kongweni Estuary and 38.90 % in Bilanhlolo Estuary. (**Figure 5.4a**).

There was a significant difference in microplastic type composition (%) in sediment between all estuaries (PERMANOVA: Pseudo-F = 14.10, df = 2, $p < 0.001$). Square-root transformed microplastic composition (%) was significantly different in sediment samples between Bilanhlolo Estuary and Kongweni Estuary (PERMANOVA pairwise comparison: t = 3.77, $p = 0.001$), between Bilanhlolo Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 5.34, $p = 0.001$), and between Kongweni

Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 1.87, *p* = 0.022) (**Figure 5.4c and 5.4d**). SIMPER analysis showed an average dissimilarity of microplastic type composition in sediment of 29.11 % between Bilanhlolo Estuary and Kongweni Estuary, mostly as a result of dissimilarities in film (31.95 %), fibres (28.55 %), and fragments (19.15 %). There was an average of 37.75 % dissimilarity in microplastic composition in sediment between Bilanhlolo Estuary and Kongweni Estuary, the microplastics types that contributed the most to the dissimilarity were fibres (33.46 %), film (29.27 %), and fragments (19.60 %). There was an average dissimilarity in microplastic type composition in sediment of 23.4 % between Kongweni Estuary and Mhlangeni Estuary. This dissimilarity was largely explained by the dissimilarities of fragments (25.61 %), film (24.32 %), and fibres (20.99 %). In a similar pattern to surface water samples, fibres were the most dominant microplastic type in sediment across estuaries (72.10 %), contributing 67.40 %, 78.30 %, and 78.40 % to the total microplastic composition in Bilanhlolo Estuary, Kongweni Estuary and Mhlangeni Estuary respectively (**Figure 5.4c and Figure 5.4d**). Microplastic film was the second most dominant microplastic type within sediment samples (13.90 %), contributing 18.30 %, 8.30 % and 8.00 % to microplastic composition in Bilanhlolo Estuary, Kongweni Estuary, and Mhlangeni Estuary respectively (**Figure 5.4c and 5.4d**).

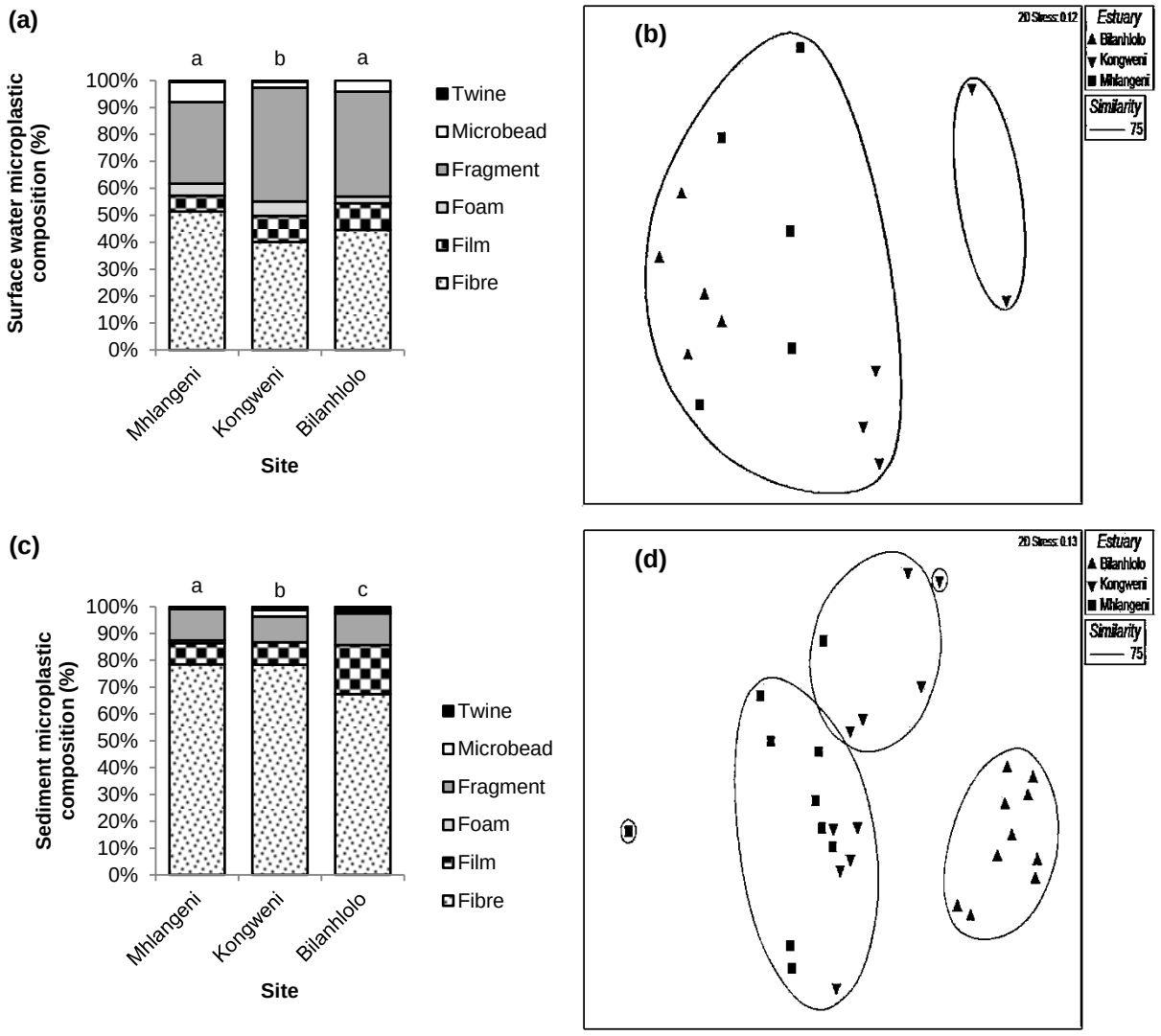

Figure 5.4: Relative proportion of microplastic type composition (%) found within estuarine surface water (a) and estuarine sediment (c) of Mhlangeni Estuary, Kongweni Estuary and Bilanhlolo Estuary. Lower case letters indicate significant differences in microplastic type composition (%) (PERMANOVA pairwise comparison). Non-metric Multidimensional scaling plots are displayed for estuarine surface water (b) and sediment (d). Cluster analysis is set at 75 % similarity.

5.4.3. Estuarine microplastic size class distribution

There was no significant difference in microplastic size class (*µ*m) distribution (%) among estuaries (surface water and sediment combined) (PERMANOVA: Pseudo-F = 0.64, df = 2, *p* = 0.705). Microplastics in the size class 300 – 999 *µ*m were the most dominant in combined surface water and sediment data across all estuaries (51.40 %), contributing 53.30 % in Bilanhlolo Estuary, 53.00 % in Kongweni Estuary, and 46.20 % in Mhlangeni Estuary to the total microplastic size class distribution within each estuary (**Figure 5.5**). Slightly larger microplastics (1000 - 2499 *µ*m) were the second most dominant microplastic size class (22.00 %), contributing 20.40 %, 23.80 %, and 23.60 % to the total microplastic size distribution in Bilanhlolo Estuary, Kongweni Estuary, and Mhlangeni Estuary respectively (**Figure 5.5**).

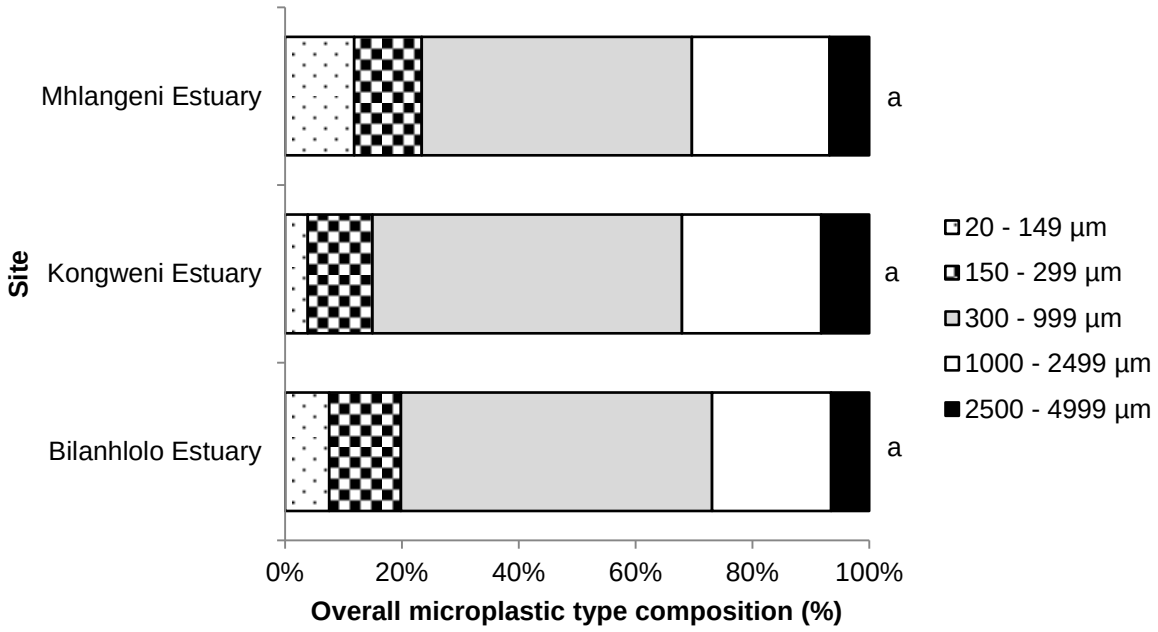

Figure 5.5: Microplastic size (*µ*m) class distribution (%) in combined surface water and sediment data among Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary. Lowercase letters indicate significant difference between microplastic size class (*µ*m) distribution (%) between estuaries (PERMANOVA pairwise comparison).

There was a significant difference in square-root transformed microplastic size class (μm) distribution (%) between surface water and sediment across all estuaries (PERMANOVA: Pseudo-F = 18.57, df = 1, $p < 0.001$) (PERMANOVA pairwise comparison: Bilanhlolo Estuary: t = 6.59, $p < 0.001$; Kongweni Estuary: t = 4.39, $p < 0.001$, Mhlangeni Estuary: t = 5.38, $p < 0.001$). Microplastic size class (μm) distribution (%) was significantly different in estuarine surface waters among the three estuaries (PERMANOVA: Pseudo-F = 30.81, df = 2, $p < 0.001$). Although the nMDS plot did not reflect a similar pattern (**Figure 5.6b**), there was a significant difference in microplastic size class (μm) distribution (%) between Bilanhlolo Estuary and Kongweni Estuary (PERMANOVA pairwise comparison: t = 7.95, $p = 0.012$), between Bilanhlolo Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 3.115, $p = 0.009$), and between Kongweni Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 4.28, $p = 0.005$) (**Figure 5.6a**).

Surface waters in estuaries were dominated by microplastics in the size range 300 – 999 μm (42.30 %), contributing 39.30 %, 54.00 %, and 40.30 % to the surface water microplastic size distribution in Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary respectively (**Figure 5.5a**). SIMPER similarity percentage analysis showed that the majority of the average 27.48 % dissimilarity in the microplastic size class (μm) distribution (%) of surface water between Bilanhlolo Estuary and Kongweni Estuary was largely due to the 300 – 999 μm size class (35.25 %). Between Bilanhlolo Estuary and Mhlangeni Estuary surface water, the average dissimilarity (10.48 %) in microplastic size class (μm) distribution (%) was attributed to the large dissimilarity of the size class 2500 – 4999 μm (44.25 %). Between Kongweni Estuary and Mhlangeni Estuary, the average dissimilarity in microplastic size class (μm) distribution (%) in surface water (18.18 %) was mostly due to the dissimilarity in the size class 300 – 999 μm (45.23 %).

Microplastic size class (μm) distribution (%) was significantly different in estuarine sediment among the three estuaries (PERMANOVA: Pseudo-F = 20.39, df = 2, $p < 0.001$). There was a significant difference in microplastic size class (μm) distribution (%) between Bilanhlolo Estuary and Kongweni Estuary (PERMANOVA pairwise comparison: t = 5.37, $p < 0.001$), between Bilanhlolo Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 5.61, $p < 0.001$), and between Kongweni

Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 2.22, *p* = 0.004) (**Figure 5.6c and Figure 5.6d**). SIMPER similarity percentage analysis determined that the majority of the dissimilarity in sediment microplastic size class distribution (%) between Bilanhlolo Estuary and Kongweni Estuary (average dissimilarity = 25.36 %) was largely attributed to the dissimilarity in the 300 – 999 μm size class (39.64 %). The average dissimilarity in sediment size class distribution (%) between Bilanhlolo Estuary and Mhlangeni Estuary (33.96 %) was largely due to the dissimilarity of the 300 – 999 μm size class (37.31 %). The 19.84 % average dissimilarity between Kongweni Estuary and Mhlangeni Estuary was mostly due to the dissimilarity in the 1000 – 2499 μm size class (26.15 %). Microplastics in the size class 300 – 999 μm were the most abundant in sediment across all estuaries (59.20 %), contributing 63.20 %, 52.30 %, and 56.30 % to the total microplastic size class distribution in Bilanhlolo Estuary, Kongweni Estuary, and Mhlangeni Estuary respectively (**Figure 5.6c**).

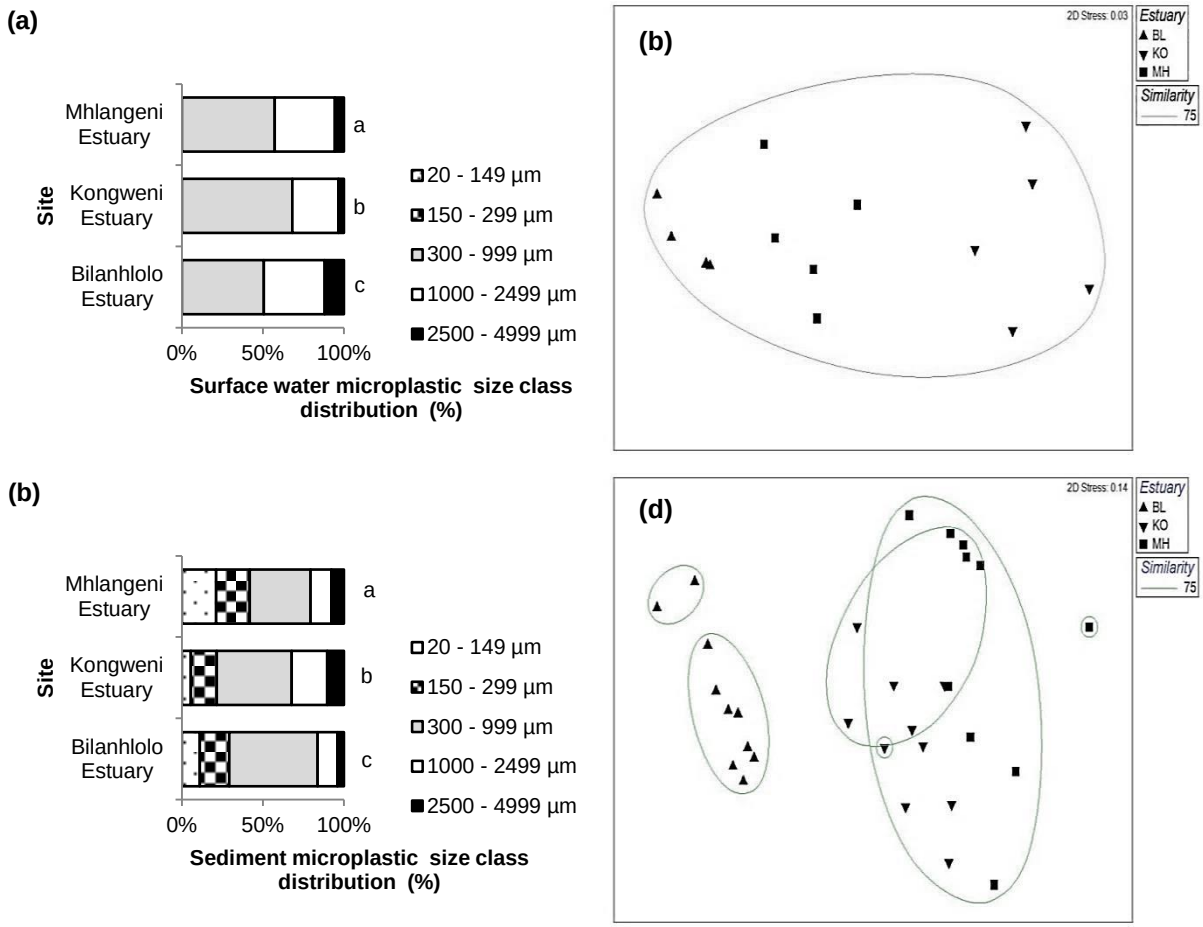

Figure 5.6: Microplastic particle size (*µ*m) class distribution (%) in estuarine surface water (a) and sediment (c) within Mhlangeni Estuary, Kongweni Estuary and Bilanhlolo Estuary. No size class < 300 *µ*m for surface water due to sampling equipment mesh size. Lower case letters indicate significant differences in microplastic size class (*µ*m) distribution (%) (PERMANOVA pairwise comparison). Non-metric Multidimensional scaling plots are displayed for estuarine surface water (b) and sediment (d) within Bilanhlolo Estuary (BL), Kongweni Estuary (KO), and Mhlangeni Estuary (MH). Cluster analysis is set at 75 % similarity.

5.5. Discussion

Microplastic abundances (microplastics.m^{-2}) were significantly different in both surface water (**Figure 5.1a**) and sediment (**Figure 5.1b**) between Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary. The Present Ecological State (PES) category for each estuary as described by DWA (2013) was not a good indicator for the microplastic pollution status of the estuaries in this investigation. It was expected that Kongweni Estuary (PES category D) would contain more microplastics as it has a higher degree of anthropogenic disturbances than Bilanhlolo Estuary (PES category C) and Mhlangeni Estuary (PES category C). Bilanhlolo Estuary had the highest microplastic abundance in both surface water (5.98 ± 0.46 microplastics.m^{-2}) and sediment (4.22 x 10^4 ± 2.17 x 10^3 microplastics.m^{-2}) (**Figure 5.1**) out of all the estuaries. Kongweni Estuary, although showing the lowest microplastic abundance in surface water (2.34 ± 0.23 microplastics.m^{-2}) in comparison to the other estuaries (**Figure 5.1a**), displayed the second highest microplastic abundance in sediment (1.89 x 10^4 ± 2.31 x 10^3 microplastics.m^{-2}) (**Figure 5.1b**).

