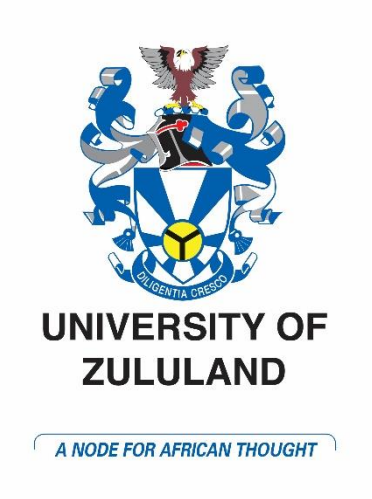
Characterisation of exposure and biological effects of contaminant of emerging concerns in Thukela and Mhlathuze River systems



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LIST OF ABBREVIATIONS

µg/g-Microgram per gram

µg/L-Microgram per litre

µg/L-Micrograms per litre

ABS-Acrylonitrile butadiene styrene

ACh- Acetylcholine

AChE-Acetylcholinesterase

AgNPs- Nanoparticles of silver

Ag-Silver

Al_2O_3NPs –Aluminium oxide nanoparticles

Al-Aluminium

ARVs-Antiretrovirals

ATR–Attenuated total reflectance

CECs-Contaminants of Emerging Concerns

Co-Cobalt

Cr-Chromium

Cu-Copper

CuONPs-Cupper oxide nanoparticles

DDD-Dichlorodiphenyldichloroethane

DDE-Dichlorodiphenyldichloroethylene

DDT-Dichlorodiphenyltrichloroethane

DEA-Desethylatrazine

DIA-Deisopropylatrazine

DWAF-Department of water affairs and forestry

DWS-Department of Water and Sanitation

E2-17β- estradiol - natural steroidal oestrogen

EC_{50} -Concentration observed to cause an adverse biological effect on 50% of test organisms

EDCs- Endocrine disrupting compounds

EDX- Energy-dispersive X-ray spectroscopy

EE2-17α- ethinylestradiol -synthetic oestrogen

ENMs-Engineered nanomaterials

ERL - Effect Range Low

EU-European union

EVA + poly (cyclohexanone)-Poly(ethylene vinyl acetate) poly (cyclohexanone)

EVA- Poly(ethylene vinyl acetate)

Fe_2O_3NPs-Iron oxide nanoparticles

Fe-Iron

FTIR-Fourier transform infrared spectroscopy

g- Gram

GC X GC - HRTOF- MS - 2-dimensional gas chromatography coupled to high-resolution time-of-flight mass spectrometry

GC-MS - Gas Chromatography-Mass Spectrometry

GC-MSD- Single quadrupole Gas Chromatography – Mass Spectrometry

GF – AAS – Graphite furnace atomic absorption spectrometry

HDPE- High-density polyethylene

HIV – Human immunodeficiency virus

HPGe – High purity germanium

HPLC – High performance liquid chromatography

HPLC –MS/MS - High-performance liquid chromatography coupled to a mass spectrometer

ICP- MS – Inductively coupled plasma mass spectrometry

ICP-OES – Inductively coupled plasma atomic emission spectroscopy

K-Kilogram

K_{ow} -Octanol partition coefficient

KZN-KwaZulu Natal

LC_{50} -Lethal concentration that is fatal to 50% of the test organisms

LDPE - Low-density polyethylene

LLE –Liquid/liquid extraction

L-Litre

LX-Latex

m^{-2} – Per square metre

mg/L –Milligram per litre

Min-Minutes

ml- Millilitre

Mn-Manganese

MPs-Microplastics

NEPs-Nano enabled products

ng/L-Nanogram per gram

ng/L-Nanogram per litre

nm-Nanometer

NMTP-National monitoring toxicity programme

NOAA-National oceanic and atmospheric administration

NPs-Nanoparticles

NSAIDs -Nonsteroidal anti-inflammatory drugs

NTA-Nanoparticle Tracking analysis

NT-Nitrile

NWRS-National water resources strategy

NY-Nylon

PBA-Poly butyl acrylate

Pb-Lead

PC- Polycarbonate

PCA- Principal Component Analysis

PET -Poly ethylene terephthalate

POPs-Persistent organic pollutants

PP-Polypropylene

PS-Polystyrene

PU-Polyurethane

PVC-Polyvinyl chloride

SA-South Africa

SD-Standard deviation

SE- SOXHELET EXTRACTION

SE-Standard error

Si-Silicon

TB-Tuberculosis

TEL - Threshold Effect Level

TiO_2NPs -Nanoparticles of titanium dioxide

Ti-Titanium

TPs-Transformation products

UHPLC-MS/MS - ultrahigh-pressure liquid chromatography-tandem mass spectrometry

UNEP-United nations environment programme

UPLC/TQD-MS - ultrahigh performance liquid chromatography and triple-quadrupole -mass spectrometer

USA-United State of America

UV-Ultraviolet

WHO -World health organisation

WMA -Water management area

WWTWs -Wastewater treatment works

Zn-Zinc

ABSTRACT

Rivers, estuaries, and oceans are exposed to a cocktail of pollutants, including contaminants of emerging concern (CECs). This study aimed to examine the extent of contamination and/or pollution of selected contaminants of emerging concern in the uMhlathuze and uThukela River-estuaries, following the source-to-sea approach. Microplastics, organic pollutants, engineered nanomaterials (ENMs) and metals were analysed in surface water samples, and accumulation of metals and biomarker response (acetylcholinesterase enzyme) were examined in the mud crabs *Chiromantes eulimene*. Thirty-eight organic compounds were detected in surface water samples from the uMhlathuze River-Estuary, which belonged to 14 classes of pharmaceuticals and lifestyle drugs, and 20 compounds were found in the uThukela River-Estuary, belonging to 13 therapeutic compound classes. The differences in detection frequency of compounds illustrated differences in pollution sources and consumption rates, due to differing land-use activities and disease burdens. Pesticides were also found in surface water samples, with a total of 13 herbicides, insecticides and fungicides in the uMhlathuze and 14 in the uThukela Estuary. Herbicides were the dominant pesticide class in both systems. The presence of pesticides was linked to agricultural activities in both catchments.

Microplastic concentrations decreased towards the sea, and wastewater treatment works (WWTWs) did not appear to be a major source. Microplastics were detected in all surface water samples and were composed of fibres and films. Fibres were numerically dominant. Poly(propylene) (PP), polycarbonate (PC), nylons (NY), low-density poly(ethylene) (LDPE), high-density poly(ethylene) (HDPE), poly (ethylene terephthalate) (PETE), poly (ethylene vinyl acetate) + poly (cyclohexanone) (EVA + poly(cyclohexanone)), poly (butyl acrylate) (PBA) were the dominant plastic polymers found in the uMhlathuze River-Estuary. In uThukela River-Estuary, PETE, NY, PP, PBA and low-density poly(ethylene) (LDPE) were dominant polymers. Engineered nanomaterials (ENMs) such as titanium dioxide (TiO_2), silicon dioxide (SiO_2), zinc oxide (ZnO), iron oxide (Fe_2O_3) and copper oxide (CuO) nanoparticles were also recovered from surface water samples. ENMs were irregular, sheet-like, spherical or hexagonal in morphology. The ENMs were possibly from the use of various domestic

nano-enabled products, for example, products manufactured for bathing, laundry washing and personal care products.

The concentrations of zinc (Zn), chromium (Cr), aluminium (Al), lead (Pb), nickel (Ni), copper (Cu) and lead (Pb) detected in the studied systems exceeded the DWAF threshold values for freshwater and marine environments. High metal concentrations could be attributed to various industrial and agricultural activities dominating within these systems. Metals also accumulated in crabs, and accumulation varied between seasons. Chromium, Ni and Fe accumulation was also gender specific, with accumulation in females generally higher than in males in the uThukela Estuary. The effluent from domestic use, industries and other related activities are probable sources of metal contamination in these regions. Overall, AChE activity in the gills of *C. eulimene* was reduced in both systems, which was a negative effect deduced to be caused by contaminants. In conclusion, this study confirmed the occurrence of CECs in surface water samples, and contaminants such as metals were observed to bioaccumulate in crabs.

Keywords: Contaminant of emerging concern, occurrence, accumulation, WWTWs and effect.

DECLARATION ON ORIGINALITY OF THE WORK

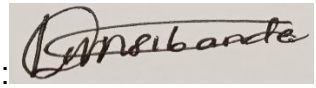
I acknowledge that I have read and understood the University's policies and rules applicable to postgraduate research, and I certify that I have, to the best of my knowledge and belief, complied with their requirements.

In particular, I confirm that I did obtain an ethical clearance certificate for my research (Certificate Number: UZREC 171110-030 PGM 2020/25) and that I have complied with the conditions set out in that certificate.

I further certify that this dissertation is original, and that the material has not been published elsewhere, or submitted, either in whole or in part, for a degree at this or any other university.

I declare that this dissertation is, save for the supervisory guidance received, the product of my own work and effort. I have, to the best of my knowledge and belief, complied with the University's Plagiarism Policy and acknowledged all sources of information in line with normal academic conventions.

I have subjected the document to the University's text-matching and/or similarity-checking procedures during the course of the research and dissertation preparation.

Candidate signature: 

Date: April 2022

CHAPTER 1:

GENERAL INTRODUCTION

1.1. Introduction

Advancing manufacturing and analytical capability has resulted in ever-increasing production and application of chemicals, which in turn has increased their potential for release into aquatic environments at various life cycle stages (manufacturing, usage, and disposal). The continuous release of these chemicals adds to the long list of contaminants of emerging concern in aquatic systems worldwide. Contaminants of emerging concern (CECs) are defined as chemicals that have been detected in the environment, and whose fate, behaviour, and toxicological effects are poorly known, and currently are not included in routine monitoring programs (La Farre *et al*., 2008). CECs have gained more research attention due to their environmental occurrence at trace concentrations, coupled with their potential to exert adverse effects (Miraji *et al.*, 2016). CECs encompass a wide array of contaminants, categorized according to their chemical structure or mode of action, which include pharmaceuticals, lifestyle drugs, engineered nanomaterials (ENMs), perfluorinated compounds, microplastics, brominated flame retardants and their transformation products (La Farre *et al*., 2008).

1.2. Environmental concern of CECs in South Africa

South Africa, as a water-stressed country, pays attention to pollution of water resources by a variety of contaminants, including CECs (Petersen, 2016). The situation is likely to become worse due to the rapid population growth rate and the growth of industrialization in South Africa. In particular, scientific advancements have resulted in a rise in the manufacturing and application of chemicals to meet demands for food production and disease management in South Africa (Petersen, 2016, Bouwman *et al*., 2018, Ngqwala and Muchesa, 2020). Consequently, there is rising potential for environmental exposure from the use of old [chemicals that were long existing in environment but their environmental concern was not recognised, such as pesticides, persistent organic pollutants (POPs)] and new [chemicals that are recent introduced and recognised for their negative impact in the environment, i.e. microplastics and ENMs] chemicals (Agüera *et al*., 2013). This is exacerbated by

inappropriate use and disposal practices (Bolong *et al.*, 2009). As many CECs are not routinely monitored, this may lead to their release into water resources via point and non-point sources (Kroon *et al*., 2020), and they often occur at very low concentration ranges, for example, ng/L to µg/L which are known to pose environmental health problems (Swartz *et al.,* 2016, Wanda *et al*., 2017). Some CECs, predominantly organic forms, have been shown to persist and bioaccumulate at different trophic levels (Swartz *et al.,* 2016).

South Africa's current knowledge regarding elimination and monitoring strategies for CECs in aquatic compartments is not well developed. Some studies have produced a list of priority compounds, which have led to the prioritization of endocrine disrupting compounds (EDCs) (Burger, 2005), as well as a list of 130 compounds of industrial chemicals, pesticides, disinfection by-products, polymeric residues, cyanotoxins, pharmaceuticals and personal care products (PPCPs) to be monitored in drinking water (Ncube *et al.*, 2012). Some studies have reported the extent of CECs in different aquatic compartments in various parts of South Africa. For example, pesticide compounds (atrazine, simazine, propazine, ametryn and prometryn) were detected in Hartbeespoort Dam (Rimayi, 2018), ARV compounds (abacavir, efavirenz, lopinavir and nevirapine) have been recorded in wastewater treatment plants (WWTPs) in the eThekwini Municipality (Abafe *et al*., 2018), and stimulants, antiepileptics, anti-psychotic and antibiotic drugs have been detected in wastewater, surface water and sediments in the uMsunduzi River (Matongo *et al*., 2015b). The occurrence of microplastics has been reported in various provinces (Lamprecht, 2013, Naidoo *et al*., 2015, Nel and Froneman, 2015, Bouwman *et al*., 2018, Weideman *et al*., 2019). Although CECs have been reported in various parts of the country, most of the information is from metropolitan areas, with little study of non-metropolitan areas.

1.3. Framework for managing CECs in South Africa

South Africa's limited water resources are under threat from pollution, including from CECs. The National Water Act of South Africa (Act 36 of 1998) forms the basis for water resources management, including guiding the protection against pollution for present and future generations (National Water Act, 1998). The Department of Water and Sanitation (DWS) is the custodian of water resources in South Africa, and

operates an array of water resource monitoring programs, including the National Toxicity Monitoring Program (NTMP), which has an interest in monitoring some CECs. The NTMP focuses predominantly on legacy and toxic persistent organic pollutants (POPs) (Bouwman, 2004). Various strategies, such as National Water Resource Strategy I and II (NWRS), have raised concerns regarding emerging water quality issues such as antimicrobial resistance, pharmaceuticals, and engineered nanomaterials (ENMs) (Department of Water and Sanitation, 2012). More detailed information on hazards and risks posed by CECs is needed to aid in the implementation of strict policies and regulations of water bodies.

1.4. Justification of this study

This study investigated the presence (and detection) of selected pharmaceuticals and lifestyle drugs, pesticides, microplastics, ENMs and metals in river-estuary systems, as well as their interaction with biota.