The differences in microplastic abundance between surface water and sediment within each estuary may be as a result of microplastics accumulating in sediment (Santana *et al*., 2016), although the differences may be attributed to the sampling gear used for surface water, as any microplastics smaller than 300 μm would not be sampled (MSFD, 2013). Rainfall has been cited as a major influence on microplastic abundances in estuaries (Zhao *et al*., 2015). Since all samples were collected during the same period in a rainy season, the differences in results could not be attributed to differences in rainfall. The period of time each estuary mouth was open for prior to sampling may be a factor influencing microplastic abundances within each estuary. During periods of mouth closure, plastics may accumulate in the estuary, and are washed out of the estuary once the mouth opens. During this study, the period that each mouth was open was not taken in to account, therefore the results reported should be used only as what microplastic pollution was within each estuary at the time of sampling.

The catchment size of the river leading in to the estuary may also influence the quantity of microplastics in the estuary (Zhao *et al*., 2015). The greater the catchment

size, the greater the quantity of water and potential microplastic pollution flowing through the estuary. The results presented (**Figure 5.1**) showed that catchment size did not seem to have an effect on the quantity of microplastic pollution in each estuary. Bilanhlolo Estuary and its associated river has a relatively smaller catchment size (19.8 km^2) (DWA, 2013) as compared to the other studied estuaries and the associated rivers (Mhlangeni Estuary: 37.2 km^2, Kongweni Estuary: 7.9 km^2) (DWA, 2013), but displayed the greatest microplastic abundance. This information seems to highlight that the actual sources of microplastic pollution within each river and estuarine system have a greater effect on the microplastic abundance than the river catchment size. The differences in microplastic abundance may be as a result of the different inputs of microplastic pollution into each estuary (Naidoo *et al*., 2015). Microplastics may be introduced into each estuary by fragmenting plastics from within each estuarine system (Gallagher *et al*., 2016), oceans during an open mouth phase (Vermeiren *et al*., 2016), in addition to domestic and commercial activities surrounding each estuary (Lima *et al*., 2015).

Treated sewage has been cited as a significant source of microplastic pollution in river and estuarine environments (Lebreton *et al*., 2017). However, the results of this study contradict that statement as Kongweni Estuary, which receives treated sewage discharge (DWA, 2013) displayed lower microplastic abundance than Bilanhlolo Estuary, which does not receive treated sewage discharge (DWA, 2013). This contradiction was also shown by Nel *et al*. (2018), where microplastic abundances were found to be similar up and downstream of a wastewater treatment plant in the Bloukrans River near the town of Grahamstown in Eastern Cape province, South Africa, in both summer and winter seasons.

Bilanhlolo River and associated estuary receives unintentional overflow from the nearby Oatlands domestic landfill site leachate dam during periods of high rainfall and consequential leachate dam overspill. Landfill sites, often regarded as sinks for plastic waste, have recently been confirmed as sources of microplastics to the environment (Mahon *et al*., 2017). Due to the physio-chemical degradation processes occurring in landfills, plastic products are fragmented and transported out of the landfill via leachate production. Landfill leachate is the product of rainfall percolating through decomposing landfill waste and is collected in dams on the landfill site before

treatment and disposal, often directly in to rivers or sewage (Kilponen, 2016). In periods of heavy rainfall, leachate dams sometimes overflow in to nearby freshwater systems. In Ireland, Mahon *et al*. (2017) described microplastic content in raw landfill leachate of up to 49600 ± 18385 particles.m^{-3}, with an insignificant decrease after reverse osmosis treatment (45000 ± 4242 particles.m^{-3}). Although the present study does not attempt to identify landfill leachate dams as a source of microplastic pollution, these results highlight the potentially large input of microplastic pollution from landfill leachate to freshwater systems and should be a focus of future research.

Increased microplastic pollution abundance in estuaries is cause for concern as it allows for greater interaction frequency with lower trophic level organisms that live in the estuaries (Zhao *et al*., 2015). The increased ingestion of microplastics by these organisms may decrease the survival and reproductive fitness of important lower trophic level animals, potentially causing the ecosystem dynamics of organisms to change (Lima *et al*., 2015). The increased microplastic consumption may increase the transfer of harmful toxicants throughout estuarine food webs (Chua *et al*., 2014). In addition, estuaries are a major source of microplastics to the marine environment (Bakir *et al*., 2014), so the quantification of microplastic abundances within estuaries allows for a greater understanding of microplastics inputs in to the marine environment.

Although a wide range of microplastic types were found in estuarine surface water and sediment samples (**Figure 5.2** and **Figure 5.3**), microplastic fibres were the most dominant plastic type within the surface water and sediment in all studied estuaries (**Figure 5.2**). Microplastic fibres were significantly more abundant in sediment (72.10 %) than in surface water (51.54 %), however this result may be due to the different sampling methods used. The results of this investigation are similar to Zhao *et al*. (2014), who found that microplastic fibres were the most dominant microplastic type (79.1%) in the surface water of the Yangtze Estuary system, China. Microplastic fibres were also the most dominant plastic type (> 90 %) in Jiaojiang, Ouijiang, and Minjiang Estuaries in China (Zhao *et al*., 2015). Microplastic type is important to quantify as it may provide insight of microplastic pollution origins within freshwater systems (Rodríguez-Seijo and Pereira, 2017). Zhao *et al*. (2014) suggested that microplastic fibres are an indication that most microplastic pollution in a particular

area is derived from land-based debris, as microplastic fibres are the result of synthetic polymer clothing being washed in washing machines and the resulting wastewater transported into natural water courses (Browne *et al*., 2011). In addition, microplastic type is important to quantify as negative impacts on organisms that ingest microplastics have been shown to be associated with microplastic particle shapes (Wright *et al*., 2013). Microplastic fibres have a higher surface area to volume ratio than other microplastic shapes, potentially allowing for increased toxicant accumulation on the surface of the microplastic, increasing the possibility of increased toxicant transfer to animals which ingest the microplastic fibres (Chua *et al*., 2014).

The results of this investigation show that there was no overall difference in microplastic size class (*µ*m) distribution (%) between studied estuaries (**Figure 5.5**), therefore the hypothesis stating that there will be a difference in microplastic size class distribution between the studied estuaries was rejected. However, there was a significant difference in the microplastic size classes (*µ*m) between surface water and sediment among estuaries (**Figure 5.6**). This may be as a result of the sampling gear used, as no microplastic particles smaller than 300 *µ*m were sampled in the surface water samples due to the net mesh size. Smaller microplastics were more abundant in all samples as opposed to larger microplastics (**Figure 5.5**). These results are similar to previous studies, indicating that smaller microplastics in estuarine surface water and sediment are more abundant than larger microplastics (Naidoo *et al*., 2015; Zhao *et al*., 2015).

Smaller microplastics may be more frequently encountered and consumed by benthic and pelagic estuarine organisms (Zhao *et al*., 2014), especially lower trophic level organisms. Foekema *et al*. (2013) found that smaller microplastics are more frequently found in filter feeders as opposed to larger carnivorous taxa. Filter feeders in estuarine environments have been shown to ingest microplastics the same size and shape as their natural prey (Wright *et al*., 2013). The large quantities of smaller microplastics in estuarine systems may lead to more frequent ingestion by lower trophic level organisms, such as filter-feeders, and in turn, potentially increase the bioaccumulation of microplastics and the associated toxicants throughout the food web (Ivar do Sul and Costa, 2014).

5.6. Conclusion

The results of Chapter 5 show that microplastic pollution was present in both surface water and estuarine sediment of three studied estuaries during the sampling period. The differences in microplastic abundances among estuaries may be due to different inputs of microplastic pollution into each estuarine system; however the source of microplastic pollution in each estuary may only be speculated. Microplastic fibres dominated throughout all samples, indicating that domestic sources of microplastics (i.e. washing machines) and fisheries may largely contribute to the microplastic pollution within estuaries. The differences between surface water and sediment microplastic abundances may be due to differences in sampling technique, which demonstrates the importance to sample both sediment and surface water in estuaries when investigating microplastic pollution levels. The microplastic pollution within each estuary highlights the potential contribution of estuaries as conduits of microplastic pollution transfer from land-based sources to the marine environment. The most anthropogenically disturbed estuary (PES) (Kongweni Estuary) had lower levels of microplastic pollution than the more ‘pristine’ Bilanhlolo Estuary and Mhlangeni Estuary. This information shows that the PES category (DWA, 2013) may not be a good predictor of microplastic pollution levels in the studied estuaries. Future research should include the seasonal sampling of TOCEs. As TOCEs are only intermittently open to the marine environment, the effluents derived from these estuaries are only transported into the marine environment in seasonal periods of increased rainfall (wet season). Seasonal sampling can allow for identification of potential spatial and temporal patterns of microplastic pollution from estuaries, which eventually may be used to identify major point sources of microplastic pollution into individual estuaries.

CHAPTER 6: TEMPORARILY OPEN/CLOSED ESTUARIES AS SOURCES OF MICROPLASTIC POLLUTION TO KWAZULU-NATAL COASTAL ENVIRONMENTS

6.1. Abstract

Microplastic (< 5 mm in maximum dimension) pollution monitoring has only recently become the focus of a large body of research, and as such, there is limited data regarding microplastic pollution in South African coastal environments. Estuaries have been described as important sources of microplastic pollution to marine environments, however there is still a lack of knowledge regarding this phenomenon in a South African context. The aim of this study was to determine and compare spatial differences in beach sediment microplastic pollution originating from selected KwaZulu-Natal temporarily open/closed estuaries (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) during an open mouth phase at distances 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth. Sediments from sites near the Mhlangeni Estuary displayed lower mean levels of microplastic pollution ($8.76 \times 10^3 \pm 2.39 \times 10^3$ microplastics.m^{-2}) than sites near Kongweni Estuary ($1.21 \times 10^4 \pm 3.02 \times 10^3$ microplastics.m^{-2}). Sediment from sites near Bilanhlolo Estuary displayed the highest microplastic pollution levels ($2.03 \times 10^4 \pm 6.44 \times 10^3$ microplastics.m^{-2}). Beach sediment displayed higher abundances of microplastics (microplastics.m^{-2}) at sites nearer to each estuary mouth than sites further away. Microplastics in beach sediment were found to largely consist of microplastic fibres (79.90 %), indicating that land-based microplastic pollution is a significant source of marine microplastic pollution. The results of this study are the first to investigate microplastic pollution in beach sediment in these areas of South Africa and add new knowledge of microplastic pollution levels in South Africa.

Keywords: microplastics, beach sediment, temporarily open/closed estuaries, KwaZulu-Natal, South Africa, baseline survey

6.2. Introduction

The majority of studies on plastic pollution in the marine environment have focused largely on macro-plastics and their negative effects on marine organisms (Setälä *et al.*, 2014). However, in comparison to macroplastics, there has been far less research on microplastics (Barnes *et al.,* 2009; Andrady, 2011). Microplastics particles are those which are < 5 mm in maximum size dimension and have commonly been described as major marine pollutants (Watts *et al*., 2014). They are classified as being either primary or secondary microplastics (Cole *et al.,* 2011). Primary microplastics are those which are manufactured to be smaller than 5 mm, commonly for domestic uses such as exfoliating face washes, and for industrial uses such as 'sand-blasting' (Teuten *et al.,* 2007; Ivar do Sul and Costa, 2014). Secondary microplastics are those which are derived from the degradation and/or fragmentation of larger plastic items (Ivar do Sul and Costa, 2014).

Microplastics are considered to be ubiquitous throughout the marine environment (Andrady, 2011), with reports of microplastic pollution found in sediment, in the water column, as well as within organisms throughout the globe (Browne *et al.,* 2011). Techniques of microplastic pollution monitoring have often involved the sampling and analysis of microplastics deposited in benthic sediments which has been successfully applied to beach, estuarine and sea-floor sediments (Solomon and Palanisami, 2016). Microplastic deposition on sandy beaches has previously been used to extrapolate microplastic pollution levels in a particular coastal area (Nel and Froneman, 2015). Microplastic abundances are commonly investigated in sandy beaches due to the ease of accessibility (Van Cauwenberghe *et al*., 2015). Microplastics from the ocean are primarily deposited in inter-tidal zones of beaches, most commonly on strand or drift lines (Moreira *et al*., 2016). Patterns of microplastic distributions in beach sediment are subject to numerous influencing factors and are therefore considered highly dynamic (Besley *et al*., 2017). The evaluation of microplastics found in the inter-tidal zone would therefore be appropriate to determine the amount of microplastic input from the ocean (Moreira *et al*., 2016) and not necessarily the amount of accumulated microplastics over time. When an estuary mouth is open the water flowing from the estuary will mix with the ocean water, often resulting in the pollution of the nearby coastal environments. For this reason, the

analysis of sediment samples collected from the inter-tidal zone of sandy beaches near estuary mouths may be useful to determine the input of microplastic pollution from estuaries into the nearby coastal environments.

To expand the understanding of microplastic pollution in South African marine environments a case study was performed at three beaches near temporarily open/closed estuaries (TOCEs) in KwaZulu-Natal. The aim of this investigation was to determine and compare spatial differences in beach sediment microplastic pollution originating from selected KwaZulu-Natal temporarily open/closed estuaries (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) during an open mouth phase at distances of 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth. Objectives were to compare 1) microplastic abundances (microplastics.m^{-2}) in beach sediment at stations 500 m, 1000 m, and 2000 m North/South on the coastline adjacent to each estuary mouth during an open mouth phase, 2) microplastic type composition (%) in beach sediment at stations 500 m, 1000 m, and 2000 m North/South on the coastline adjacent to each estuary mouth during an open mouth phase, and 3) microplastic size class (μm) distribution (%) in beach sediment at stations 500 m, 1000 m, and 2000 m North/ South on the coastline adjacent to each estuary mouth during an open mouth phase. It was hypothesized that there will be a difference in microplastic abundances (microplastics.m^{-2}), microplastic type composition (%), and microplastic size class (μm) distribution (%) in beach sediment at stations 500 m, 1000 m, and 2000 m North/ South on the coastline adjacent to each estuary mouth during the open mouth phase.

6.3. Methods and Materials

6.3.1. Data collection

All data collection took place during a three day period in January 2017 at low tide. Sediment samples for microplastic analysis were collected at six beach stations (500 m, 1000 m, and 2000 m North and South) adjacent to each estuary mouth (**Chapter 1: Figure 1.1**) (Geographic coordinates: **Appendix A: Table A 2**). At each station, five replicate sediment cores of the top five cm of sediment were taken at the hightide

mark at a minimum distance of one metre apart using a plastic corer (45 mm internal ⌀). If multiple drift lines were present, the samples were collected from the highest observable drift line (Naidoo *et al*., 2015). Each core sample was sieved through a five mm stainless steel mesh on site and thereafter transferred in to individual ziplock bags. Personnel collected samples downwind of each site to minimize airborne microplastic contamination of samples from clothing (MSFD, 2013). Sediment samples were transported and stored in a freezer at -20 °C until laboratory processing.

Prior to sample analysis, sediment samples were removed from the freezer and allowed to thoroughly thaw at room temperature (± 25 °C). Thereafter, sediment samples were transferred to aluminium 'boats' and covered with additional aluminium foil and oven dried at 60 ºC for 48 hours until constant mass (Naidoo *et al*., 2015). Density separation was used to separate microplastics from sediment. A fully saturated sodium chloride (NaCl) solution was prepared by mixing 360 g of commercially available table salt (First Value®) with one L of distilled water using a magnetic stirrer. The NaCl solution was thereafter vacuum-filtered through Whatman® borosilicate glass microfibre filters (47 mm ⌀, 0.7 *µ*m pore size) (Sigma-Aldrich©) to remove any potential microplastic contaminants. The supernatant was collected and the process was repeated until the required quantity of NaCl solution was obtained. Once sediment samples were dried to constant mass, each sample was mixed in a glass beaker with 200 mL of the saturated NaCl solution with a glass rod for approximately two minutes. The mixture was allowed to stand for one hour before the supernatant was filtered through a 5.0 *µ*m polycarbonate track-etched ISOPORE™ membrane filter (47 mm ⌀) (Merck Millipore Ltd.). The sediment sample was replenished with a further 200 mL of filtered NaCl solution and the extraction procedure was repeated three times per sediment sample. Filters were placed in to new, clean petri dishes, covered loosely with aluminium foil, and oven dried at 60 ºC for 24 hours. Blank samples (with no sediment) were included to account for any airborne microplastic contamination during sample processing.

6.3.2. Microplastic analysis

Filtered samples were viewed up to 40 X magnification using a Nikon© AZ100 stereomicroscope. Microplastics were distinguished from non-plastic particles using previously recommended procedures (**Chapter 3: Table 3.3**). Once a particle had been identified as plastic, the maximum size dimension of each particle was measured (μm), and microplastic type was recorded. Microplastic abundances (microplastics.m^{-2}) were calculated for each sediment sample as follows:

$$Microplastic\ abundance\ (particles.m^{-2}) = \frac{Number\ of\ particles}{area\ of\ core\ (m^2)}$$

6.3.3. Statistical analyses

Statistical significance was set at α = 0.05. Absolute *p* values were reported for all values > 0.001. Univariate statistics were conducted using IBM SPSS Statistics® (version 23 for Microsoft® Windows® 10). A fully-nested ANOVA was used to compare microplastic abundance (microplastics.m^{-2}) among sediment sites near each estuary, direction (North/South) of each estuary mouth, and distances (0 m, 500 m, 1000 m, and 2000 m) within directions after $\log_{10}(x)$-transformed data met assumptions of normality of residuals (Shapiro Wilk test statistic = 0.99, $p > 0.05$), and homogeneity of variances (Levenes test statistic = 1.17, $p > 0.05$). Post hoc Tukey HSD comparisons were used to determine significant differences of microplastic abundances (microplastics.m^{-2}) among sites. Multivariate statistics were conducted using Plymouth Routines in Multivariate Ecological Research (PRIMER) version 6 and permutational multivariate analysis of variance (PERMANOVA) package to determine significant differences in microplastic type composition (%) in beach sediment stations among estuaries, directions and distances. A Bray Curtis matrix of similarity was constructed from square-root transformed data. A nested PERMANOVA and PERMANOVA pairwise comparisons were performed on the Bray Curtis similarity matrix of square-root transformed data. A similarity percentage analysis (SIMPER) was used to determine percentage similarities and dissimilarities between microplastic types in the different estuaries. The multivariate statistical

analysis was repeated for microplastic size class (μm) distribution (%) in beach sediment samples.

6.4. Results

6.4.1. Beach sediment microplastic abundance

Microplastics were recorded in all beach sediments samples. A total of 3413 individual microplastics were recorded at beach sediment stations. A fully-nested ANOVA showed a significant difference in $\log_{10}$(x)-transformed microplastic abundance overall between estuary beach sites (Nested ANOVA: $F_{(2, 72)} = 390.53$, $p < 0.001$), between directions North and South of each estuary mouth (Nested ANOVA: $F_{(3 ,72)} = 78.10$, $p < 0.001$), and a significant difference in microplastic abundance at increasing distances North and South away from each estuary mouth (Nested ANOVA: $F_{(12, 72)} = 17.84$, $p < 0.001$).

Log_{10}(x)-transformed microplastic abundance (microplastics.m^{-2}) was significantly different between estuarine systems (Tukey HSD post hoc comparison: $p < 0.001$ for all interactions) (**Figure 6.1**). Sediments from sites near the Mhlangeni Estuary displayed lower mean levels of microplastic pollution ($8.76 \times 10^3 \pm 2.39 \times 10^3$ microplastics.m^{-2}) than sites near Kongweni Estuary ($1.21 \times 10^4 \pm 3.02 \times 10^3$ microplastics.m^{-2}). Sediment from sites near Bilanhlolo Estuary displayed the highest microplastic pollution levels ($2.03 \times 10^4 \pm 6.44 \times 10^3$ microplastics.m^{-2}). Tukey HSD post hoc comparisons also showed that microplastic abundance was significantly different between North and South sites within all estuary systems ($p < 0.05$ for all interactions). Tukey HSD post hoc comparisons showed that sites further away from each estuary mouth had significantly less microplastic abundances than sites closer to each estuary mouth (**Figure 6.1**). All sites displayed decreased microplastic abundance with increase in distance away from each estuary mouth, except for site 2000 m North of the Mhlangeni Estuary mouth which was significantly similar to sites closer to the estuary mouth (**Figure 6.1**).

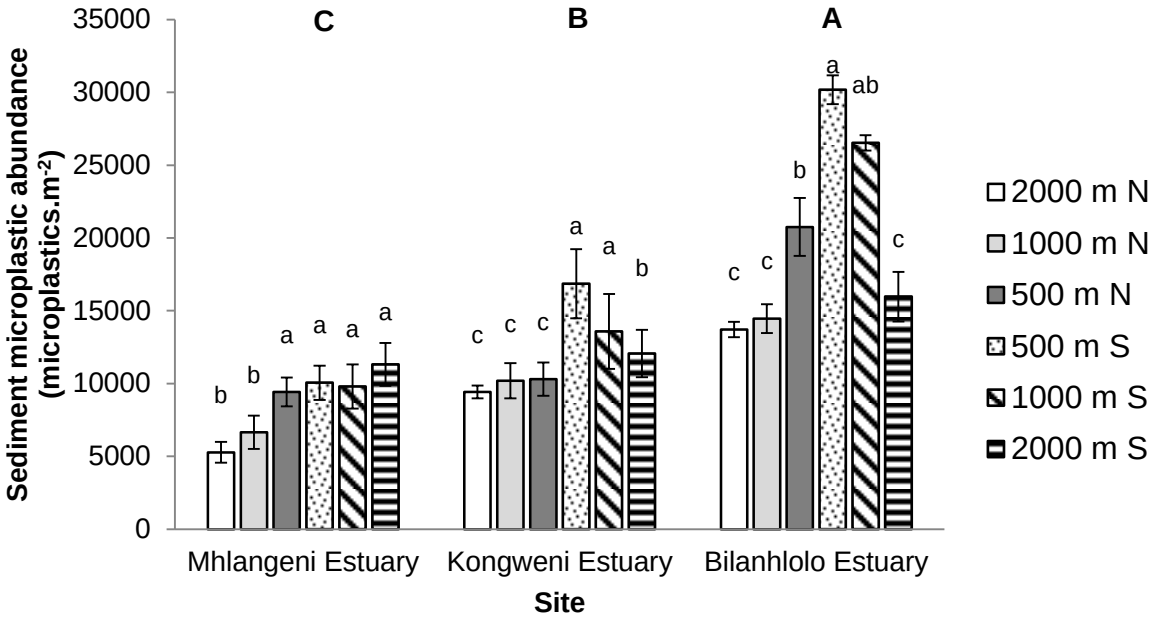

Figure 6.1: Mean microplastic abundance (microplastic.m^{-2}) in beach sediments at increasing distances (m) North (N) and South (S) away from each estuary mouth (n= 5). Uppercase letters indicate significance differences between estuarine systems. Lowercase letters indicate Tukey HSD post hoc significance differences between sites within each estuary system. Error bars represent ± 1 SD.

6.4.2. Beach sediment microplastic type composition

Square-root-transformed microplastic type composition (%) was significantly different overall in each estuary (PERMANOVA: Pseudo-F = 37.00, df = 2, $p < 0.001$), within North/South stations in each estuary (PERMANOVA: Pseudo-F = 12.78, df = 3, $p < 0.001$), and between distances nested in direction between all estuaries (PERMANOVA: Pseudo-F = 3.27, df = 12, $p < 0.001$).

Square-root transformed microplastic type composition (%) was significantly different between sediments combined between Bilanhlolo Estuary and Kongweni Estuary (PERMANOVA pairwise comparison: t = 5.93, $p < 0.001$), Bilanhlolo Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 8.49, $p < 0.001$), and Kongweni Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 2.94, $p < 0.001$) (**Figure 6.2**). Fibres were the most abundant microplastic type

between all beach sediment sites across estuaries (79.90 %), contributing 75.30 %, 88.10 %, and 79.20 % to the total microplastic type composition (%) in Bilanhlolo Estuary, Kongweni Estuary, and Mhlangeni Estuary respectively (**Figure 6.2**). Beach sediment sites near Bilanhlolo Estuary showed a higher proportion of microplastic film (12.20 %) as compared to Kongweni Estuary (6.50 %), and Mhlangeni Estuary (5.80 %) (**Figure 6.2**). Beach sediment sites near Mhlangeni Estuary had the highest proportion of microbeads (3.50 %) relative to the total microplastic type composition (%) in comparison to Kongweni Estuary (0.90 %), and Bilanhlolo Estuary (0.30 %) (**Figure 6.2**). Similarity percentage analysis (SIMPER) showed that the average dissimilarity (27.29 %) between Bilanhlolo Estuary and Kongweni Estuary was largely due to dissimilarities in fragments (30.45 %), film (26.06 %), and fibres (25.09 %). The average dissimilarity (36.53 %) between Bilanhlolo Estuary and Mhlangeni Estuary was largely attributed to dissimilarities in fragments (29.32 %), fibres (27.41 %), and film (27.11 %). The average dissimilarity between Kongweni Estuary and Mhlangeni Estuary (26.26 %) was as a result of the main dissimilarities between film (28.77 %), fibres (27.42 %), and fragments (25.96 %).

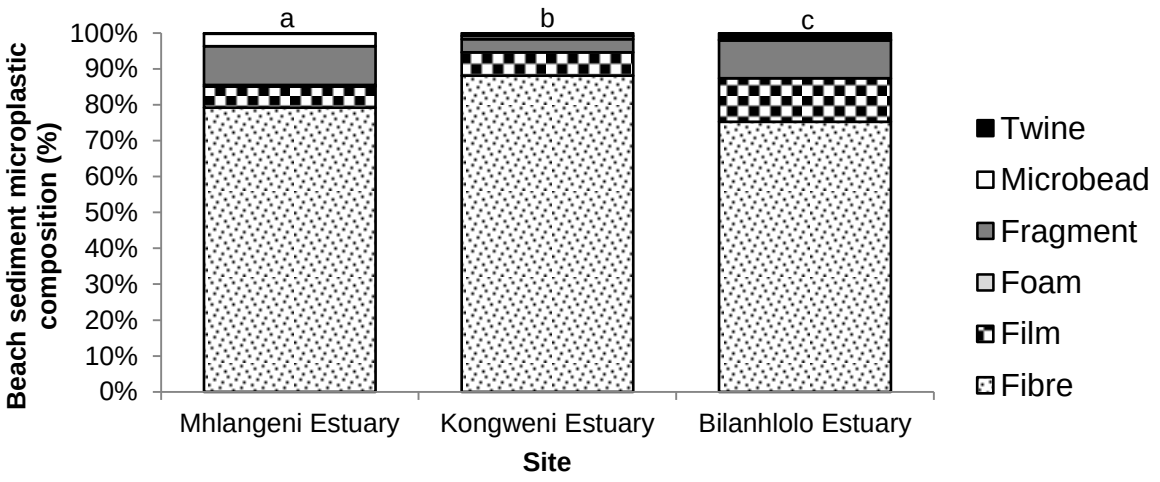

Figure 6.2: Overall microplastic type composition (%) in beach sediment sites near each estuary (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary). Lowercase letters indicate PERMANOVA pairwise comparison significant differences.

Square-root transformed microplastic type composition (%) was significantly different between North and South sites within each estuary (PERMANOVA pairwise comparison: Bilanhlolo Estuary: t = 3.17, $p < 0.001$; Kongweni Estuary: t = 2.92, $p = 0.002$; Mhlangeni Estuary: t = 4.18, $p < 0.001$) (**Figure 6.3**). Square-root microplastic type composition (%) was generally not significantly different between beach sediment sites at increasing distances (500 m, 1000 m, and 2000 m) North and South away from each estuary mouth (**Figure 6.3**), except for a few beach stations (**Figure 6.3**). Beach sediment sites near Mhlangeni Estuary had similar proportions of microplastics fibres (**Figure 6.3a**), except for the site 2000 m South of Mhlangeni Estuary which had a larger proportion of fragments (30.80 %), and relatively smaller proportion of fibres (50.00 %) than other sediment sites near the Mhlangeni Estuary mouth (**Figure 6.3a**). Beach sediment sites near Kongweni Estuary mouth had similar proportions of microplastic fibres (**Figure 6.3b**), but the site at 2000 m North had a larger proportion of microplastic film (10.20 %) than other sediment sites near Kongweni Estuary mouth, while the site 1000 m North had a larger proportion of fragments (8.00 %) (**Figure 6.3b**). Beach sediments sites near Bilanhlolo Estuary mouth also had a similar proportion of microplastic fibres throughout (**Figure 6.3c**). The site 1000 m North of the Bilanhlolo Estuary mouth showed the largest proportion of fragments (21.70 %) relative to other beach sites (**Figure 6.3c**)

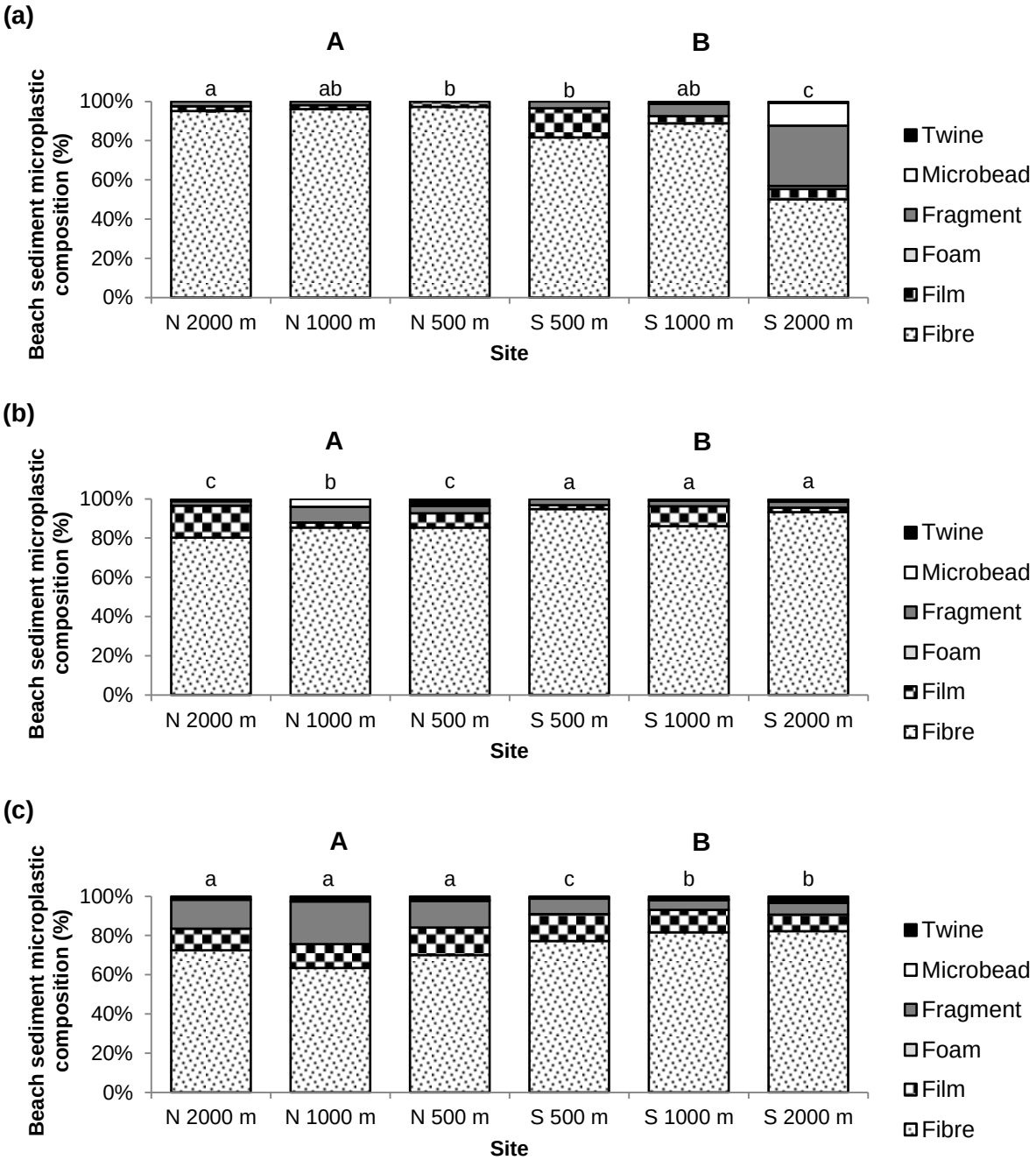

Figure 6.3: Microplastic type composition (%) in beach sediment from sites at increasing distances (500 m, 1000 m, and 2000 m) North (N) and South (S) away from each estuary mouth. Mhlangeni Estuary sites (a), Kongweni Estuary sites (b) and Bilanhlolo Estuary sites (c). Uppercase letters indicate PERMANOVA significant differences between sites North and South. Lowercase letters indicate significant PERMANOVA pairwise comparison significant differences between sites at increasing distance (500 m, 1000 m, 2000 m) away from each estuary mouth.

6.4.3. Beach sediment microplastic size class distribution

Square-root transformed microplastic size class (*μ*m) distribution (%) was significantly different among combined sediment sites near each estuary mouth (PERMANOVA: Pseudo-F = 17.16, df = 2, $p < 0.001$), among sites North and South of each estuary mouth (PERMANOVA: Pseudo-F = 6.66, df = 3, $p < 0.001$), and among sites at increasing distances (500 m, 1000 m, and 2000 m) North and South of each estuary mouth (PERMANOVA: Pseudo-F = 2.33, df = 12, $p < 0.001$).

Square-root transformed microplastic size class (*μ*m) distribution (%) was significantly different in combined sediments sites between Bilanhlolo Estuary and Kongweni Estuary (PERMANOVA pairwise comparison: t = 4.44, $p < 0.001$), between Bilanhlolo Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 5.31, $p < 0.001$) and between Kongweni Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 2.33, $p = 0.003$). Microplastics in the size class 300 – 999 *μ*m were the most abundant for all beach sediment sites near estuaries, contributing 60.90 %, 52.20 %, and 53.70 % to the total microplastic size class distribution in Bilanhlolo Estuary, Kongweni Estuary, and Mhlangeni Estuary respectively (**Figure 6.4**). The beach sediments in sites near Mhlangeni Estuary had a greater abundance (5.60 %) of smaller microplastics (20 – 149 *μ*m) relative to total microplastic size distribution than Bilanhlolo Estuary (0.70 %), and Kongweni Estuary (0.90 %) (**Figure 6.4**). Similarity percentage analysis (SIMPER) showed that the average dissimilarity between Bilanhlolo Estuary and Kongweni Estuary (22.89 %) was largely due to the dissimilarity in the size classes 300 – 999 *μ*m (30.27 %), 150 – 299 *μ*m (25.68 %), and 2500 – 4999 *μ*m (21.39 %). The average dissimilarity between Bilanhlolo Estuary and Mhlangeni Estuary (30.21 %) was predominantly as a result of the dissimilarity in the size classes 300 – 999 *μ*m (30.39 %), 150 – 299 *μ*m (25.73 %), and 2500 – 4999 *μ*m (19.07 %). The average dissimilarity between Kongweni Estuary and Mhlangeni Estuary (23.98 %) was largely attributed to dissimilarities between the size classes 2500 – 4999 *μ*m (27.48 %), 150 – 299 *μ*m (24.26 %), and 1000 – 2499 *μ*m (19.65 %).