Pharmaceuticals are prescription drugs used for the curing and prevention of diseases, as well as adding value to human and animal life. Recently there has been a rapid increase in the volumes of new and old chemicals used in the pharmaceutical industry worldwide, which has been driven primarily by a growing disease burden globally, including in South Africa (Tijani *et al.*, 2016, Ebele *et al*., 2017, Ngqwala and Muchesa, 2020). There is a lack of data regarding the quantities of pharmaceuticals consumed in South Africa. However, the high usage of pharmaceuticals in South Africa is assumed, owing to the exponential increase of chronic and acute diseases in South Africa (StatsSA, 2019). For example, South Africa has been fighting the epidemics of TB and HIV/AIDs for decades and recently COVID-19. The prevalence of these diseases in South Africa means that a high volume of pharmaceuticals such as antitubercular drugs, antiretrovirals (ARVs) and antimicrobial drugs are consumed to manage and treat such diseases. This is in addition to other lifestyle diseases such as hypertension, diabetes, and depression, which require chronic medication to control. Therefore, a cocktail of drugs is potentially released into the environment daily (Ngqwala and Muchesa, 2020).

Agriculture forms a crucial component of South Africa’s economy in terms of job creation and poverty alleviation. Pesticides are used to protect food production against

various pests, or to manage pests that affect livelihoods. However, they pose negative consequences to environmental health as they may end up in non-target environments where they may still be biologically active (Horak *et al*., 2020). By design, pesticides are biologically active and persistent, and so pose human and environmental health risks (Horak *et al*., 2020). The environmental toxicity of numerous pesticides is a priority of concern, with some commonly used pesticides being confirmed carcinogens and/or endocrine-disrupting agents (Huang *et al.*, 2014, Zhu *et al*., 2014b, Horak *et al*., 2020). There is uncertainty about the volume of pesticides used in South Africa as such data is not publicly available, but it is estimated that there are over 3 000 registered pesticides with 500 active ingredients (Horak *et al*., 2020) currently used in South Africa. Currently, South Africa is the largest pesticide consumer in sub-Saharan Africa (Dalvie and London, 2009, Ansara-Ross *et al*., 2012). Approximately 6 800 tonnes of pesticides were applied to grapes, pome, stone fruit, potato, and wheat crops in 1999 (Dalvie and London, 2009). About 60–70% of the applied pesticide does not reach the target recipient, ending up in non-target areas (Al-Rajab and Hakami, 2014). Microplastics are generally defined as plastic fragments with a size smaller than 5 mm (Nel and Froneman, 2015, Li *et al*., 2018). They are extensively produced for various applications, including household products, personal care products, and the aerospace, maritime, and auto industries, due to their high durability and lightweight and malleable characteristics (Bouwman *et al*., 2018, Pereao *et al*., 2020). Global plastics production increased sharply from 0.35 million tons during the 1950s to 359 million tons in 2018, which in turn has played a part in the increase of microplastics in the environment. Further increases in the quantities of plastic is expected due to the current COVID-19 coronavirus disease pandemic, which requires a lot of protective equipment composed of plastics (such as masks, gloves, and protective gowns) (Govender *et al*., 2020). South Africa also has a vibrant plastics manufacturing industry and public use of plastic is high. However, it has low levels of recycling and managing waste strategies (Verster *et al.,* 2017). Currently, South Africa is ranked 11th on top of the top 20 countries for its highly mismanaged plastic waste (Verster *et al*., 2017, Govender *et al*., 2020). South Africa is a water-stressed, developing country, in the middle of growing disease burden and poor sanitation management, water pollution by microplastics receives little attention. However, studies on microplastics have increased over the past ten years (Browne *et al.*, 2011, Lamprecht, 2013, Naidoo *et al*., 2015, Nel and Froneman, 2015, Nel *et al*., 2017, Bouwman *et al*., 2018, Govender

et al., 2020). It is worth noting that much of the microplastics research in South Africa has focused on marine environments (Browne *et al*., 2011, Nel and Froneman, 2015, Naidoo *et al*., 2015, Nel *et al*., 2017, La Daana *et al*., 2017, Govender *et al.*, 2020) while freshwater environments have received little attention (Bouwman *et al*., 2018, Weideman *et al.,* 2019, Dahms *et al*., 2020). The present study addresses this knowledge gap, and therefore has focused on the freshwater environment to contribute to the body of knowledge regarding the extent of microplastic pollution.

Engineered nanomaterials (ENMs) are defined as materials of any state of matter with at least one dimension, that ranges from 1–100 nm in size (Borm *et al.*, 2006). The desirable properties of ENMs (e.g., magnetic, optical, mechanical, electrical, and reactive) attract novel applications in various sectors (Moeta *et al*., 2019). For example, nanoparticles of silver (AgNPs) have a well-documented antibacterial action and have high thermal and electrical conductivity, and so they are extensively used in the sterilization of products and equipment in hospitals, as well as coatings of household appliances (Fabrega *et al*., 2011). Nanoparticles of titanium dioxide (TiO_2NPs) are largely inert, photocatalytic, enhance performance on UV filtering and are opaque, and are therefore applied to colouring agents in paints, paper, inks, UV filtration and water purification (Lovern and Klaper, 2006, Kiser *et al*., 2009, Shi *et al*., 2013, Nam *et al*., 2014, Nthwane *et al*., 2019). The wide application of ENMs and usage of nano-enabled products (NEPs) may increase the presence of ENMs in the environment, with wastewater systems being priority recipients, and the situation could be heightened by the persistent poor performance of the wastewater treatment works (WWTWs).

Exposure to metal contamination can affect the survival of aquatic animals through direct and indirect toxicity. Over the past few decades many issues have been associated with the presence of metals in different aquatic compartments (Miller *et al.*, 2019). In particular, anthropogenic activities (agricultural, mining and other industries) along the east coast of KwaZulu-Natal (KZN) release various metals during production processes which have an impact on both freshwater and estuarine organisms. Some impacts of the release of metals have been investigated on various organisms in different regions (Newman *et al*., 2015, Miller *et al*., 2019, Mzimela *et al*., 2003, Mzimela *et al*., 2014, Adeleke *et al*., 2020, Majola *et al*., 2020). Despite this existing information regarding KZN estuarine systems, very little is known about the current

situation regarding the dynamics of contaminants in northern KZN freshwater systems, predominantly the relationship between CECs and biomarkers (acetylcholinesterase). These are currently of great concern, taking into account their potential adverse environmental and human health effects for non-target recipients (Osunmakinde *et al*., 2013). Therefore, these factors provided the motivation for the present study, to generate more information that could help to inform the national agenda to protect the aquatic environment for present and future purposes, by focusing on semi-urban catchments which have remained largely unexplored in this context.

1.5. Problem statement

UMhlathuze and uThukela River systems flow through the most populated areas of the uMhlathuze and uThukela District municipalities in KwaZulu-Natal (KZN). These municipalities are earmarked for the expansion of industrial zones, as well as rising agricultural activities, following a rise in human population (MhlathuzeIDP, 2019, ThukelaIDP, 2019). UMhlathuze Municipality had an estimated population of 410 465 in 2017, which is expected to increase to approximately 500 000 by 2030. Approximately 58% of this population lives within tribal areas which are predominately active in commercial agriculture, mainly sugar cane farming, and rely on the uMhlathuze River for domestic and agricultural purposes. In addition, bigger towns such as Empangeni and Richards Bay also depend on the uMhlathuze River for various purposes (MhlathuzeIDP, 2019).