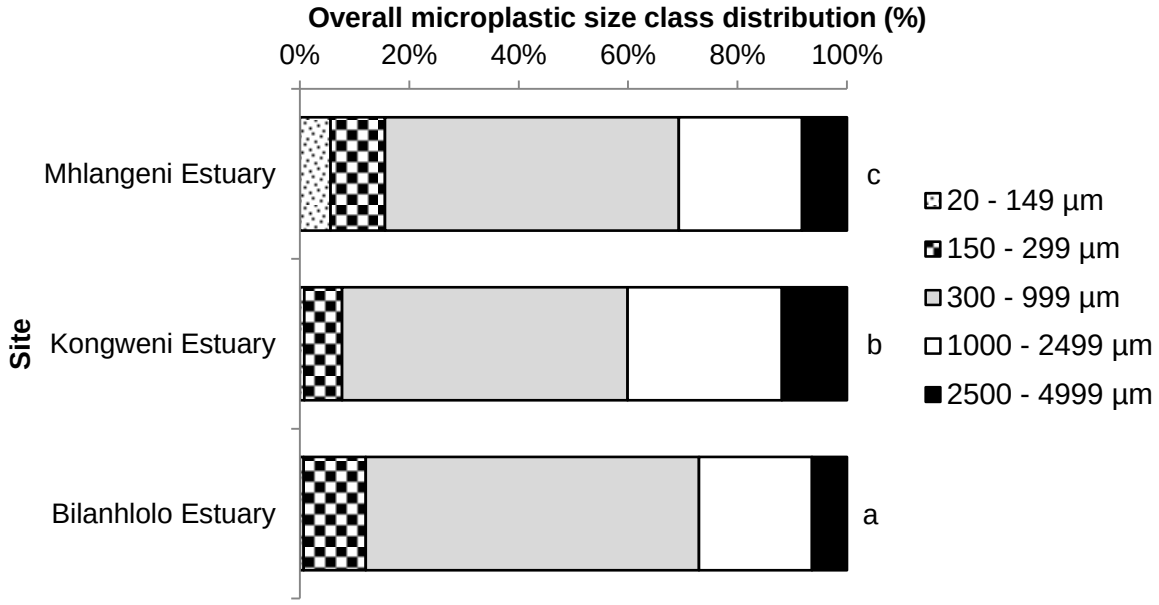

Figure 6.4: Overall microplastic size class (*µ*m) distribution (%) (sites at increasing distance North and South of estuary mouth combined) in beach sediment samples near estuary (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary). Lowercase letters indicate PERMANOVA pairwise comparison significant differences.

Square-root transformed microplastic size class (*µ*m) distribution (%) was significantly different between North and South sites within each estuary (PERMANOVA pairwise comparison: Bilanhlolo Estuary: t = 2.62, $p < 0.001$; Kongweni Estuary: t = 2.52, $p < 0.001$; Mhlangeni Estuary: t = 2.60, $p < 0.001$) (**Figure 6.5**). Square-root transformed microplastic size class (*µ*m) distribution (%) showed that microplastic size class (*µ*m) distribution was also significantly different in some sediment sites at increasing distances North and South of each estuary mouth (**Figure 6.5**). Microplastic size class distribution (%) was not significantly different at increasing distances (m) North or South of Mhlangeni Estuary (**Figure 6.5a**), except at site 2000 m S of the Mhlangeni Estuary mouth which was significantly different in terms of microplastic size class distribution (%) than the sites closer to the estuary mouth (500 m South) (**Figure 6.5a**), showing a much greater relative proportion of microplastics in the size class 20 – 149 *µ*m (18.50 %), and 150 – 299 *µ*m (22.3 %) than other sites near the Mhlangeni Estuary mouth (**Figure 6.5a**). Sediment sites North of the Kongweni Estuary mouth showed no significant difference in microplastic

size class (%) with an increase in distance away from the estuary mouth (**Figure 6.5b**). All sediment sites near Kongweni Estuary mouth showed a similar proportion of microplastics in the size class 300 – 999 μm (**Figure 6.5b**), however the site 500 m S of the Kongweni Estuary mouth had a higher proportion of larger microplastics (2500 – 4999 μm) than other sites (**Figure 6.5b**). Sediment sites near Bilanhlolo Estuary mouth showed significant differences in microplastic size class distribution (%) at distances (m) increasing North of the estuary mouth (**Figure 6.5c**), but the site nearest to the estuary mouth (500 m North) displayed a greater proportion (3.5 %) of smaller microplastics (20 – 149 μm) (**Figure 6.5c**). Sediment sites further South of the Bilanhlolo Estuary mouth were significantly different to sediment sites closer to the estuary mouth (**Figure 6.5c**), but all showed a relatively large proportion of microplastics in the size class 300 – 999 μm (**Figure 6.5c**).

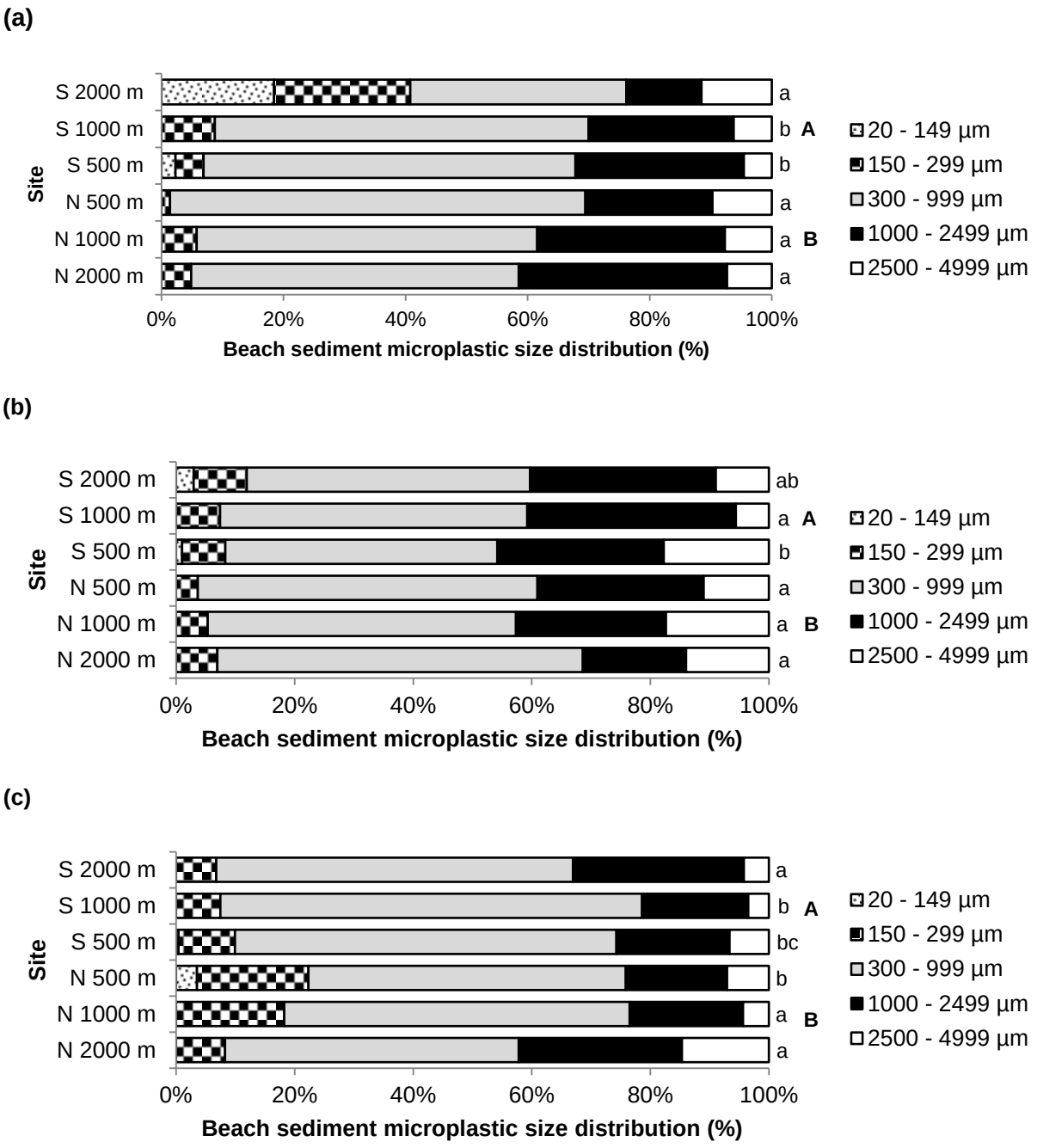

Figure 6.5: Microplastic size class (*µ*m) distribution (%) in beach sediment from sites at increasing distances (500 m, 1000 m, and 2000 m) North (N) and South (S) away from each estuary mouth. Mhlangeni Estuary sites (a), Kongweni Estuary sites (b) and Bilanhlolo Estuary sites (c). Uppercase letters indicate PERMANOVA significant differences between North and South stations. Lowercase letters indicate PERMANOVA pairwise comparison significant differences between stations at increasing distances North and South away from each estuary mouth.

6.5. Discussion

Beach sediment displayed higher abundances of microplastics (microplastics.m^{-2}) at sites nearer to each estuary mouth than sites further away within North and South groups (**Figure 6.1**). Therefore, the hypothesis stating that microplastic abundance (microplastics.m^{-2}) will change with increasing distance from each estuary mouth was accepted. The only exception to this trend was the higher microplastic abundance at the site 2000 m South of the Mhlangeni Estuary mouth (**Figure 6.1**). This may be due to the site being located adjacent to the Vungu Estuary mouth (approximately 250 m North), which may also be a source of microplastics to the marine environment. The increase of microplastic abundances in beach sediment sites closer to each estuary mouth than sites further away indicate that estuaries may be a point source of microplastic pollution to the marine environment. Similar patterns of microplastic abundances in beach sediment decreasing further North and South of the study areas were reported for selected eThekwini estuary mouths and adjacent beach environments (Naidoo *et al*., 2015). Naidoo *et al.* (2015) reported higher quantities of microplastic pollution at sites 500 m North and South on the adjacent coastline of estuary mouth than at sites further away. Increased microplastic abundances in sediment at sites South of each estuary mouth as opposed to sites North of each estuary mouth, may be due to the influence of the prevailing Agulhas current which flows in a southerly direction along the KwaZulu-Natal coastline (Driver *et al*., 2004). However, the predominant inshore current along KwaZulu-Natal flows in a northerly direction (Guastella, 1994), indicating that the distribution of microplastics along the studied beaches cannot be inferred from the nearby inshore currents.

Estimates of microplastic abundances in **Chapter 6** (8760.72 ± 3024.01 - 20267.04 ± 6439.03 microplastics.m^{-2}) (**Figure 6.1**) were much larger than estimated in previous investigations. Nel and Froneman (2015) reported microplastic abundances in beach sediment of 688.9 ± 348.2 – 3308 ± 1449 microplastics.m^{-2} at 21 sites along the south eastern coast of South Africa. In a more recent study, Nel *et al*., (2017) reported beach sediment microplastic abundances between 86.67 ± 48.68 to 754.7 ± 393 particles.m^{-2} between 16 sites along the entire South African coastline. Further abroad, Fok and Cheung (2015) found average microplastic abundances of 5595 items/m^2 from 25 beach sediment sites near the Pearl River Estuary, China.

Comparisons of the results of **Chapter 6** with those of previous studies are difficult due to the different sampling methodologies and units reported (Besley *et al*., 2017). In addition, the density separation technique used may not have extracted microplastics with a density greater than 1.2 $g.cm^{-3}$ (Van Cauwenberghe *et al*., 2015). However, the results obtained provide important insight into previously unreported microplastic pollution loads along a small stretch of KZN coastline adjacent to estuary mouths.

Beach sediments have been cited as an important sink of microplastic pollution (Hildago Ruz *et al*., 2012; Nel and Froneman, 2015, Besley *et al*., 2017). Microplastic abundances in beach sediment may be influenced by a number of factors, such as tide (Santana *et al*., 2016), wind (Besley *et al*., 2017), and distance from point source (Wang *et al*., 2016). Microplastics may be resuspended from sediment in to the water column during events such as storm surges (Wegner *et al*., 2012). There are some models of microplastic deposition in beach sediment that are currently in development (Wang *et al*., 2016). However, due to the large number of factors that may influence microplastic deposition in beach sediment, it is currently not feasible to make any inferences regarding the patterns seen in this study (**Figure 6.1**).

The results of **Chapter 6** show that microplastic fibres contribute a large proportion of microplastic types in beach sediment (79.90 %) (**Figure 6.2**). These results are similar to previous investigations of microplastic pollution in beach sediment. Nel and Froneman (2015) found that microplastic fibres contributed a large proportion to the total microplastic type composition in 21 sites along the south eastern coastline of South Africa. Naidoo *et al*. (2015) also found a large proportion of microplastic fibres in the beach sediment adjacent to five eThekwini estuaries. International studies have shown similar results, with large quantities of microplastic fibres found in Solvenia shores (75 %) and infratidal regions (90 %) (Laglbauer *et al*., 2014). Lots *et al*. (2017) found that the majority of microplastics in beach sediment across 23 European beaches consisted mostly of fibrous microplastics (98.7 %). Microplastic fibres were more abundant in sites nearer to each estuary mouth (**Figure 6.3**). The hypothesis that microplastic type composition (%) will change with increasing distance (m) from the estuary mouth is therefore accepted. The microplastic fibre pollution in beach sediment near each estuary mouth can be as a result of sewage

input rather than fragmentation of larger plastic items in the marine environment (Alomar *et al*., 2016). However, links between microplastic shape and source can only be alluded to with these results; further testing is required to determine microplastic polymers and their definite sources. The results of **Chapter 6** show that microplastic sizes in beach sediment range between relatively short distances (**Figure 6.5**). Therefore, the hypothesis that microplastic size class distribution differs between sediment sites at sites up to 2000 m North and South of each estuary mouth is accepted. An important observation from these results is that microplastic abundances, types, and sizes vary significantly within relatively short distances in beach sediment.

6.6. Conclusion

Microplastic abundances in sediment vary between relatively short distances within beaches, highlighting the highly dynamic factors influencing microplastic particle deposition and turnover within these areas. Microplastic abundances were significantly different at sites at increasing distances North and South of each estuary mouth. Based on the results of **Chapter 6**, in conjunction with those of Naidoo *et al*. (2015), it is recommended that future investigations sample sites 500 m North and South of estuary mouths to determine levels of microplastic input from estuary mouths into coastal environments. Larger distances between sample sites are cautioned against, particularly along the KZN coastline, for the following reasons: 1) unstudied or undocumented water sources (e.g. stormwater runoff) and 2) other estuaries may have an impact on microplastic loading in a particular area (such as the site 2000 m South of Mhlangeni Estuay, which had increased quantities of microplastics due to the close proximity to Vungu Estuary mouth).

Microplastics in beach sediment were found to largely consist of microplastic fibres, indicating that land-based microplastic pollution is a significant source of marine microplastic pollution. Although sediment is considered to be a sink of microplastic pollution, microplastic particles may re-enter marine environments via resuspension, potentially increasing microplastic pollution in marine water columns in a particular area. Patterns of microplastic pollution in the water column and sediments are erratic as they are influenced by a number of abiotic factors such as wind, tidal actions, and

ocean currents (Santana *et al*., 2016). Microplastics may accumulate in sediment in densities far greater than those in the water column and these values cannot be used to extrapolate microplastic abundances for what is available to organisms for ingestion. The limitations and lack of standardisation throughout the use of microplastic sampling methodologies highlight the need for a SOP for microplastic sampling, or the development of a new rapid and accurate procedure of microplastic sampling.

CHAPTER 7: RAPID BIOASSESSMENT OF MICROPLASTIC POLLUTION IN KWAZULU-NATAL COASTAL ENVIRONMENTS USING THE BROWN MUSSEL, *PERNA PERNA* (LINNEAUS, 1758)

7.1. Abstract

Microplastic pollution has become the focus of a large body of research due to the ubiquity throughout marine environments and potential danger to organisms which ingest them. As microplastic pollution research is a relatively new area of interest, procedures of microplastic sampling are limited and not harmonized. A recent recommendation was made to potentially use mussels as biomonitors of microplastic pollution in marine environments as opposed to monitoring abiotic matrices. Mussels have already been successfully used to monitor heavy metal pollution in South African marine environments due to their sedentary lifestyle and filter-feeding strategy. The aim of this study was to determine and compare spatial differences in microplastic pollution in the mussel species, *Perna perna*, originating from selected temporarily open/closed estuaries (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) during an open mouth phases at increasing distance 500 m, 1000 m, and 2000 m North and South on the coastline adjacent to each estuary mouth. The overall mean microplastic abundance per mussel was 2.22 ± 0.79 microplastics.g^{-1} tissue (w/w). Microplastics were significantly more abundant in mussels from sites nearer to estuary mouths than those further away. Microplastic fibres were the most dominant microplastic type found in *P. perna* (61.80 %). The results of this study highlight that microplastic pollution is entering South African marine food webs. The results of this study also highlight the potential for *P. perna* to be used as biomonitors of coastal microplastic pollution in marine environments. However, further research is required to develop standardised international protocols for mussel microplastic monitoring.

Keywords: microplastics, *Perna perna*, biomonitoring, South Africa, baseline survey

7.2. Introduction

Due to their sedentary lifestyles and filter-feeding strategy, bivalve molluscs, including mussels, have been used worldwide as biomonitors of marine pollutants (Degger *et al*., 2011). Marine mussels are found along rocky shore habitats and are considered as one of the best biological indicators of environmental degradation (Kacar *et al*., 2016). In South Africa, mussels have been used to monitor levels of heavy metal pollution in the marine environment since 1974 (Greenfield *et al*., 2014). Their sedentary lifestyle, filter-feeding strategy, and extensive range along the South African coastline allow for spatial and temporal trends of marine pollutants to be rapidly assessed (Degger *et al*., 2011; Vosloo *et al*., 2012). Mussel Watch Programs (MWPs) in South Africa almost exclusively focus on heavy metal pollution in marine environments (Greenfield *et al*., 2014). Due to the enormous success of marine pollution biomonitoring using mussels, there have been recommendations to use mussels as biomonitors of microplastic pollution in marine environments.

Microplastics are any plastic particles < 5 mm in largest size dimension (Lusher *et al*., 2017) and have been shown to be ubiquitous throughout the marine environment. Microplastics are small enough to become available for ingestion by marine invertebrates such as mussels (Li *et al*., 2015). Numerous studies have investigated microplastic content in several species of wild mussels (De Witte *et al*., 2014; Mathalon and Hill, 2014; Van Cauwenberghe and Jannsen 2014; Van Cauwenberghe *et al*., 2015). As mussels are ecologically important and a major seafood resource (Li *et al*., 2015), it is important to classify microplastics in mussels in the interest of ecosystem and human health (Vandermeersch *et al*., 2015; Phuong *et al*., 2017). Mussels may also be useful to monitor smaller microplastics which recommended procedures (**Chapter 3**) may underestimate (Lusher *et al*., 2017).