The population of uThukela District Municipality was estimated to be 196 227 in 2011 and had risen to 215 182 in 2016. Concurrently, there has been a rise in industrial activities such as manufacturing, fabrics, and other related activities in the Mandeni area (ThukelaIDP, 2019). Therefore, concerns regarding water pollution by industrial, agricultural, and domestic activities have increased. To date, most research on CECs has focused on metropolitan areas in South Africa (Swartz *et al*., 2016, Petersen, 2016, Wanda *et al*., 2017, Rimayi, 2018, Rimayi *et al*., 2018), neglecting systems in non-metropolitan areas. Therefore, this study addresses this paucity of information by focusing on the occurrence and potential biological effects of CECs (including metals) in the highly populated and industrial intensive catchments of uMhlathuze and uThukela Rivers in northern KZN.

1.6. PROJECT AIM AND OBJECTIVES

1.6.1. Aim

The study aimed to examine the extent of contamination and/or pollution by selected contaminants of emerging concern in the uMhlathuze and uThukela River-Estuary systems, using the source-to-sea approach.

1.6.2. Objectives

The objectives of this study are:

i. To qualitatively screen for the occurrence of pharmaceuticals, lifestyle drugs and pesticides in surface water samples collected from the uMhlathuze and uThukela River-Estuary systems.
ii. To characterize microplastics and ENMs in surface water samples collected from the uMhlathuze and uThukela River-Estuary systems.
iii. To examine bioaccumulation and biomarker response (acetylcholinesterase enzyme) to assess contaminant exposure to the mud crabs *Chiromantes eulimene* in the uMhlathuze and uThukela Estuaries.

1.7. Dissertation outline

The dissertation contains six chapters, and the contents of each chapter are described below.

CHAPTER 1: Provides a background, justification of the study, problem statement, aim, and objectives.

CHAPTER 2: Reviews literature on pharmaceuticals, lifestyle drugs, pesticides, microplastics, engineered nanomaterials (ENMs), metal sources and pathways, their distribution and the extent of occurrence, as well as ecotoxicological effects on aquatic organisms.

CHAPTER 3: Describes the study areas, the methodology followed in this study and how data was analysed.

CHAPTER 4: Provides an exposure assessment of contaminants of emerging concern in the uMhlathuze and uThukela River-Estuary systems. This chapter addresses the first and second objectives.

CHAPTER 5: Describes the bioavailability of dissolved metals in mud crabs *Chiromantes eulimene* from the two study areas using a biomarker assay. This chapter addresses the third objective.

CHAPTER 6: Summarises findings, provides conclusion and recommendations.

REFERENCE

CHAPTER 2:

LITERATURE REVIEW

2.1. Introduction

Recent advancements in chemical science and technology have allowed the discovery of new chemicals, new applications of old chemicals and their increased production. This has increased the potential for environmental release, which may lead to chemical pollution. Environmental exposure arises from the use of chemicals that are indispensable for the standard of living in the 21st century, and some of this exposure this are classified as contaminants of emerging concern (CECs). In addition, due to rising chemical use, the environmental release of CECs (both parental compounds and their transformation products (TPs) is increasing, which might escalate environmental impacts (Kroon *et al.*, 2020). It is suggested that the global production of CECs in general has increased by one million to 500 million tons per year over the last decade (Rasheed *et al*., 2019). These staggering production volumes are an indication of CECs that might eventually reach the environment (Petersen, 2016, Rasheed *et al*., 2019, Kroon *et al*., 2020). The presence of CECs in water bodies has been associated with possible adverse consequences in aquatic ecosystems (Rasheed *et al*., 2019). As a result, attention has increased worldwide, focussing on the investigation of the occurrence and their toxic effects on environmental compartments such as surface water and sediments, as well as in drinking water (Daughton and Ternes, 1999, La Farre *et al*., 2008, Bolong *et al*., 2009, Farré *et al*., 2012, Meador *et al*., 2016, Swartz *et al*., 2016, Wanda *et al*., 2017).

In South Africa, the existing knowledge gaps hinder the establishment of environmental policy and regulation efforts specific to CECs. However, some information can be adapted from global multilateral organizations such as the European Union (EU), World Health Organisation (WHO), and United Nations Environment Programme (UNEP) (Barbosa *et al*., 2016, Petersen, 2016). South Africa, as a signatory of the Stockholm convention (Bouwman, 2004), benefits from the progress made regarding the reduction of the environmental effects of persistent

organic pollutants (POPs) worldwide. However, some local and specific information is still required regarding the sources of CECs, their environmental fate, behaviour, and toxicity in aquatic ecosystems, to justify inclusion in routine monitoring programmes and regulatory efforts.

This review focuses on select groups of CECs, namely, pharmaceuticals, lifestyle drugs, pesticides, microplastics, engineered nanomaterials (ENMs) and metals. The ubiquity of these CEC parameters has been reported in various aquatic environmental compartments (Ansara-Ross *et al*., 2012, Murray *et al*., 2010, Wanda *et al.*, 2017, Bouwman *et al.*, 2018, Moeta *et al.,* 2019, Nthwane *et al.*, 2019, Pereao *et al*., 2020). These CECs are also extensively applied in a variety of commercial sectors, owing to their benefits to society and the economy (Rasheed *et al*., 2019).

2.2. Organics: pharmaceuticals, lifestyle drugs and pesticides

There is a wide application of pharmaceuticals in South Africa for human and veterinary purposes, owing to epidemics facing the country (such as HIV, TB and COVID-19). For example, an estimated 100 000–200 000 tons of antibiotics (Petersen, 2016) and 1.27 million kg of antiretroviral drugs are consumed each year (Schoeman *et al.*, 2017). It is estimated that a high percentage of the consumed pharmaceutical compounds are excreted in urine or faeces form. Consequentially, these compound residues end up in sewage systems, where they are inefficiently removed (Schoeman *et al*., 2017, Ngqwala and Muchesa, 2020). Eventually these residues spread across different environmental media. The presence of pharmaceuticals in the environment is worrisome, as some of these compounds are persistent and known for their endocrine disruption, antimicrobial resistance, and low environmental degradability (Petersen, 2016). The broad-scale application of pesticides in South Africa plays an important role in the country's economy with regards to job creation, poverty alleviation, and food security and quality (Horak *et al*., 2020). Despite the far-reaching benefits of their use, pesticides have become the focus of growing ecological concern due to the high percentage of applied pesticides that reaches non-target areas. The major concern is that they are sometimes persistent, carcinogenic, and endocrine-disrupting agents (Horak *et al*., 2020). This section focuses on the sources, pollution extent, distribution and ecotoxicological effects of selected pharmaceuticals, lifestyle drugs, and pesticides.

2.2.1. Sources and pathways of pharmaceuticals, lifestyle drugs and pesticides

In aquatic environments, the presence of pharmaceuticals and lifestyle drugs can be attributed mostly to the inefficiency of wastewater treatment works (WWTWs) in removing them (Osunmakinde *et al.,* 2013, Ngumba *et al*., 2016). Additional sources include direct discharge of effluent from pharmaceutical industries and hospitals (Ngumba *et al.,* 2016). Moreover, improper disposal of unused waste, rinsing of dermally applied products, runoff from landfill leachates and other impervious surfaces are probable sources (Agunbiade and Moodley, 2014, Petersen, 2016, Ngqwala and Muchesa, 2020). In the case of pesticides, aquatic environment exposure arises predominantly from agricultural runoff, leaching, spray drift, inadequate agricultural waste management practices and discharge from WWTWs (Yahaya *et al*., 2017, Rimayi, 2018, Horak *et al*., 2020). Rasheed *et al*., (2019) presented the generalised environmental pathway for this group of CECs (Figure 2.1).