A large majority of terrestrial-based effluents reach rocky shore environments via rivers and estuaries where mussel populations are concentrated (Greenfield *et al*., 2014). Marine mussels in rocky shore habitats can therefore be used as biomonitors of marine microplastic pollution derived from estuaries (Santana *et al*., 2016). Several methodologies exist to monitor microplastic pollution in marine environments by sampling of abiotic matrices (e.g. water and sediment) (Hildago-Ruz *et al*., 2012;

Besley *et al*., 2017). However, spatial and temporal patterns of microplastic pollution can vary extensively due to the large number of influencing abiotic variables, such as wind, climate and tides (Santana *et al*., 2016). The use of mussels as biomonitors of microplastic pollution in marine environments enables the identification of the microplastics most likely ingested by mussels (Santana *et al*., 2016), as well as the potential risk these mussels pose to humans who utilize the mussels as a food source. The brown mussel *Perna perna*, belonging to the family Mytilidae, is indigenous to South Africa (Zardi *et al*., 2006) and dominates the KwaZulu-Natal coast line along inter-tidal zones (Zardi *et al.*, 2006). *P. perna* typically have quicker growth rates than other mussel species (Oliveira *et al*., 2016) and are harvested from natural populations on the KwaZulu-Natal (KZN) coast by subsistence fishermen throughout the year (Yap *et al*., 2004).

The aim of **Chapter 7** was to determine and compare spatial differences in microplastic pollution in the mussel species, *P. perna*, originating from selected temporarily open/closed estuaries (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary) during an open mouth phase at increasing distances of 500 m, 1000 m, and 2000 m North/South on the coastline adjacent to each estuary mouth. Objectives included comparisons of 1) microplastic abundances (microplastics.g^{-1} tissue w/w) in *P. perna* at stations 500 m, 1000 m, and 2000 m North/South on the coastline adjacent to each estuary mouth during an open mouth phase, 2) microplastic type composition (%) in *P. perna* at stations 500 m, 1000 m, and 2000 m North/ South on the coastline adjacent to each estuary mouth during an open mouth phase, and 3) microplastic size class (μm) distribution (%) in *P. perna* at stations 500 m, 1000 m, and 2000 m North/South on the coastline adjacent to each estuary mouth during an open mouth phase. It was hypothesized that there would be a difference in microplastic abundances (microplastics.g^{-1} tissue w/w), microplastic type composition (%), and microplastic size class (μm) distribution (%) in *Perna perna* at stations 500 m, 1000 m, and 2000 m North/South on the coastline adjacent to each estuary mouth during an open mouth phase.

7.3. Methods and Materials

7.3.1. Data collection

All data collection took place during a three day period in January 2017 at low tide. *Perna perna* specimens (50 – 60 mm) were collected from the rocky shores at stations 500 m, 1000 m, and 2000 m North and South adjacent to each estuary mouth (**Chapter 1: Figure 1.1**) (Geographic coordinates: **Appendix A: Table A 2**). Mussel samples were collected in accordance with the field permit [RES2017/71] for the purposes of scientific investigations or practical experiment in terms of Section 83 of the Marine Living Resource Act (Act No. 18 of 1998) issued by the Department of Agriculture, Forestry and Fisheries of the Republic of South Africa (**Appendix B**). The mussels were detached from the rocks by severing the byssal threads with a titanium blade and placed into individual plastic bags. A total of five *P. perna* specimens were collected from each site. Due to a lack of rocky shore habitat, no mussels were collected at the station 500 m North of Kongweni Estuary mouth. Mussels were preserved immediately on-ice after collection to minimize microplastic loss via gut evacuation (Lusher *et al*., 2017). Once collected, the mussels were transported, transferred to and stored in a freezer (-20 °C) for a minimum of twenty-four hours. The freezing of the mussels helps to break down tissue, aiding in greater digestion efficiency (Catarino *et al*., 2016). Before analysis, mussels were removed from the freezer and individually placed into aluminium ‘boats’ and allowed to completely thaw at room temperature (< two hours) (Catarino *et al*., 2016). Mussels were removed of all fouling organisms and detritus using a titanium blade and stainless steel scrubbing brush to reduce the possibility of contamination of samples of micoplastics not present within the mussel tissue. The byssal threads of each mussel were removed as microplastics may adhere to the byssal threads, thereby leading to overestimations of microplastics within mussel tissue (Phuong *et al*., 2017). Mussels were weighed whole to the nearest 0.001 g. Soft tissue was dissected out of the mussel (Phuong *et al*., 2017). Shell tissue was thereafter weighed to determine wet weight of soft tissue by difference of mass (Catarino *et al*., 2016). A 10 % (w/w) potassium hydroxide (KOH) solution was prepared by dissolving 10 g KOH pellets (The Great Supply (Pty) Ltd., South Africa) in a glass beaker containing 90 g (90 mL) distilled water and thoroughly mixed with a previously acid-

washed glass rod until all the KOH had visibly dissolved. The process was repeated until the desired quantity of 10 % (w/w) KOH solution was prepared. The soft tissue of each mussel was placed in individual jars containing 250 mL of 10 % (w/w) KOH solution. Five blank samples (containing no mussel tissue) were also subjected to the same procedure to account for microplastic contamination (Catarino *et al*., 2016; Lusher *et al*., 2017). The jars were sealed and placed in an oven 60 ºC for twenty-four hours (Dehaut *et al*., 2016). Thereafter, the contents of each jar was vacuum-filtered (Catarino *et al*., 2016) through a 5.0 *µ*m polycarbonate track-etched ISOPORE™ membrane filter (47 mm ⌀) (Merck Millipore Ltd.). Filters were each placed into clean plastic petri dishes and covered with aluminium foil (Catarino *et al*., 2016). The samples were dried in an oven at 60 ºC for twenty-four hours (Catarino *et al*., 2016), thereafter removed from the oven and stored at room temperature until microscope analysis.

7.3.2. Microplastic analysis

Filtered mussel samples were viewed up to 40 X magnification using a Nikon© AZ100 stereomicroscope. Microplastics were distinguished from non-plastic particles using previous recommendations (**Chapter 3: Table 3.3**). Once a particle had been identified as plastic, the maximum size dimension of each particle was measured (*µ*m), and microplastic type was recorded. Microplastic abundances (microplastics.g^{-1} tissue w/w) were calculated for each sediment sample as follows:

$$(particles.g^{-1}\ tissue\ (w/w)) = \frac{Number\ of\ particles}{Soft\ tissue\ mass\ (g\ wet\ weight)}$$

7.3.3. Statistical analyses

Statistical significance was set at α = 0.05. Absolute *p* values were reported for all values > 0.001. Where *p* values were less than 0.001, the significance was reported as $p < 0.001$. Univariate statistics were conducted using IBM SPSS Statistics® (version 23 for Microsoft® Windows® 10). A fully-nested analysis of variance (ANOVA) was used to compare microplastic abundance in *P. perna* (microplastics.g^{-1}

tissue w/w) among rocky shore sites near each estuary, direction (North/South) of each estuary mouth, and distances (500 m, 1000 m, and 2000 m) within directions after $log_{10}(x)$-transformed data met assumptions of normality of residuals (Shapiro Wilk test statistic = 0.89, $p > 0.05$), and homogeneity of variances (Levenes test statistic = 1.31, $p > 0.05$). Post hoc Tukey HSD comparisons were used to determine significant differences of microplastic abundances in *P. perna* (microplastics.g^{-1} tissue w/w) among sites. A Pearson correlation was used to correlate microplastic abundances in mussels (microplastics.g^{-1} tissue w/w) with microplastic abundances in sediment (microplastics.m^{-2}) (**Chapter 6**) at each site. To compare variability of microplastic abundance data between sediment (**Chapter 6**) and mussels, coefficients of variation (%) were calculated for each data set and compared using a modified One-way ANOVA. The coefficient of variance is the ratio of the standard deviation (SD) to the mean, allowing the variability of data to be compared between two data sets with different unit values.

Multivariate statistics were conducted using Plymouth Routines in Multivariate Ecological Research (PRIMER) version 6 and permutational multivariate analysis of variance (PERMANOVA) package to determine significant differences in microplastic type composition (%) in *P. perna* at rocky shore stations between estuaries, directions and distances. Data was square root transformed to weight the contributions of common and 'rare' microplastic types. A Bray Curtis matrix of similarity was constructed with the square root transformed data. A nested PERMANOVA and post hoc PERMANOVA pairwise comparisons were conducted on the Bray Curtis matrix of square root transformed data to determine if microplastic types in mussels were significantly different among sites. A similarity percentage analysis (SIMPER) was used to determine percentage similarities and dissimilarities between microplastic types in mussels among sites. The multivariate statistical analysis was repeated for microplastic size class (μm) distribution (%) in rocky shore *P. perna* samples.

7.4. Results

7.4.1. Microplastic abundance in Perna perna

Microplastics were recorded in all sampled *P. perna* individuals. $Log_{10}(x)$-transformed microplastic abundances in *P. perna* (microplastics.g^{-1} tisse w/w) were significantly different among rocky shore sites near each estuary mouth (ANOVA: $F_{(2, 84)} = 149.43$, $p < 0.001$), between directions North and South of each estuary mouth (ANOVA: $F_{(3, 68)} = 28.08$, $p < 0.001$), and at increasing distances North and South away from each estuary mouth (ANOVA: $F_{(11, 68)} = 34.44$, $p < 0.001$). Mussels at rocky shore sites near Kongweni Estuary mouth contained greater mean quantities of microplastics (3.10 ± 0.34 microplastics.g^{-1} tissue w/w) than mussels near Mhlangeni Estuary (2.10 ± 0.21 microplastics.g^{-1} tissue w/w) and Bilanhlolo Estuary (1.67 ± 0.26 microplastics.g^{-1} tissue w/w) (Tukey HSD post hoc comparison: $p < 0.05$ for all interactions) (**Figure 7.1**). Mussels from sites North and South of the Kongweni Estuary mouth showed no significant differences in microplastic abundance (Tukey HSD post hoc comparison: t = -166, $p = 0.56$) (**Figure 7.1**), but there was significant differences in microplastic abundance in mussels from sites North and South of Mhlangeni Estuary mouth (Tukey HSD post hoc comparison: t = 7.73, $p < 0.001$), and Bilanhlolo Estuary mouth (Tukey HSD post hoc comparison: t = -4.65, $p < 0.001$) (**Figure 7.1**). Microplastic abundances were significantly greater at sites South of the Mhlangeni Estuary mouth, and greater at sites North of the Bilanhlolo Estuary mouth (**Figure 7.1**). Microplastic abundance within mussels tended to decrease with an increase in distance (m) North and South of each estuary mouth (**Figure 7.1**), except for the site 2000 m South of Mhlangeni Estuary mouth, where mussels showed an increased microplastic abundance (microplastics.g^{-1} tissue w/w) than sites nearer to the estuary mouth (**Figure 7.1**). There was no correlation between $log_{10}(x)$-transformed sediment and mussels abundances (Pearson's correlation = 0.306, $p > .05$) between sites within each estuary system.

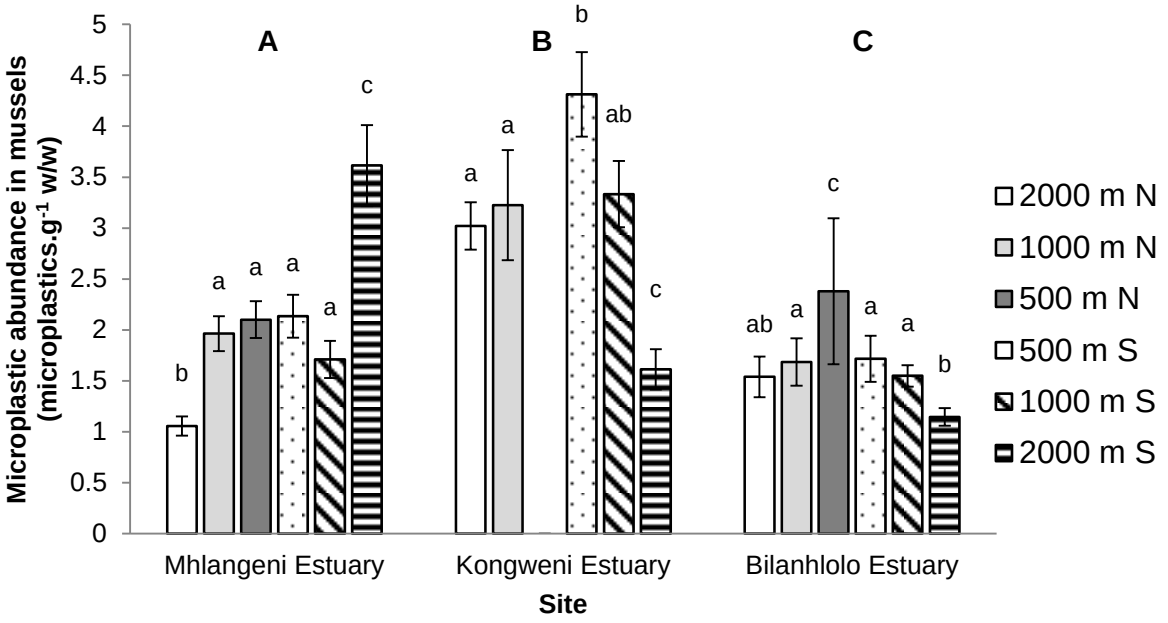

Figure 7.1: Mean microplastic abundance (microplastic.m^{-2}) in *Perna perna* specimens at increasing distances (m) North (N) and South (S) away from each estuary mouth (n = 5). Uppercase letters indicate significance differences between estuarine systems. Lowercase letters indicate Tukey HSD post hoc significance differences between sites within each estuary system. Error bars represent ± 1 SD. No data at site 500 m North of Kongweni Estuary mouth due to absence of rocky shores.

The coefficients of variance were significantly different between sediment and mussels (ANOVA: F = 3.99, df = 1, p = 0.047) (**Figure 7.2**). Mean coefficients of variation were greatest for microplastic abundance in sediment (38.32 ± 30.65 %) in comparison to mussels (30.69 ± 17.86 %). Microplastic abundances in sediment were far more variable than in mussels, with a larger coefficient variation range and a greater number of outliers within samples (**Figure 7.2**). The calculated coefficients of variance for total data sets were 48.97 % for sediment (14046.14 ± 6878.01 microplastics.m^{-2}), and 35.45 % for mussels (2.22 ± 0.79 microplastics.g^{-1} w/w).

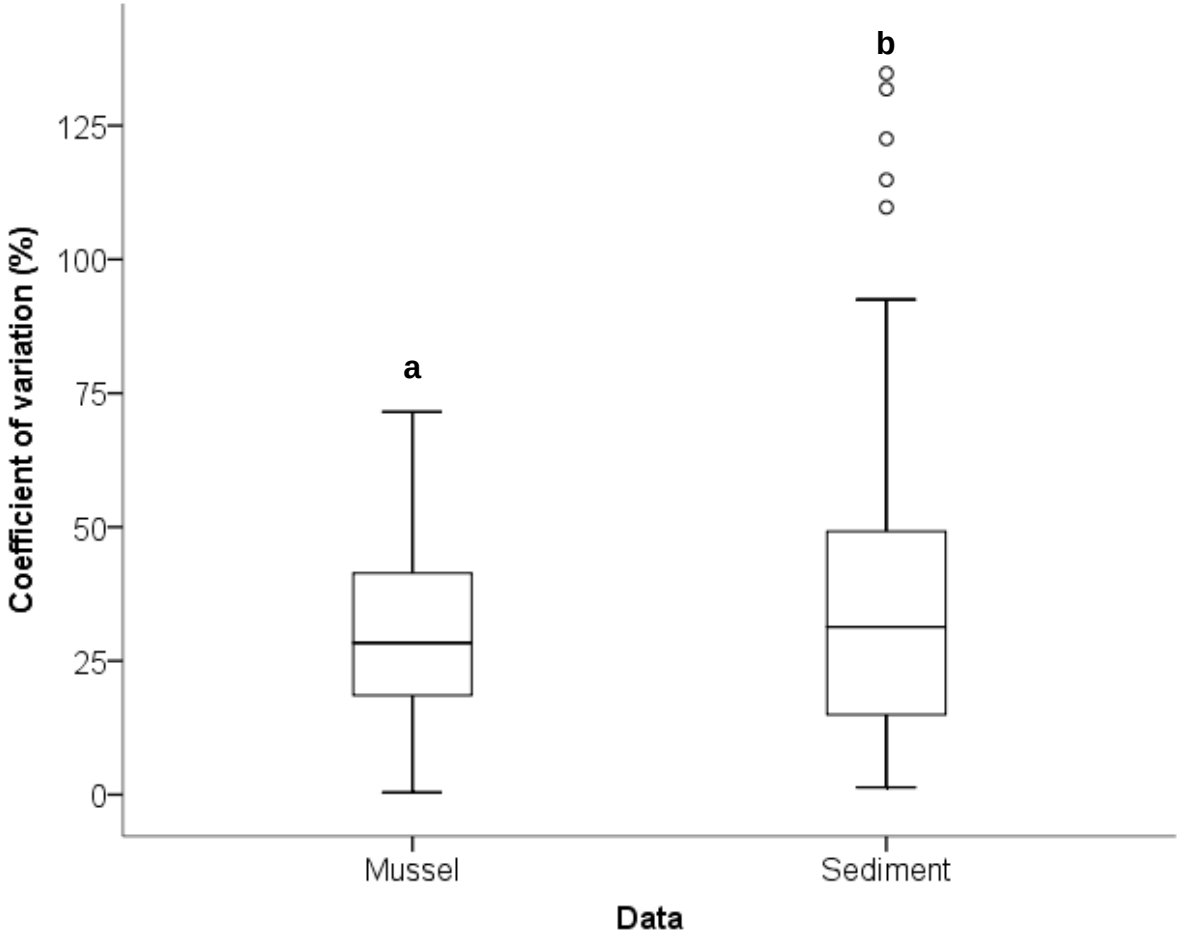

Figure 7.2: Coefficients of variance (%) of microplastic abundances in mussels (n = 85) and sediment (n = 90). Lowercase letters indicate significant differences (ANOVA).