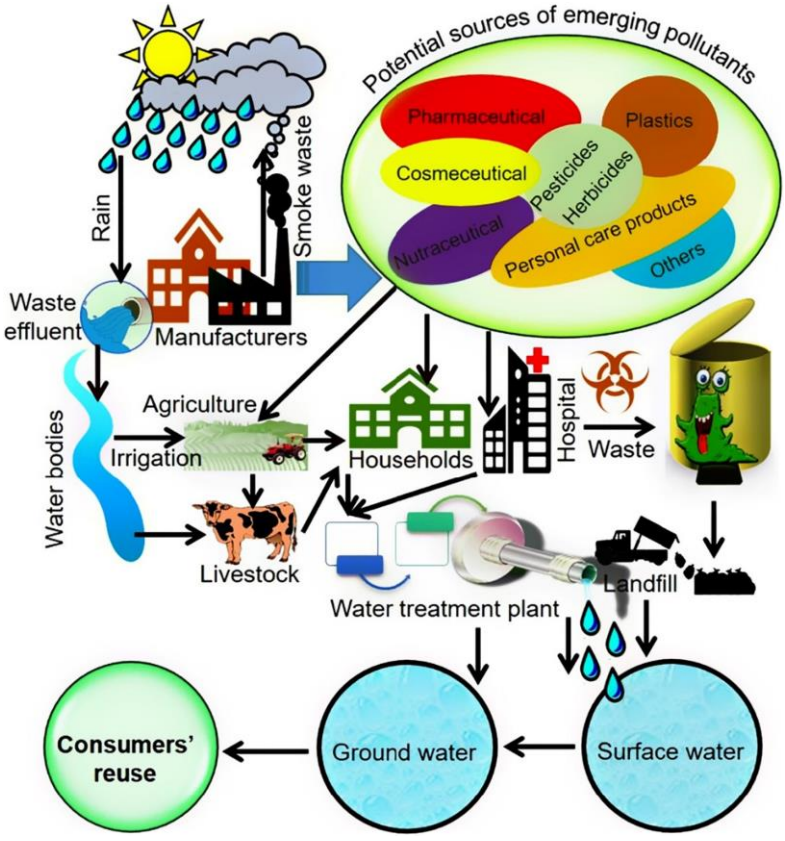


Figure 2.1: Conceptual environmental pathways of pharmaceuticals, lifestyle drugs and pesticides (adopted from Rasheed *et al*., 2019).

2.2.2. Environmental distribution and occurrence of pharmaceuticals, lifestyle drugs and pesticides

Wastewater treatment works (WWTWs) in South Africa do not effectively remove organic compounds (Osunmakinde *et al*., 2013, Wanda *et al*., 2017, Madikizela *et al*., 2017), although tertiary processes such as activated charcoal and ozone treatment can be incorporated to improve the treatment of organic pollutants in general. Consequently, organic compounds pass through WWTWs at detectable concentrations and can spread to receiving water resources. This is exacerbated by their hydrophobicity, persistency, partial degradation, and low volatility (Wanda *et al*., 2017). Therefore, they are distributed in water bodies such as oceans (Biel-Maeso *et al.,* 2018), groundwater (Barnes *et al*., 2004), estuaries (Thomas and Hilton, 2004), rivers (Bagnis *et al*., 2020) and lakes (Blair *et al*., 2013) and are further detected in drinking water (Osunmakinde *et al*., 2013, Wanda *et al.,* 2017).

There are growing reports about the ubiquitous nature of pharmaceuticals in different compartments of aquatic environments worldwide, for example, in Germany (Daughton and Ternes, 1999), Canada (Kleywegt *et al*., 2011), Kenya (K'oreje *et al*., 2012, Bagnis *et al*., 2020), China (Bu *et al*., 2013), United State of America (Blair *et al*., 2013), Portugal (Barbosa *et al*., 2016) and South Africa (Olujimi *et al*., 2010, Agunbiade and Moodley, 2014, Tijani *et al*., 2016). Likewise, pesticide distribution has been reported in USA (Stehle *et al*., 2019), Brazil (de Souza *et al*., 2008), Belgium (Tu *et al.*, 2009), Singapore (Bayen *et al*., 2004) and South Africa (Pick *et al.*, 1981, Pick *et al*., 1992, Du Preez *et al*., 2005, Ansara-Ross *et al*., 2012, Horak *et al*., 2020).

The mean concentrations of organics in South African aquatic environments have varied greatly within different matrices across different areas or systems (Tables 2.1 and 2.2). This could be attributed to several parameters such as different consumption rates, input variations, sampling times, seasonal effect, varying analytical methods, hydrological factors, natural attenuation factors, and proximity of the system to the sources such as agricultural activity, industries and livestock (Madikizela *et al.,* 2017, Bagnis *et al*., 2020).

Various drugs have been recorded in various aquatic media in South Africa. For example, ibuprofen has been detected in water, sediments, and WWTWs (Table 2.1) at high concentrations (Amdany *et al*., 2014, Matongo *et al.,* 2015a). This indicates high public usage of ibuprofen, as it is an over the counter drug, requiring no prescription by a medical doctor (Madikizela *et al*., 2017). Compounds such as

caffeine have been detected in influents (0.62–4.48 µg/L) and effluents (0.99–1.74 µg/L) at high concentrations (Table 2.1). Caffeine is known to be a useful marker of wastewater contamination (Bagnis *et al*., 2020). From the study conducted by Matongo *et al*., (2015a) in the Umgeni River, the concentration of caffeine recorded in the sediments was much higher than that in the surface waters (Table 2.1). This was ascribed to the negative partition coefficient of caffeine (log K_{ow} -0.07) which allows it to adsorb onto organic matter.

Carbamazepine (from contamination related to medical prescriptions and their persistence, and considered to be a wastewater marker) has been recorded in Darvil WWTWs, the surface waters of Msunduzi River, KwaZulu Natal and from Hartbeespoort Dam (North West) at different concentrations. These studies demontrate the wide occurrence and distribution of pharmeceuticals in aquatic environments across the country (Matongo *et al*., 2015b, Wanda *et al*., 2017). Nevirapine, an antiretroviral drug (ARV) was recorded in the surface waters of Roodeplaat Dam (Wood *et al*., 2015), where its prevalence was ascribed to high therapeutic usage, coupled with its persistence in the environment, indicating a high chance of inefficient removal by WWTWs. However, some of ARV compounds, such as lamivudine, stavudine, and abacavir can be removed with greater than 80% efficiency (Wood *et al*., 2015).

Research indicates that some compounds may occur in either original or metabolite form. For example, dichlorodiphenyl trichloroethane (DDT) may also be present in the form of dichloridiphenyl dichloroethylene (DDE) or 1,1 dichloro-2,2-bis(p-chlorophenyl) ethane (DDD). Atrazine may be detected in samples with their metabolites, such as terbuthylazine, simazine and propazine (Table 2.2).

To date, local research has demonstrated the widespread distribution of organics in aquatic environments. Their presence in waterways has been linked with various toxic effects. However, many of the studies emerge from systems in or near to metropolitan areas, while there is comparatively little known about water systems in non-urban areas such as the uMhlathuze and uThukela River systems.

Table 2.1: Mean concentrations of pharmaceuticals in different systems in South Africa

Matrix	Class	Compound	Locations	Method	Concentrations	Reference
WWTWs/ WWTP	NSAIDS	Naproxen	Goudkopples WWTP	HPLC	Influent (55.0 µg/L) Effluent (13.5 µg/L)	(Amdany *et al*., 2014)
			Northern WWTP	HPLC	Influent (52.3 µg/L) Effluent (20.4 µg/L)	(Amdany *et al*., 2014)
		Ibuprofen	Goudkopples	HPLC	Influent (39.8 µg/L) Effluent (12.6 µg/L)	(Amdany *et al*., 2014)
			Northern WWTP	HPLC	Influent (111.9 µg/L) Effluent (24.6 µg/L)	(Amdany *et al*., 2014)
	Steroid hormone	Testosterone	Darvill WWTW, KZN	Enzyme-linked immunosorbent assay	Influent (343 ng/L) Effluent (11 ng/L)	(Manickum and John, 2014)
		Progesterone	Darvill WWTW, KZN	Enzyme-linked immunosorbent assay	Influent (408 ng/L) Effluent (9 ng/L)	(Manickum and John, 2014)
	Stimulants	Caffeine	Darvill WWTP, KZN	HPLC-MS/MS	Influent (0.62-4.48 µg/L) Effluent (0.99-1.74 µg/L)	(Matongo *et al.,* 2015b)
	Anti-epileptic	Carbamazepine	Darvill WWTP, KZN	HPLC-MS/MS	Influent (2.21 µg/L) Effluent (0.91 µg/L)	(Matongo *et al.,* 2015b)

	Antiretrovirals	Nevirapine	Roodeplaat Dam system,	UHPLC-MS/MS	Effluent (973 ng/L)	(Wood *et al*., 2015)
Surface water	NSAIDS	Ibuprofen	uMgeni River	HPLC-MS/MS	0.23-6.20 µg/L	(Matongo *et al*., 2015a)
	Steroid hormone	Testosterone	Msunduzi River	Enzyme-linked immunosorbent assay	10 ng/L	(Manickum and John, 2014)
		Progesterone	Msunduzi River	Enzyme-linked immunosorbent assay	7-13 ng/L	(Manickum and John, 2014)
	Antibiotic	Sulfamethaxazole	uMgeni River	HPLC-MS/MS	0.22–6.02 ng/L	(Matongo *et al*., 2015a)
		Sulfamethazine	uMgeni River	HPLC-MS/MS	0.05–1.2 µg/L	(Matongo *et al*., 2015a)
		Erythromycin	uMgeni River	HPLC-MS/MS	0.03–0.26 µg/L	(Matongo *et al*., 2015a)
	Antipyretic	Acetaminophen	uMgeni River	HPLC-MS/MS	1.13–1.78 µg/L	(Matongo *et al*., 2015a)
			Msunduzi River	HPLC-MS/MS	0.99–1.74 µg/L	(Matongo *et al*., 2015b)
	Stimulants	Caffeine	uMgeni River	HPLC-MS/MS	0.41–9.25 µg/L	(Matongo *et al*., 2015a)
			Msunduzi River	HPLC-MS/MS	0.11–3.32 µg/L	(Matongo *et al*., 2015b)
		Nicotine	Gauteng River	UPLC/TQD-MS	154.3–245.5 ng/L	(Archer *et al.,* 2017)
	Antidepressants	Venlafaxine	Gauteng River	UPLC/TQD-MS	35.4–94.6 ng/L	(Archer *et al.,* 2017)
	Diabetes	Metformin	Gauteng River	UPLC/TQD-MS	73.3–174.6 ng/L	(Archer *et al*., 2017)
	Antiepileptic	Carbamazepine	Msunduzi River	HPLC-MS/MS	0.14–3.24 µg/L	(Matongo *et al*., 2015b)
			Hartbeespoort Dam, North West	GCxGC-HRTOFMS	8.58 ng/L	(Wanda *et al*., 2017)
	Industrial compounds	Bisphenol A	Hartbeespoort Dam, North West	GC XGC – HRTOFMS	81.24 ng/L	(Wanda *et al.,* 2017)

Sediment	NSAIDs	Ibuprofen	uMgeni River, KZN	HPLC-MS/MS	6.53–41.41 ng/g	(Matongo *et al.,* 2015a)
	Antibiotic	Sulfamethaxazole	uMgeni River, KZN	HPLC-MS/MS	15.95–17.2 ng/g	(Matongo *et al*., 2015a)
		Erythromycin	uMgeni River, KZN	HPLC-MS/MS	0.12–1.57 ng/g	(Matongo *et al*., 2015a)
	Antipyretic	Acetaminophen	uMgeni River	HPLC-MS/MS	6.03–8.96 ng/g	(Matongo *et al*., 2015a)
			Msunduzi River	HPLC-MS/MS	6.33–15.8 ng/g	(Matongo *et al*., 2015b)
	Stimulants	Caffeine	uMgeni River	HPLC-MS/MS	1.87–224.35 ng/g	(Matongo *et al*., 2015a)
			Msuduzi River	HPLC-MS/MS	1.32 ng/L	(Matongo *et al*., 2015b)
Tap water	Stimulants	Caffeine	Gauteng	UHPLC-MS/MS	263 ng/L	(Wood *et al.,* 2015)
	Antiretrovirals	Zidovudine	Hartbeesport Dam, North West	UHPLC-MS/MS	72.7 ng/L	(Wood *et al.*, 2015)

HPLC (High-performance liquid chromatography); HPLC-MS/MS (high-performance liquid chromatography coupled to a mass spectrometer); GCxGC-HRTOFMS (2-dimensional gas chromatography coupled to high-resolution time-of-flight mass spectrometry); UHPLC-MS/MS (ultrahigh-pressure liquid chromatography-tandem mass spectrometry); UPLC/TQD-MS (ultrahigh performance liquid chromatography and triple-quadrupole-mass spectrometer)

Table 2.2: The mean concentrations of pesticides in different systems in South Africa

Matrix	Class	Compound	Locations	Method	Concentrations	References
Surface water	Insecticides	DDT	Jukskei River	Activated carbon technique	0.11–0.80 µg/L	(Okonkwo *et al.*, 2007)
			Eastern Cape freshwater systems	LLE	<0.006–0.26 µg/	(Awofolu and Fatoki, 2003)
			Limpopo Vhembe district	GC-MS	<0.10–0.30	(Bornman *et al*., 2010)
		DDE	Limpopo Vhembe district	GC-MS	0.06–1.21 µg/L	(Okonkwo *et al*., 2007)
		DDE	Eastern Cape freshwater systems	LLE	<0.008–0.24 µg/L	(Awofolu and Fatoki, 2003)
		DDE	Laurens River, Western Cape	GC-MS	0.01 µg/L	(Bollmohr *et al*., 2007)
	Herbicide	Atrazine	Transvaal		0.73–8.0 µg/L	(Pick *et al*., 1992)
			Hartbeespoort Dam	GC-MS	<5–923 ng/L	(Rimayi, 2018)
		Propazine	Hartbeespoort Dam	GC-MS	65–208 ng/L	(Rimayi, 2018)
		Terbuthalazine	Viljoenskroom (Free State), Pottchefstroom	GC-MSD	1.04–4.10 µg/L	(Du Preez *et al.,* 2005)
		Simazine	Viljoenskroom (Free State), Pottchefstroom	GC-MSD	1.00–3.20 µg/L	(Du Preez *et al*., 2005)