7.4.2. Microplastic type composition in Perna perna

Square-root transformed microplastic type composition (%) was significantly different in *P. perna* among combined sites near each estuary mouth (PERMANOVA: Pseudo-F = 8.24, df = 2, $p < 0.001$), between sites North and South of each estuary mouth (PERMANOVA: Pseudo-F = 4.14, df = 3, $p < 0.001$), and among sites at increasing distances (500 m, 100 m, and 2000 m) North and South of each estuary mouth (PERMANOVA: Pseudo-F = 3.72, df = 11, $p < 0.001$). There was a significant difference in microplastic type composition (%) in *P. perna* specimens between all sites (PERMANOVA pairwise comparison: Bilanhlolo Estuary and Kongweni Estuary: t = 3.51, $p < 0.001$; Bilanhlolo Estuary and Mhlangeni Estuary: t = 2.81, $p = 0.003$; Kongweni Estuary and Mhlangeni Estuary: t = 1.97, $p = 0.013$) (**Figure 7.3**).

Microplastic fibres were the most dominant microplastic type in sites within combined sites near estuaries (61.80 %), contributing 55.60 %, 55.20 %, and 77.30 % to the total microplastic abundance in sites near Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary respectively (**Figure 7.3**). Fragments were the second most abundant microplastic type in mussels (26.20 %), with a relatively small proportion of microplastic film in mussels (8.0 %) (**Figure 7.3**). Similarity percentage analysis (SIMPER) showed that the average dissimilarity between combined sites near Bilanhlolo Estuary and Kongweni Estuary (33.76 %) was largely attributed to the dissimilarity in fragments (41.98 %), fibres (22.00 %), and film (21.15 %). The average dissimilarity between combined sites near Bilanhlolo Estuary and Mhlangeni Estuary (32.72 %) was largely as a result of fragments (39.35 %), fibres (24.89 %), and film (24.02 %). The average dissimilarity in combined sites near Kongweni Estuary and Mhlangeni Estuary (26.26 %) was due to the dissimilarities between fragments (28.00 %), fibres (26.06 %), and film (25.15 %).

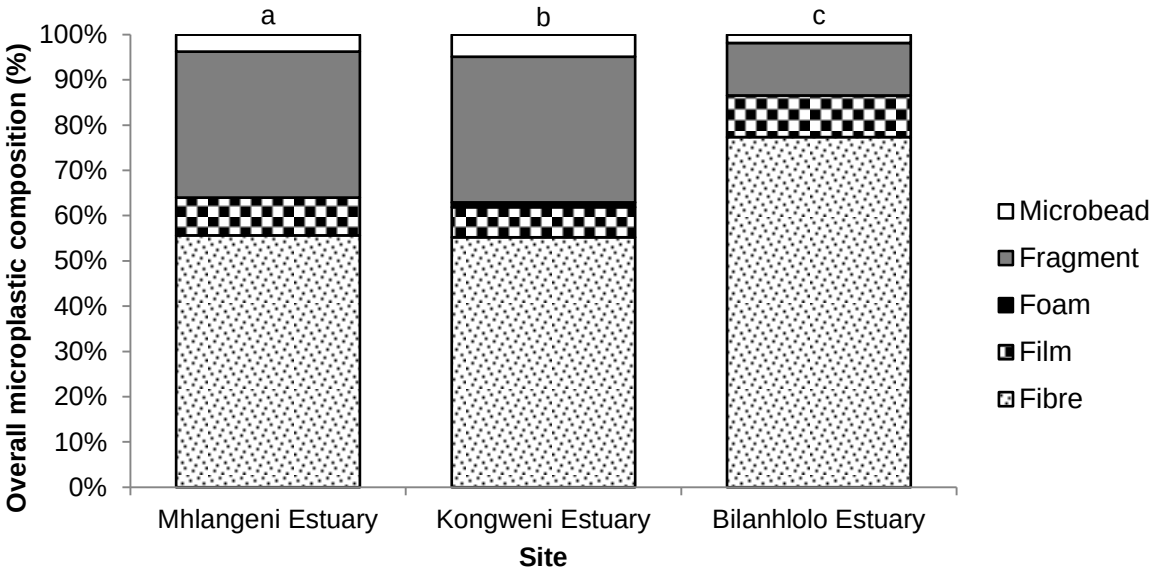

Figure 7.3: Overall microplastic type composition (%) in *Perna perna* from rocky shore sites at increasing distances (500 m, 1000 m, and 2000 m) North and South of estuary mouth combined (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary). Lowercase letters indicate significant differences in microplastic type composition (%) (PERMANOVA).

There was a significant difference in microplastic type composition (%) between directions North and South of Bilanhlolo Estuary mouth (PERMANOVA pairwise comparison: t = 2.92, *p* = 0.005), but no difference in microplastic type composition (%) between directions North and South of Kongweni Estuary mouth (PERMANOVA pairwise comparison: t = 1.22, *p* = 0.256), and between directions North and South of Mhlangeni Estuary mouth (PERMANOVA pairwise comparison: t = 0.61, *p* = 0.688) (**Figure 7.4**). There was no significant differences in microplastic type composition (%) between sites North of Mhlangeni Estuary (PERMANOVA pairwise comparison: *p* > 0.05 for all interactions), or between sites 2000 m and 1000 m South of Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 1.66, *p* = 0.09) (**Figure 7.4a**). There was a significant difference in microplastic type composition (%) between sites 1000 m and 500 m South (PERMANOVA pairwise comparison: t = 2.32, *p* = 0.04) and between sites 2000 m and 500 m South (PERMANOVA pairwise comparison: t = 2.53, *p* = 0.02) of Mhlangeni Estuary (**Figure 7.4a**). There was no significant difference in microplastic type composition (%) between sites 1000 m and 2000 m North of Kongweni Estuary mouth (PERMANOVA pairwise comparison: t = 1.18, *p* = 0.27) (**Figure 7.4b**), but there was a significant difference in all sites South of the Kongweni Estuary mouth (PERMANOVA pairwise comparison: *p* < 0.05 for all interactions) (**Figure 7.4b**). There was no significant differences in microplastic type composition (%) in *P. perna* in rocky shore sites North of Bilanhlolo Estuary or between sites 2000 m and 500 m South, and sites 1000 m and 500 m South (PERMANOVA pairwise comparison: *p* > 0.05 for all interactions) (**Figure 7.4c**), but there was a significant difference in sites 1000 m and 2000 m South of Bilanhlolo Estuary mouth (PERMANOVA pairwise comparison: t = 3.08, *p* = 0.03) (**Figure 7.4c**).

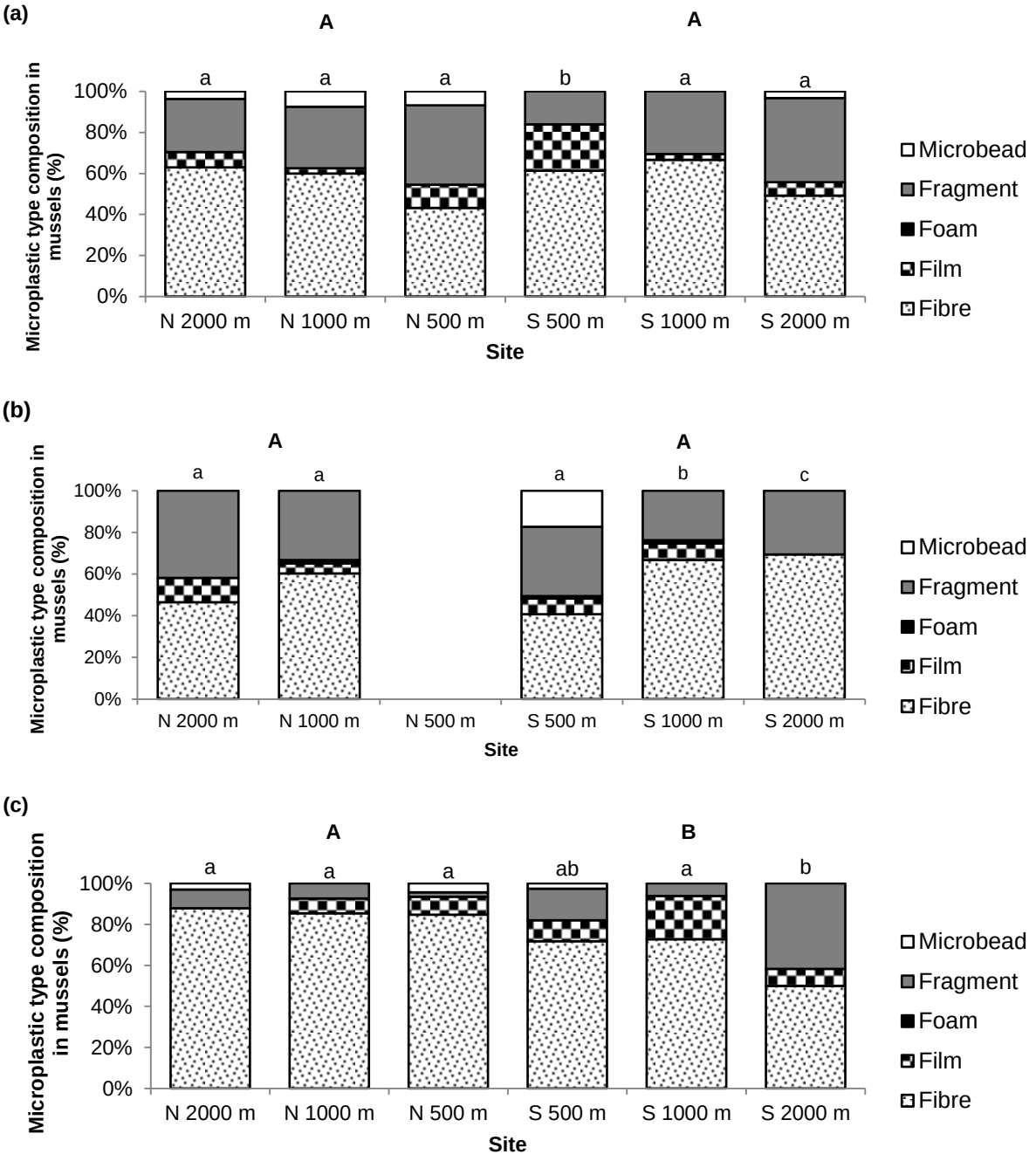

Figure 7.4: Microplastic type composition (%) in *Perna perna* from sites at increasing distances (500 m, 1000 m, and 2000 m) North (N) and South (S) away from each estuary mouth (Mhlangeni Estuary sites (a), Kongweni Estuary sites (b), and Bilanhlolo Estuary sites (c)). Uppercase letters indicate significant differences between North and South sites (PERMANOVA pairwise comparison). Lowercase letters indicate significant differences between sites at increasing distances away from each estuary mouth within North and South groups (PERMANOVA pairwise comparison). No data at site 500 m North of Kongweni Estuary mouth due to absence of rocky shores.

7.4.3. Microplastic size class distribution in Perna perna

Square-root transformed microplastic size class (*μ*m) distribution (%) was significantly different in *P. perna* specimens among combined sites near each estuary mouth (PERMANOVA: Pseudo-F = 8.26, df = 2, $p < 0.001$), between rocky shore sites North and South of each estuary mouth (PERMANOVA: Pseudo-F = 3.81, df = 3, $p = 0.002$), and among sites at increasing distances (500 m, 1000 m, and 2000 m) North and South of each estuary mouth (PERMANOVA: Pseudo-F = 3.03, df = 11, $p < 0.001$). Microplastic size class (*μ*m) distribution (%) was significantly different in *P. perna* specimens in combined sites between Bilanhlolo Estuary and Kongweni Estuary (PERMANOVA pairwise comparison: t = 3.55, $p < 0.001$), and between Bilanhlolo Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 3.05, $p = 0.003$), but not between Kongweni Estuary and Mhlangeni Estuary (PERMANOVA pairwise comparison: t = 1.50, $p = 0.16$). Microplastics in the size class 300 – 999 *μ*m were the most abundant for all mussels in sites near estuaries, contributing 53.20 %, 38.80 %, and 35.10 % to the total microplastic size class distribution in Bilanhlolo Estuary, Kongweni Estuary, and Mhlangeni Estuary, respectively (**Figure 7.5**). The mussels in sites near Mhlangeni Estuary had a greater abundance (31.00 %) of smaller microplastics (20 – 149 *μ*m) relative to total microplastic size distribution than Bilanhlolo Estuary (14.40 %), but similar to Kongweni Estuary (30.40 %) (**Figure 7.5**).

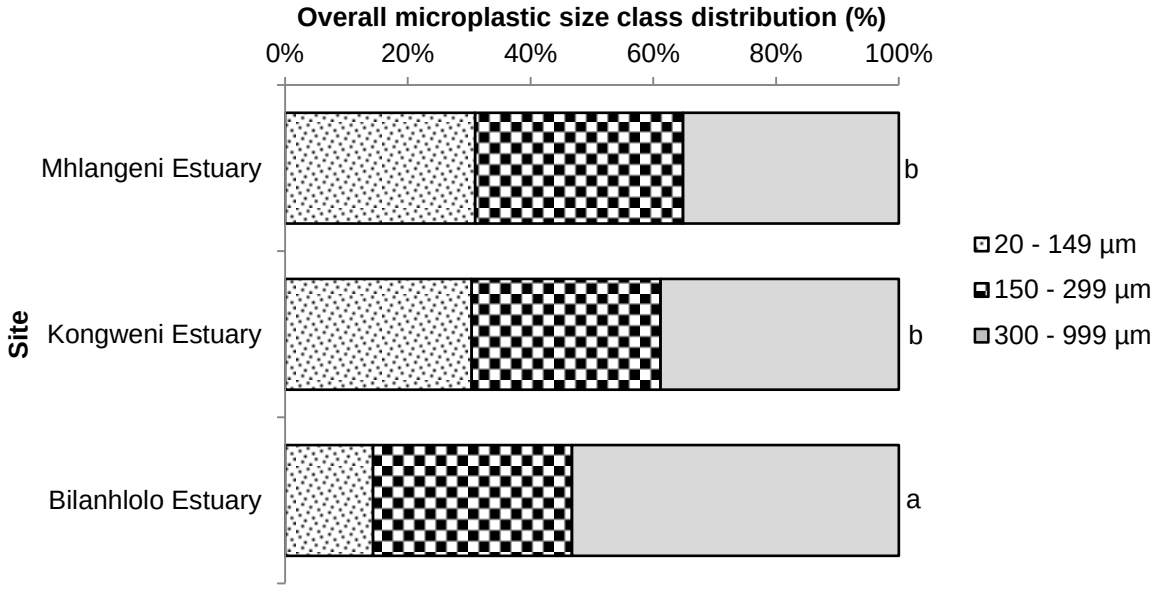

Figure 7.5: Overall microplastic size class (*µ*m) distribution (%) (sites at increasing distance North and South of estuary mouth combined) in *Perna perna* samples near estuary (Mhlangeni Estuary, Kongweni Estuary, and Bilanhlolo Estuary). Lowercase letters indicate significant differences (PERMANOVA).

There was a significant difference in microplastic size class (*µ*m) distribution (%) between sites North and South of Mhlangeni Estuary mouth (PERMANOVA pairwise comparison: t = 2.17, p = 0.04), and between sites North and South of Kongweni Estuary mouth (PERMANOVA pairwise comparison: t = 2.11, p = 0.02), but not between sites North and South of Bilanhlolo Estuary mouth (PERMANOVA pairwise comparison: t = 1.66, p = 0.11) (**Figure 7.6**). Microplastic size class (*µ*m) distribution (%) was not significantly different at increasing distances North and South away from Mhlangeni Estuary mouth (PERMANOVA pairwise comparison: $p < 0.05$ for all interactions), except at site 2000 m South of the Mhlangeni Estuary mouth (**Figure 7.6a**). This site was significantly different in terms of microplastic size class (*µ*m) distribution (%) than the sites closer to the estuary mouth (500 m South) (PERMANOVA pairwise comparison: t = 2.39, p = 0.008), showing a much greater relative proportion of microplastics in the size class 300 - 999 *µ*m (31.10 %) than other sites near the Mhlangeni Estuary mouth (**Figure 7.6a**). Mussels from sites 1000 m and 2000 m North of the Kongweni Estuary mouth showed no significant difference in microplastic size class (*µ*m) distribution (%) (PERMANOVA pairwise

comparison: t = 1.49, p = 0.15). Mussels from sites South of the Kongweni Estuary mouth showed similarly large proportions of larger microplastics (300 – 999 μm) (**Figure 7.6b**), but showed a significant difference between sites 1000 and 2000 m South (PERMANOVA pairwise comparison: t = 2.63, p = 0.04) which was largely attributed to the differences in microplastic size class of 150 – 299 μm (**Figure 7.6b**). Mussels from sites near Bilanhlolo Estuary mouth showed no significant differences in microplastic size class (μm) distribution (%) at distances (m) increasing North of the estuary mouth (PERMANOVA pairwise comparison: $p < 0.05$ for all interactions), but the furthest from the estuary mouth (2000 m North) displayed a greater proportion (15.20 %) of smaller microplastics (20 – 149 μm) (**Figure 7.6c**). Mussels from sites further South of the Bilanhlolo Estuary mouth had significantly different size class composition to mussels from sites closer to the estuary mouth (PERMANOVA pairwise comparison: t = 3.22, p = 0.004), but all showed a relatively large proportion of microplastics in the size class 300 – 999 μm (**Figure 7.6c**).