		Heptachlor	Eastern Cape freshwater systems	LLE	<0.01–0.20 µg/L	(Awofolu and Fatoki, 2003)
Sediment	Insecticides	Heptachlor	Jukskei River, Gauteng	LLE and SE	22.5–14.0 µg/kg	(Sibali *et al*., 2008)
		Carbaryl	Ubombo and Ingwavuma district, KZN	GC-MS	<0.001–50.1 µg/kg	(Sereda and Meinhardt, 2003)
		DDX	Amanzimnyama River, KZN		364.5 and 373.9 ng/g	(Newman *et al*., 2015)

DDE (Dichloridiphenyl dichloroethylene); DDT (Dichlorodiphenyl trichloroethane); DDE (Dichloridiphenyl dichloroethylene); DDD (1,1 dichloro-2,2-bis(p-chlorophenyl) ethane); LLE (Liquid/liquid extraction); SE (Soxhelet Extraction); GC-MS (Gas Chromatography-Mass Spectrometry), GC-MSD (single quadrupole Gas Chromatography -Mass Spectrometry)

2.2.3. Ecotoxicological effects of pharmaceuticals, lifestyle drugs and pesticides on aquatic organisms

Some organic pollutants have been shown to cause negative effects on aquatic organisms, such as mortality, as well as changes in physiology, behaviour, and reproduction properties. These pollutants include pesticides (insecticides, fungicides, herbicides), life-style drugs, and human and veterinary pharmaceuticals. They bioaccumulate in non-target organisms, especially in their fatty tissues, owing to their hydrophobic and lipophilic properties (Yahaya *et al.,* 2017, Ebele *et al*., 2017). Organisms can be exposed to these compounds through inhalation, ingestion, and dermal contact, and can cause health issues such as endocrine disruption, reproductive toxicity, genotoxicity, mutagenicity, carcinogenicity, chemo-sensitization, and neurotoxic effects as well as other associated acute and chronic toxicities (Table 2.3).

Table 2.3: Examples of measured effects of certain organic residues in aquatic organisms

Therapeutic groups	**Compounds**	**Adverse effects**	**Test Organism**	**Example of studies.**
Analgesics	Ibuprofen	DNA damages, and effects on biomarker responses (GST and GPX were activated and LPO induction).	*Carcinus maenas*	(Hong *et al.*, 2007, Aguirre-Martínez *et al*., 2013)
Antibiotics	Ferazolidone	Gills showed the in reduced AChE activity	*Penaeus monodon*	(Gracia *et al*., 2007, Ji *et al.*, 2010, Lacaze *et al.*, 2015)
Antidiabetics	Metformin	Endocrine-disrupting effects	*Pimephales promelas*	(Niemuth and Klaper, 2015, Godoy *et al.*, 2018)
Anti-convulsants	Carbamazepine,	Biochemical effects	*Perna viridis*	(Oetken *et al*., 2005, Qiang *et al*., 2016, Chen *et al*., 2019)
Antifungals	Vinclozolin and dicofol	Endocrine disrupting effects.	*Daphnia magna*	(Haeba *et al.,* 2008)
Beta-blockers	Propranolol	Growth and reproduction changes.	*Oryias latipes*	(Huggett *et al*., 2002, Feiner et *al*., 2014)
Psychiatric drugs	Fluoxetine, venlafaxine, paroxetine	DNA damages, genotoxicity, cytotoxicity and immunotoxicity.	*Mytilus edulis*	(Lacaze *et al.,* 2015)
Insecticides	Carbofuran	Biomarker and behavioural changes	*Dicentrarchus labrax*	(Hwang *et al*., 2004, Campos-Garcia *et al.*, 2015)
Herbicides	Atrazine	Endocrine disrupting effects.	*Carassius auratus, Pimephales promelas*	(Ralston-Hooper *et al.,* 2009)

Fungicides	Carbendazim,	DNA damages, changes in feeding rates	*Daphnia magna*	(Miracle *et al.*, 2011, Singh *et al.*, 2016, Li *et al.*, 2019)
	Chlorothalonil	Biochemical changes	*Mytilus edulis, Crassostrea gigas*	Haque *et al.,* 2009

DNA (Deoxyribonucleic acid); GST (Glutathione-S-transferase); GPX (Glutathione peroxidase); LPO (Lipid peroxidation) and AChE (Acetylcholinesterase)

2.3. Microplastics

Microplastics (particles less than 5 mm in diameter) are currently a material of choice due to their unique characteristics for novel applications in a broad range of industrial uses. Microplastics are widely used in plastic bottles, air-blasting media, synthetic clothing fabrics, skincare products and toothpaste (Peng *et al*., 2017), and their volumes have resulted in notable environmental release (Pereao *et al*., 2020). Microplastic pollution in South African waters is a matter of growing environmental concern, which is receiving attention from researchers and environmental health authorities. There is an urgent need for further research based on the pollution of microplastics, especially in freshwater environments, as much of the information generation thus far has been largely dedicated to marine environments. The next section discusses microplastic sources, distribution, occurrence extent and ecotoxicological effects.

2.3.1. Sources and pathways of microplastics

Microplastics can be categorised into primary and secondary plastics (Table 2.4), and their introduction into aquatic systems is primarily driven by a variety of anthropogenic activities (Pereao *et al*., 2020). Microplastics find their way through sewage effluents as a consequence of domestic activities (washing of clothes, use of cosmetics products containing microplastics), industrial effluents, runoff from landfills, illegal dumping sites, accidental spillage, and atmospheric deposition, as well as a breakdown of larger plastic particles to smaller ones (Bouwman *et al*., 2018, Li *et al.,* 2018, Pereao *et al*., 2020).

Table 2.4: Classification of microplastics (Anderson *et al.,* 2017, Li *et al*., 2018, Pereao *et al*., 2020)

Class	Definition
Primary microplastics	Intentionally manufactured for industrial or domestic purposes, for instance, those that are found in textiles, medicines, industrial pellets, and personal care products (e.g., facial and body scrubs).
Secondary microplastics	Formed via breakdown of larger plastics and synthetic materials through physical, biological, and chemical processes (e.g., those that originate from fishing lines, industrial resin pellets, household items, discarded plastics).