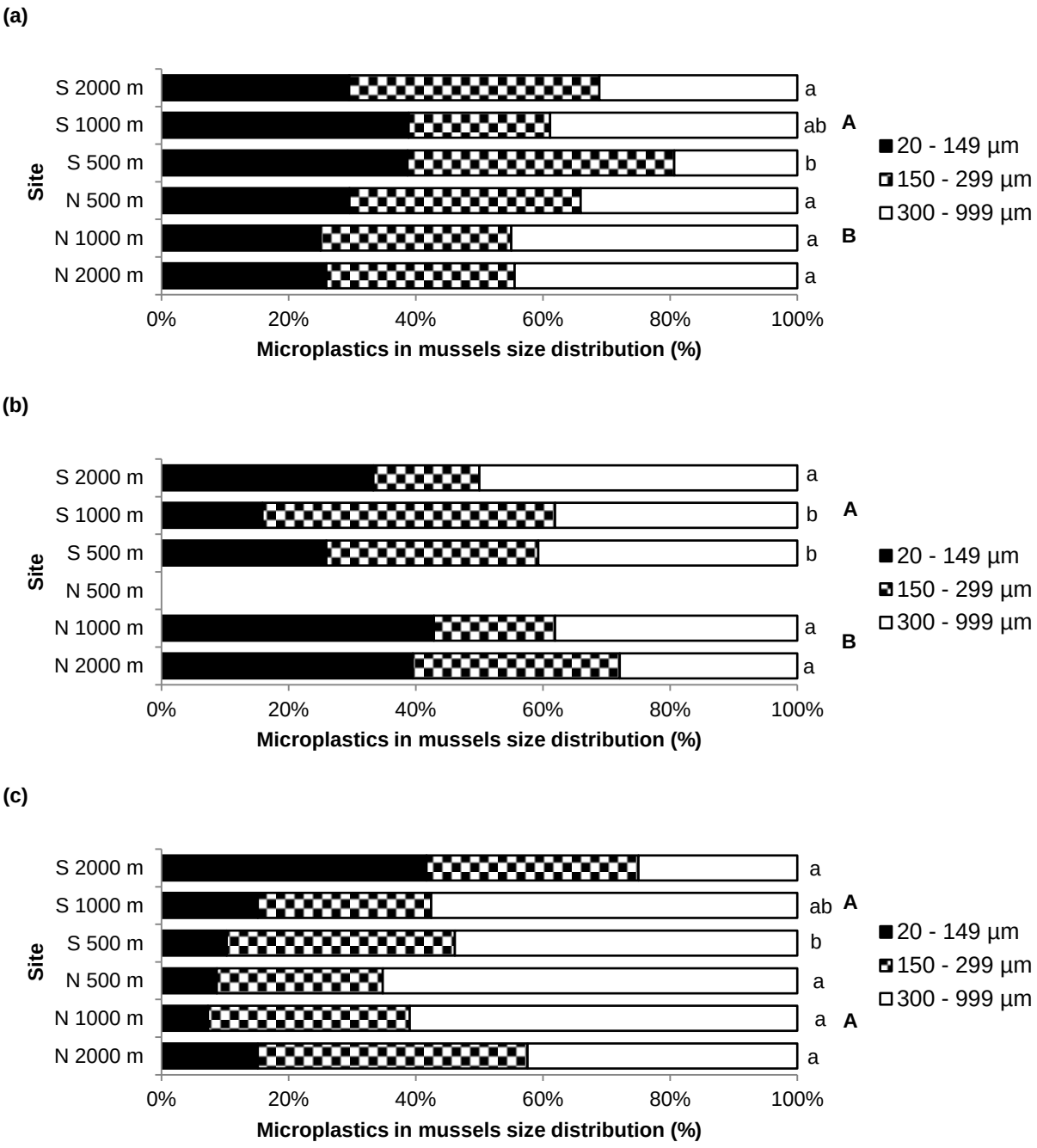

Figure 7.6: Microplastic size class (*µ*m) distribution (%) in *Perna perna* from sites at increasing distances (500 m, 1000 m, and 2000 m) North (N) and South (S) away from each estuary mouth. Mhlangeni Estuary sites (a), Kongweni Estuary sites (b) and Bilanhlolo Estuary sites (c). Uppercase letters indicate significant differences between North and South sites (PERMANOVA pairwise comparison). Lowercase letters indicate significant differences between sites at increasing distances away from each estuary mouth within North and South groups (PERMANOVA pairwise comparison). No data at site 500 m North of Kongweni Estuary mouth due to absence of rocky shores.

7.5. Discussion

The results of **Chapter 7** show that *Perna perna* (50 – 60 mm) contained significantly different mean microplastic concentrations among all sites (**Figure 7.1**). The number of microplastics,g^{-1} tissue (w/w) decreased with an increase away from each estuary mouth (**Figure 7.1**). The only exception was for the site 2000 m South of the Mhlangeni Estuary mouth, as this site was adjacent to the Vungu Estuary mouth (**Figure 7.1**). This means that the mussels from this site may be exposed to increased microplastic loads from Vungu Estuary. Pillay (2015) found a similar pattern of stable nitrogen isotopes in *P. perna*, which was greater at sites closer to Kongweni Estuary, Umtamvuna Estuary, and Mhlungwa Estuary, than sites further away. The increased microplastic pollution at the site 2000 m South of the Mhlangeni Estuary mouth was also reflected in sediment microplastic abundances (**Chapter 6**). Therefore, the hypothesis stating that microplastic abundances in mussels will differ among sites was accepted. The overall mean microplastic abundance per mussel (2.22 ± 0.79 microplastics.g^{-1} tissue w/w) was comparable to previous studies of microplastics in *Mytilus edulis* of 2.4 items/g from a fishery market in China (Li *et al*., 2015). A more recent study found similar mean microplastic abundances in wild and farmed *M. edulis* of 2.2 items/g from China (Li *et al*., 2016). Lower abundances of microplastics were reported in *M. edulis* cultivated for human consumption in Germany (0.36 ± 0.07 microplastics.g^{-1} tissue w/w) (Van Cauwenberghe and Janssen, 2014), and *M. edulis* along the French-Belgian-Dutch coastline (0.2 ± 0.3 microplastics.g^{-1} tissue w/w) (Van Cauwenberghe *et al*., 2015). These lower abundances reported in literature, may be as a result of the nitric acid digestion technique used to separate mussel tissue from microplastics. Nitric acid has shown to alter or destroy microplastic particles within samples (Catarino *et al*., 2016).

Microplastics in *P. perna* mostly consisted of fibres (**Figure 7.3**), but microplastics types varied between sites North and South of each estuary mouth (**Figure 7.4**). Therefore, the hypothesis that microplastic types will be different in mussels at sites increasing North and South of each estuary mouth was accepted. Fibres have been reported to be the most abundant microplastic type in mussels throughout the world (De Witte *et al*., 2014; Mathalon and Hill, 2014; Li *et al*., 2016). It is important to quantify microplastic type as negative impacts on organisms ingesting microplastics

have been shown to be associated with microplastic particle shapes (Wright *et al*., 2013). Microplastic fibres have a higher surface area to volume ratio than other microplastic types, potentially allowing for increased toxicant accumulation on the surface of the microplastic, increasing the possibility of increased toxicant transfer to animals ingesting microplastic fibres (Chua *et al*., 2014).

Microplastics found in *P. perna* in this study mostly consisted of smaller microplastics (**Figure 7.5** and **Figure 7.6**), however, the microplastic size class distribution did differ significantly within sites near to each estuary mouth (**Figure 7.6**). The hypothesis stating that microplastic size class (*µ*m) distribution (%) among mussels from different sites will be different was therefore accepted. The microplastic sizes found in mussels from the study are comparable to other international studies, as microplastics were found to range between 30 to 200 *µ*m in size in *M. edulis* (Phuong *et al*., 2017). Smaller microplastics have been shown to be present in greater abundances in filter feeders than in larger carnivorous taxa (Foekema *et al*., 2013). This finding was supported by Mathalon and Hill (2014), who found that smaller microplastics have higher accumulation rates in mussel tissues as opposed to larger microplastics.

The ingestion of microplastics by mussels has been shown to have negative physiological effects, such as gut blockage, a false sense of satiation, leading to malnutrition and eventual decrease in reproductive and survival fitness (Von Moos *et al*., 2012; Wright *et al*., 2013). In addition, the increased ingestion of microplastics may increase the potential of toxicant transfer from microplastics to mussels (Chua *et al*., 2014), and potential increased bioaccumulation of these toxicants along the food web (Ivar do Sul and Costa, 2014). As mussels are an important food source for a large social sector (Yap *et al*., 2004) as well as for a wide variety of other organisms, these results highlight the potential impacts of microplastic pollution on human food sources and emphasizes the need for further research on toxicant transfer mechanisms. Although microplastics may be eliminated by mussels, they constantly ingest microplastics from the marine environment. Mussels will therefore always harbour microplastics if the environment contains microplastics (Mathalon and Hill, 2014).

Mussels have previously been determined to be suitable indicators of microplastic pollution in the marine environment (Van Cauwenberghe *et al*., 2015; Li *et al*., 2016, Santana *et al*., 2016; Wesch *et al*., 2016). The use of mussels as biomonitors of microplastic pollution enables the identification of the relevant risks of certain microplastics to the mussels (Santana *et al*., 2016), as well as the potential risk these mussels pose to humans who utilize the mussels as a food source. According to definitions of what makes a good indicator of plastic pollution monitoring outlined by the United Nations Environment Programme (UNEP, 2016) (**Table 7.1**), the results of **Chapter 7** show that *P. perna* is a good monitor of microplastic pollution. In addition to microplastic pollution levels, mussels may also be useful to monitor smaller microplastics which recommended procedures (**Chapter 3**) may underestimate (Lusher *et al*., 2017). In **Chapter 7**, the smallest detectable particle was 20 *µ*m, which is much smaller than the mesh sizes used in water (approximately 300 *µ*m). There was also less variability of microplastic abundances in mussels as compared to sediment (**Figure 7.2; Chapter 6 and Chapter 7**). This information implies that while microplastic abundances vary in mussels from different sites, the variability between samples is less than that of sediment. The decreased variability between samples indicates that *P. perna* may be a more useful and reliable way to monitor microplastic pollution in marine environments than in abiotic matrices such as sediment.

Table 7.1: Attributes of a good indicator (UNEP 2016) and relevance to *P. perna* as biomonitors of microplastic pollution

	Attributes	In this study
1.	Scientifically valid	Yes – the procedure is repeatable and provides a baseline for further microplastic biomonitoring research in South Africa.
2.	Simple to understand by public and policy makers	Yes – rapid monitoring of microplastics, simple collection methods
3.	Sensitive and responsive to change	Yes – changes in microplastic pollution in the environment will be reflected in *P. perna*
4.	Cost-effective	Yes – low cost of collection and processing
5.	Policy relevant	Yes – can be useful to include microplastic pollution in the already established national Mussel Watch Program.

The results of **Chapter 7** are only relevant to *P. perna*, however, to be part of a national monitoring program it is recommended that this study be repeated with other mussel species found along South African coastlines. The selection of which mussel species to be used as biomonitors for microplastic pollution will be regionally-dependant. While *P. perna* dominates the east coast of South Africa, *Mytilus galloprovincialis* (Lamark, 1819), an invasive mussel species, dominates the west coast of South Africa (Robinson *et al*., 2005; Picker and Griffiths, 2011). On the south coast of South Africa, *M. galloprovincialis* is often found mixed in *P. perna* mussel beds (Picker and Griffiths, 2011). *M. galloprovincialis* has successfully been used in a Mussel Watch Program (MWP) to monitor heavy metal pollution along the south-western coast of South Africa (Sparks *et al*., 2014). The already established Mussel Watch Program with *M. galloprovincialis*, in combination with the baseline data for microplastic pollution in *P. perna*, provide good motivation for future microplastic monitoring using *M. galloprovincialis* in areas where *P. perna* is not naturally found.

7.6. Conclusion

The ubiquity of microplastics in *P. perna* in **Chapter 7** highlight that microplastics are indeed entering South African marine food webs. The introduction of microplastics into marine food webs in lower trophic organisms, such as *P. perna*, may have disastrous knock-on effects throughout marine ecosystems. As *P. perna* are an important subsistence food source for a large local population, the findings of **Chapter 7** may have important repercussions for food security within the subsistence sector. The results of **Chapter 7** have shown that *P. perna* are potentially useful biomonitors of microplastics due to their sedentary lifestyle and non-selective filter-feeding strategy. Using *P. perna* to monitor microplastic pollution aids in the identification of which microplastics are the most bioavailable to organisms, thus determining which microplastics may cause the most ecological damage. The use of *P. perna* to monitor microplastics may enable the quantification of smaller microplastic particles that may be underestimated in abiotic monitoring procedures. In addition, the decreased variability of microplastic abundances in *P. perna* as opposed to sediment highlight that mussels may be a more reliable way to spatially and temporally monitor microplastic pollution. The results of **Chapter 7** provide a baseline for the development of a South African microplastic MWP, but further research is required to develop standardised international protocols for mussel microplastic monitoring (Vandermeersch *et al*., 2015; Li *et al*., 2016). Nonetheless, the concept presented in **Chapter 7** of using *P. perna* as biological monitors for microplastic pollution has been shown to be feasible on a provincial scale. National scale microplastic biomonitoring needs to be further validated using *M. galloprovincialis* to develop a nationwide microplastic pollution biomonitoring program across all South African marine biomes.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

8.1. Major findings

The study found that the novel methodology using macro-based automated counting of microfibres in samples showed no significant differences in microfibre counts and measurements between manual counting by volunteers and the developed automated counting feature (**Chapter 4**). However, the automated counting feature resulted in a significant reduction in analysis time as compared to manual counting and measurement by volunteers. The reduction in analysis time is useful for the rapid assessment of microplastics within samples from both laboratory and field studies.

This study provided the first reports of microplastic pollution in three selected KZN TOCEs during an open mouth phase (**Chapter 5**). It was found that Bilanhlolo Estuary had the highest microplastic pollution levels of the studied estuaries in both surface water (surface water (5.98 ± 0.46 microplastics.m^{-2}) and sediment (42189.81 ± 2166.67 microplastics.m^{-2}). Mhlangeni Estuary and Kongweni Estuary displayed lower levels of microplastic pollution in surface water (Mhlangeni Estuary: 4.50 ± 0.59 microplastics.m^{-2}; Kongweni Estuary: 2.34 ± 0.23 microplastics.m^{-2}) and in sediment (Mhlangeni Estuary: 13266.84 ± 1524.37 microplastics.m^{-2}; Kongweni Estuary: 18862.81 ± 2314.96 microplastics.m^{-2}). It was found that the PES category of each estuary as described by DWA (2013) was not a good indicator of microplastic pollution levels in the sampled estuaries. Microplastic fibres were the most dominant microplastic type within all estuaries (60.07 %). Microplastic size class distribution did not differ significantly between the sampled estuaries within surface water and sediment combined, but significantly differed between surface water and sediment samples. The microplastic pollution within each estuary highlights the potential contribution of estuaries as conduits of microplastic pollution transfer from land-based sources to the marine environment.

The study investigating microplastic pollution in beach sediment adjacent to each estuary mouth showed that microplastic abundances (microplastics.m^{-2}) were greater

at sites near to each estuary mouth than at beach sites further away (**Chapter 6**). In conjunction with similar results by Naidoo *et al*. (2015), this information indicates that estuaries are point sources of pollution. On average, sites near Bilanhlolo Estuary had higher levels of microplastic pollution (20267.04 ± 6439.03 microplastics.m^{-2}) than Mhlangeni Estuary (8760.72 ± 2392.44 microplastics.m^{-2}), and Kongweni Estuary (12072.19 ± 3024.01 microplastics.m^{-2}). These results are not unexpected, as Bilanhlolo Estuary had the highest microplastic abundance within the estuary.

The beach sediment microplastic abundance was an order of magnitude larger than reported by Nel and Froneman (2015) (688.9 ± 348.2 – 3308 ± 1449 microplastics.m^{-2}), Nel *et al*. 2016 (86.67 ± 48.68 to 754.7 ± 393 particles.m^{-2}), and Fok and Cheung (2016) (5595 items/m^2). The data presented in **Chapter 6** implies that the greater the microplastic pollution loads in an estuary, the more microplastic pollution will be transferred to the marine environment during open mouth phases. Microplastic fibres were the most abundant microplastic type in all beach sediment samples (79.09 %). Microplastic size class distribution differed significantly between beach sites. These results provide insight of microplastic pollution in beach sediment in previously unstudied areas. In addition, these results show the high variability of microplastic pollution in relatively short distances in beach sediment near estuaries. The high variability of microplastic loads in sediment may lead to inaccurate extrapolations of microplastic pollution in particular areas. As such, alternative methods of microplastic pollution monitoring (such as mussel biomonitoring) have been suggested.

Chapter 7, which investigated microplastic pollution in *P. perna,* showed that *P. perna* in the sampled areas contained an average of 2.22 ± 0.79 microplastics.g^{-1} tissue (w/w). Mussels nearer to each estuary mouth contained greater quantities of microplastics than sites further away. The pattern of increased microplastics in mussels nearer to estuary mouth was reflected in sediment (**Chapter 6**), as well as previous investigations of stable nitrogen isotopes in mussels at sites closer to estuary mouths (Pillay, 2015). Microplastic fibres were the most common microplastic type in all mussel samples (61.80 %). The results presented in **Chapter 7** indicate that microplastic pollution is entering South African marine food webs via TOCEs. In addition, the results show that *P. perna* can successfully be used as marine biomonitors of microplastic pollution. When comparing microplastic abundances between sediment and mussels (**Chapter 6 and 7**), no correlation between

microplastic pollution loads in sediment and mussels from the same site was found. A comparison of microplastic loads in sediment and in *P. perna* showed that microplastic abundance was less variable in *P. perna* than in sediment. This indicates that while the monitoring of microplastic abundances in sediment may be useful, biomonitoring of microplastics with *P. perna* may be a more reliable procedure. The use of *P. perna* to monitor microplastics may also be useful in the quantification of smaller microplastic particles that may be underestimated in abiotic monitoring procedures. The results of this study provide a good baseline for further research of microplastic biomonitoring in South Africa and for the development of a national microplastic Mussel Watch Program (MWP).

8.2. Challenges and shortcomings

The major challenges associated with the environmental monitoring of microplastic pollution in this study were predominantly due to the lack of standardized methodologies available. As a result, the findings of this study could only be compared to a narrow range of literature.

Some of the methodologies used in the study each had their own specific limitations. For example, the density separation method used to extract microplastics from estuarine and beach sediment (**Chapter 5 and 6**) may underestimate the quantity of microplastics with a higher density than that of the saturated NaCl solution. However, the use of a saturated NaCl solution is a more cost-effective and environmentally friendly method of density separation. In addition, it is a technically simple and replicable method, with potential to be used in a variety of microplastic pollution research investigations (**Chapter 3**). The novel methodology of macro-based automated counting of microplastics in samples is currently not able to distinguish between plastic and non-plastic debris (**Chapter 4**). However, the adoption of fluorescent tagging of samples with Nile Red, together with the automated counting methodology, may aid in the rapid assessment of microplastics from both laboratory and field studies.

The monitoring of estuarine and beach sediment microplastic pollution was largely dependent on the equipment used to collect, extract, and analysis microplastics

within samples. Microplastic abundances in estuarine surface water may be underestimated due to the mesh size of the trawl used to collect samples (**Chapter 5**). In addition, only the surface water of each estuary was sampled, which may underestimate microplastics of higher densities that are not as buoyant as microplastics floating on the surface of the water.

The sampling of mussels at the specific distance of 500 m North and South of an estuary mouth may not always be possible. For example, in **Chapter 7** there was no data for the site 500 m North of the Kongweni Estuary mouth because there was no rocky shore habitat at that location. However, future research should include the next available rocky shore to be sampled, which will still show a pattern of decreased microplastic abundance with an increase in distance North/South away from each estuary mouth.

Although *Perna perna* samples collected were all in the same size class (50 – 60 mm), there may have been variations in age, sex and physiology of each individual mussel could not be determined *in situ.* These factors may cause variations in the accumulation of microplastics and other pollutants within individual mussel tissues (Degger *et al*., 2011; Lusher *et al*., 2017).

In all environmental studies in this dissertation, microplastic particles in samples were identified by morphological type and no further chemical analysis was used to determine microplastic polymer types. Microplastic polymer identification may be useful in identifying potential sources of microplastic pollution to estuarine and marine environments. However, the lack of microplastic polymer identification certainly cannot detract from the important baseline results that this study provides. However the lack of polymer identification is not necessary for rapid assessment techniques which this study uses. In future, significantly different results from a monitoring program can always be subjected to further polymer analysis if required.

8.3. Recommendations for future research

Microplastic pollution research is still a relatively new area of global interest, and as a result, a large proportion of third world countries do not have the necessary resources to replicate microplastic pollution research undertaken in developed nations (UNEP, 2016). The recommendations of microplastic monitoring techniques outlined in **Chapter 3**, and the success of the automated macro-based microplastic counting (**Chapter 4**) provide technically simple, rapid, and cost-effective methods of microplastic monitoring and analysis that are repeatable and provide data of a relatively good quality. The recommendations and novel methodology do not require large amounts of financial resources and as such, may be used in future microplastic pollution research in developing nations.

Chapter 5 and **Chapter 6** provide baseline microplastic pollution data for three previously unstudied South African estuaries and their adjacent coastlines. Future research should include the monitoring of microplastic pollution in more estuaries throughout South Africa. To date, there is only one published report on microplastic pollution within estuaries in KZN (Naidoo *et al*., 2015). The increase in microplastic monitoring in more South African estuaries and marine environments will provide a clearer picture of the status of microplastic pollution in South Africa. This research can be expanded further to include temporal microplastic pollution trends. Microplastic pollution has been shown to fluctuate between seasons (Cheung *et al*., 2016) as a result of changes in rainfall, estuarine mouth status, and general influx of people in coastal towns during holiday seasons. Future research should include the seasonal sampling of TOCEs. As TOCEs are only intermittently open to the marine environment, the effluents derived from these estuaries are only transported into the marine environment in seasonal periods of increased rainfall (wet season). Seasonal sampling can allow for identification of potential spatial and temporal patterns of microplastic pollution from estuaries, which eventually may be used to identify major point sources of microplastic pollution into individual estuaries. Building on the temporal analysis of microplastic pollution in estuaries, future research should include the longitudinal analysis of riverine systems to investigate potential sources of microplastics in to these freshwater resources.

The use of *P. perna* as biomonitors of marine microplastic pollution offers an alternative method of marine microplastic pollution monitoring (**Chapter 7**). The results of this study can be used as a baseline of microplastic pollution levels in *P. perna* for future research of microplastic bioassessments. Microplastic abundances were less variable in mussel samples among sites than sediment. This may be because microplastics accumulate in sediment, whereas mussels continually filter-feed and egest some microplastics. Microplastics within mussels also help identify which microplastic types are most bioavailable to the mussels and therefore, which microplastics are more likely to be ingested by organisms. The results of the study provide a baseline for the development of a South African microplastic MWP, but further research is required to develop standardised international protocols for mussel microplastic monitoring. While further research is required, this study has shown that *P. perna* can be used as biomonitors of microplastic pollution in marine environments. In the interest of developing a national microplastic MWP, it is recommended that this study be extended to other dominant mussel species, such as *M. galloprovincialis*, along the South African coastline. The extension of this study to other mussel species will not only provide new knowledge of microplastics entering South African marine food webs, but will also allow microplastic pollution biomonitoring across all South African marine biomes.

The findings of these future studies will potentially advise policy makers to include microplastic pollution in estuarine health surveys and potentially introduce legislation to included more stringent plastic waste management policies in South Africa. Expanding the scope of microplastic pollution monitoring in South Africa will aid in raising awareness of the potential threats of microplastic pollution within human and environmental health sectors. Whilst the prioritization of economic and social development is vital for the well-being of the people of South Africa, the potential risks posed by microplastics to human health and ecological integrity cannot be ignored. Although microplastic pollution has received little national research attention, this does not mean that the impacts of microplastic pollution are any less important. At the very least, research in microplastic pollution in South African marine and freshwater environments is the first step towards maintaining our constitutional responsibility to "*prevent pollution and ecological degradation*" and "*promote conservation*" (Constitution of the Republic of South Africa Act No. 108 of 1996,

Chapter 2, Section 24). This dissertation has not only contributed new knowledge of microplastic pollution baseline information in South African estuarine and coastal environments, but also the first steps towards establishing a nationwide Mussel Watch Program for microplastic pollution. In this context, it can be concluded that mussels are indeed, more than just food.

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APPENDIX A

Table A 1: Ecological categories of South African estuaries based on Present Ecological State (PES) (DWA, 2013).

PES	Description
A	Unmodified, natural.
B	Largely natural with a few modifications. A small change in natural habitats and biota may have taken place but the ecosystem, functions and processes are essentially unchanged.
C	Moderately modified. A loss and change of natural habitat and biota have occurred but the basic ecosystem functions and processes are still predominantly unchanged.
D	Largely modified. A loss and change of natural habitat, biota and ecosystem functions and processes have occurred.
E	Seriously modified. The loss of natural habitat, biota and basic ecosystem functions and processes are extensive.
F	Critically/Extremely modified. Modifications have reached a critical level and the system has been modified completely with an almost complete loss of natural habitat and biota. In the worst instances the basic ecosystem functions and processes have been destroyed and the changes are irreversible.

Table A 2: Geographic co-ordinates of sample stations. Estuary names noted as MH (Mhlangeni Estuary), KO (Kongweni Estuary) or BL (Bilanhlolo Estuary). Direction from estuary mouth noted as N (North) or S (South). Number denotes distance (m) away from estuary mouth. *No rocky shore present.

Station ID Code	Latitude	Longitude
MH-N-2000	30°48'13.91"S	30°24'52.25"E
MH-N-1000	30°48'45.05"S	30°24'41.82"E
MH-N-500	30°48'57.59"S	30°24'33.19"E
MH-N-0	30°49'8.92"S	30°24'17.53"E
MH-S-0	30°49'11.14"S	30°24'16.10"E
MH-S-500	30°49'28.88"S	30°24'18.94"E
MH-S-1000	30°49'45.32"S	30°24'12.23"E
MH-S-2000	30°50'5.95"S	30°23'45.70"E
KO-N-2000	30°50'58.07"S	30°23'18.76"E
KO-N-1000	30°51'20.46"S	30°22'53.59"E
KO-N-500*	30°51'30.07"S	30°22'36.86"E
KO-N-0	30°51'37.06"S	30°22'22.53"E
KO-S-0	30°51'38.07"S	30°22'21.05"E
KO-S-500	30°51'55.07"S	30°22'19.15"E
KO-S-1000	30°52'12.20"S	30°22'11.64"E
KO-S-2000	30°52'34.39"S	30°21'46.06"E
BL-N-2000	30°52'34.78"S	30°21'45.09"E
BL-N-1000	30°53'1.19"S	30°21'23.38"E
BL-N-500	30°53'14.12"S	30°21'12.74"E
BL-N-0	30°53'20.52"S	30°20'56.19"E
BL-S-0	30°53'22.15"S	30°20'54.44"E
BL-S-500	30°53'36.38"S	30°20'55.31"E
BL-S-1000	30°53'50.25"S	30°20'48.68"E
BL-S-2000	30°54'15.04"S	30°20'28.58"E

APPENDIX B: PERMIT

environmental affairs
Department:
Environmental Affairs
REPUBLIC OF SOUTH AFRICA

agriculture, forestry & fisheries
Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Enquiries: Dr Alan Boyd
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Roy Jackson
School of Life Sciences, University of KwaZulu-Natal
Private Bag X 54001
Durban
4000

Attention: Dear Mr Jackson

INTEGRATED ENVIRONMENTAL AND FISHERIES RESEARCH AND DEVELOPMENT PERMIT FOR THE PURPOSES OF A SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT NO. 18 OF 1998).

This permit is an integrated research permit issued jointly by the Department of Environmental Affairs and the Department of Agriculture, Forestry and Fisheries authorising a scientific investigation or practical experiment in terms of section 83 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act"), which experiment or investigation includes both environmental and fisheries components as part of the project.

I, the undersigned, Acting Chief Director: Fisheries Research and Development, Branch: Fisheries Management, Department of Agriculture, Forestry and Fisheries (the Chief Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Agriculture, Forestry and Fisheries as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby jointly issue this permit, in terms of Section 83 of the Act, to the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below:

I, the undersigned, Director: Biodiversity and Coastal Research, Branch Oceans and Coasts, Department of Environmental Affairs (the Director) acting in pursuance of the delegated authority conferred upon me by the Honourable Minister of Environmental Affairs as contemplated in terms of Section 79 of the Marine Living Resources Act of 1998 (Act No. 18 of 1998) ("the Act") hereby jointly issue this permit, in terms of Section 83 of the Act, to the following person(s)/institution to engage in the scientific investigation or practical experiment referred to below:

PERMIT REFERENCE NUMBER: RES2017/71
PERSON(S) INSTITUTION: Roy Jackson, School of Life Sciences, University of KwaZulu-Natal
SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT: Collection, possession, transportation and housing of marine species for research and educational purposes,
subject to the following conditions:

1. **SPECIFIC CONDITIONS FOR THE DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES**

1.1. This permit allows the collection, possession, transportation and housing of marine species for the purposes of *bona fide* research and educational projects of the School of Life Sciences at the University of KwaZulu-Natal, as authorized by the Head of School.

1.2. A certified copy of this permit shall be carried by staff during collections and must be shown to a Fishery Control Officer or any other authorized person on demand. Staff undertaking collections shall identify themselves, if requested to do so, by means of an identification document issued by the Head of the Department.

1.3. A maximum of five hundred (500) invertebrates per species may be collected on any given day to a maximum of five thousand (5 000) specimens per species per annum, with the following exceptions:

a) A maximum of one thousand (1 000) specimens per species of amphipods, polychaetes, copepods, isopods, mysids, shrimps, white mussels, brown and black mussels, barnacles, Nemertea, sipunculids, sea-squirts, sand- and mud-prawns may be collected per day up to a maximum of ten thousand (10 000) specimens per species per annum.
b) Adult barnacles and mussels are subject to the general limit, but spat are unlimited by any number.
c) A maximum of ten thousand (10 000) littorinid snails may be collected per annum.
d) A maximum of ten (10) colonies/ specimens of corals, seafans, seapens and pansy shells may be collected per annum.
e) For large, long-lived colonial species only small branch fragments may be removed, leaving the colony in place and largely undisturbed.
f) A maximum of twenty (20) per species of cephalopods (including chokka squid *Loligo reynaudi*, and any other squid or octopus species) may be collected per annum.
g) A maximum of ten (10) mud crabs (*Scylla serrata*) may be collected per annum.
h) A maximum of fifty (50) per species of rock lobsters may be collected per annum.
i) A maximum of ten (10) specimens per species of abalone (*Haliotis* spp.) may be collected per annum.
j) Zooplankton and phytoplankton species may be collected and are not restricted by any quantity.

1.4. A maximum of one thousand (1 000) bony fish specimens per species may be collected per annum, with the following exceptions:

a) A maximum of ten thousand (10 000) specimens per species of small shoaling fishes (such as those of the Families Ambassidae, Clupeidae, Mugilidae, Atherinidae,etc.) may be collected per annum.
b) A maximum of ten (10) specimens per species of IUCN 'Endangered' or 'Critical' fish species may be collected per annum.

1.5. No shark, ray or skate species may be collected.

1.6. No harmful chemicals are to be used when collecting marine species. Limited use of fish anaesthetics (including rotenone) is permitted if no other suitable technique is available to collect fishes, and should be kept to a minimum. Local authorities should be advised when rotenone is to be used to collect fish.

1.7. Any installations must be removed on termination of the project(s).

1.8. The report, as required under Condition 1.15, should provide details of the dates, locations, species and quantities collected. The report should further provide a summary of the major research findings and outcomes.

1.9. The report, as required under Condition 3.15, should provide details of the dates, locations, species and quantities collected. The report should further provide a summary of the major research findings and outcomes.

2. SPECIFIC CONDITIONS FOR THE DEPARTMENT OF ENVIRONMENTAL AFFAIRS

2.1. No marine mammals, turtles or seabirds may be collected or disturbed.

CONDITIONS PERTAINING TO RESEARCH ACTIVITIES WITHIN MARINE PROTECTED AREAS

2.2. This permit does not allow collection within Marine Protected Areas.

3. GENERAL CONDITIONS

3.1. This permit is issued subject to the provisions and regulations of the following laws:

(a) The Marine Living Resources Act, 1998 (Act No. 18 of 1998) ("the Act"), and all regulations published in terms thereof;
(b) The National Environmental Management Act, 1998 (Act No. 107 of 1998) ("NEMA"), and in particular, the regulations that control vehicle use in the coastal zone (as amended);
(c) The National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
(d) The National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
(e) The Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973);
(f) The Prevention of Pollution from Ships Act (Act No. 2 of 1986);
(g) The National Environmental Management Integrated Coastal Management Act, 2008 (Act No. 24 of 2008); and
(h) Any other relevant law.

3.2. If, in the opinion of the Chief Director or Director there are sound reasons for doing so, the Chief Director or Director may amend the relevant conditions of the permit.

3.3. Any reference to the Permit Holder in these permit conditions includes the entity or person, his/her or its employees (whether permanent, full-time or part-time), his/her or its contractors, agents or advisers, being cognisant of the course and scope of their contractual relationship.

3.4. A breach of the provisions of the Acts, regulations or these permit conditions by the Permit Holder may result in the initiation of legal proceedings (civil or criminal). A breach includes:

(a) furnishing information to which the Department of Environmental Affairs (the Department) is entitled, which is not true or complete;
(b) contravening or failing to comply with a permit condition or with the provisions of the Acts; or
(c) being convicted of an offence in terms of the Acts.

3.5. The Permit Holder shall store at their registered place of business/residence the original permit issued. The Permit Holder shall at all times, have available a true certified copy of this permit which should be produced on demand by any law enforcement official.

3.6. This permit shall only be utilized by the individual/organisation whose name appears on the permit.

3.6. This permit shall only be utilized by the individual/organisation whose name appears on the permit.

3.7. If the permit is in the name of an institution/company/close corporation the individual utilizing the permit shall in addition to a certified copy of this permit, be in possession of identification and/or letter which identifies the individual as an authorized person or employee of the permit holder.

3.8. Any individual utilizing this permit shall in addition to the above conditions have a certified copy of any other permit/exemption required in terms of other legislation including any permit or exemption in terms of the Marine Living Resources Act, 1998 (Act No. 18 of 1998).

3.9. An application for a future permit may be refused if the conditions of this permit are not adhered to.

3.10. In terms of the Act, the permit holder is obliged to report to the Minister any contravention of the provisions of the Act by any other person.

3.11. The Permit Holder must safely store all inorganic waste material, garbage and pollutants on board the vessel or at the site of research activities if on land. Should the Permit Holder discard any inorganic waste material, garbage or pollutants into the sea or the coastal environment, the Department may institute legal proceedings (civil or criminal) which may include suspension of the permit for a period determined by the Department and the Permit Holder shall take those steps considered necessary in terms of relevant legislation to remedy any pollution caused.

3.12. This permit does not in any way absolve the holder from the obligations of and adhering to the remainder of the provisions and conditions of the Acts, regulations or any other law.

3.13. Specimens collected in terms of this permit shall not be sold or offered for sale.

3.14. No vehicle may be used in the coastal zone in terms of this permit unless the permit holder is in possession of a valid permit to use a vehicle in the coastal zone in terms of the Regulations for the Control of Use of Vehicles in the Coastal Zone (GNR 1426 of 7 December 2004).

3.15. Reports as stipulated in the 'Specific Conditions' must be submitted to the Chief Director: Fisheries Research and Development, Department of Agriculture, Forestry and Fisheries, Branch: Fisheries Management (Attention: Dr Kim Prochazka), Private Bag X2, Roggebaai, 8012 and to The Director: Dr Alan Boyd, Biodiversity and Coastal Research, Department of Environmental Affairs, Branch: Oceans and Coasts, Private Bag X4390, Cape Town, 8000. Such reports must be submitted within the timeframes provided, before any renewal of this permit or application for any other permit will be considered.

PERMIT REFERENCE NUMBER: RES2017/71
PERSON(S) INSTITUTION: Roy Jackson, School of Life Sciences, University of KwaZulu-Natal
SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT: Collection, possession, transportation and housing of marine species for research and educational purposes, subject to conditions

On behalf of
MR JUSTICE MATSHILI
ACTING CHIEF DIRECTOR: FISHERIES RESEARCH AND DEVELOPMENT
DEPARTMENT OF AGRICULTURE, FORESTRY AND FISHERIES
DATE: 13/12/2016

DR ALAN BOYD
DIRECTOR: BIODIVERSITY AND COASTAL RESEARCH
DEPARTMENT OF ENVIRONMENTAL AFFAIRS
DATE: 12/12/16