2.3.2. Environmental distribution and occurrence of microplastics

Microplastics are widely dispersed throughout all environmental matrices (Anderson *et al*., 2017). They have been documented in the open oceans (Law and Thompson, 2014, Jamieson *et al.,* 2019), coastal areas (Nel and Froneman, 2015, Setälä *et al.*, 2016), estuarine systems (Naidoo *et al*., 2015), rivers (Bouwman *et al*., 2018), lakes (Free *et al*., 2014), WWTWs (Conley *et al*., 2019) and drinking water (Bouwman *et al*., 2018). However, the distribution of microplastics in freshwater environments is still largely unknown, particularly in South Africa (Figures 2.3 and 2.4; Eerkes-Medrano *et al.,* 2015, Bouwman *et al.*, 2018, Reynolds and Ryan, 2018, Weideman *et al*., 2019). WWTWs are inefficient in removing microplastics (Peng *et al*., 2017, Anderson *et al*., 2017, Pereao *et al.,* 2020). Microplastics in effluents can easily spread into receiving surface water and sediments (Naidoo *et al*., 2015, Nel and Froneman, 2015), seep into groundwater or find their way into drinking water (Bouwman *et al*., 2018).

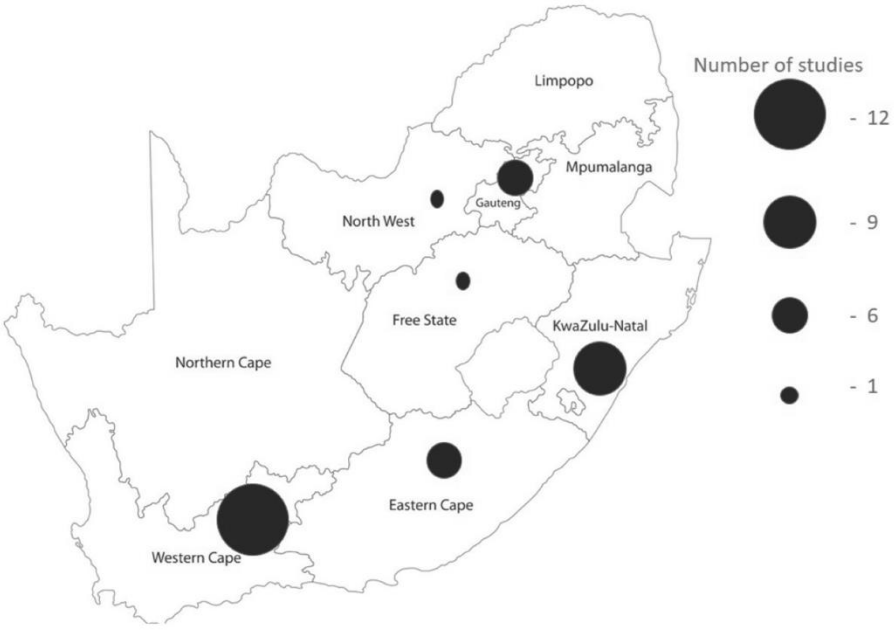


Figure 2.2: South African studies on microplastics occurrence and distribution in different provinces. Data obtained on Web of Science and Google scholar during November 2020 using search line: occurrence or presence of microplastics in South Africa aquatic systems or waterways

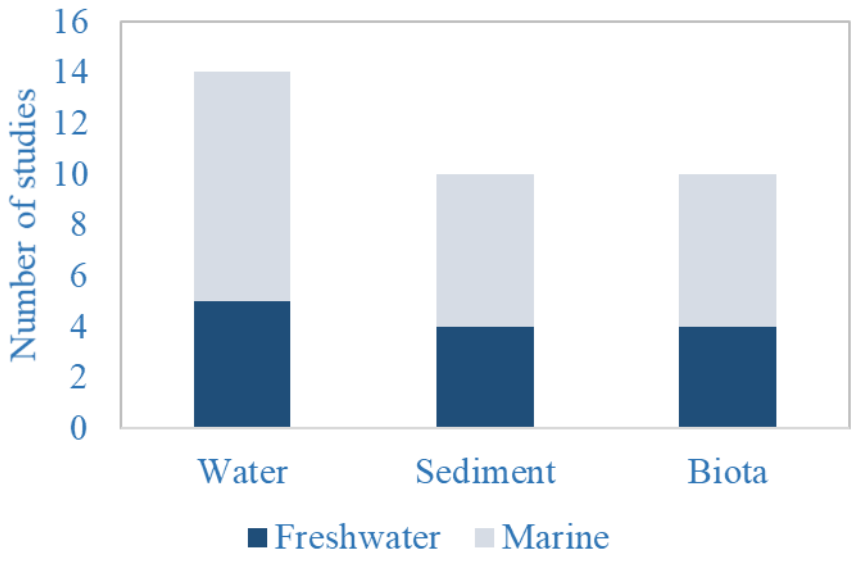


Figure 2.3: Comparative analysis on microplastics contamination between various environmental compartments in marine and freshwater ecosystems. Data obtained on Web of Science and Google scholar during November 2020 using search line: occurrence or presence of microplastics in South Africa aquatic systems or waterways

The body of knowledge on the extent of microplastics pollution has largely focused on marine environments, and few reports exist on freshwater environments (Li *et al.*, 2018). Table 2.5 summarises some of the South African studies on the distribution and extent of occurrence of microplastics in aquatic environments. The microplastics densities found in these studies varied greatly. This can be attributed to varying sampling locations, anthropogenic and production rates between regions, inherent natural conditions, sampling approaches and varying analytical methods (Eerkes-Medrano *et al*., 2015, Li *et al*., 2018, Pereao *et al*., 2020). Many land-based activities contribute substantial volumes of microplastics to the aquatic environment. For example, large quantities of microplastics discharged into WWTWs end up in natural waters (Table 2.5). Moreover, domestic, and industrial activities such as washing of clothes, and leakage through the manufacturing of plastics in industries, are also notable sources (Bouwman *et al*., 2018). A study conducted by Naidoo *et al.,* (2015) in five estuaries in KZN reported a higher concentration of microplastics detected in estuaries near urban areas of Durban, peaking in Durban Harbour, with the concentrations decreasing further from the urban areas of Durban. This clearly indicated a high exposure potential in urban area settings.

Table 2.5: Ubiquitous occurrence of microplastics across South Africa

Matrix	Location	Sampling method, processing	MP densities	References
Water (surface, ocean, tap water)	Orange-Vaal River	Bulk water, gravity-filtration, and identification	0.23 ± 0.27 particles per L^{-1} (mean, SE)	(Weideman *et al*., 2019)
	Orange-Vaal River	Neuston net, gravity-filtration, and identification	0.04 ± 0.16 particles.m^{-2} (mean, SE)	(Weideman *et al*., 2019)
	South-eastern coastline of South Africa	Filtration, density separation and identification	257.9 ± 53.36 -1215 ± 276.7 particles.m^{-3} (mean, SE)	(Nel and Froneman, 2015)
	Cape town	Filtration and identification	1.15 ± 1.45 particles m^{-3} (mean, range)	(La Daana *et al.,* 2017)
	Gauteng and North-West Province	Density separation, filtration, counting, and FTIR	1.9 -5.2 particles per L (mean, maximum)	(Bouwman *et al*., 2018)
	Isipingo Estuary	Conical plankton net, filtration, and identification	50.6 ± 56.0 particles per L (mean, SD)	(Govender *et al.,* 2020)
	Isipingo Estuary	Conical zooplankton net, filtration, and identification	31.1 ± 11.1 particles per L (mean, SD)	(Naidoo *et al.*, 2015)
	uMgeni Estuary	Conical zooplankton net, filtration, and identification	25.3 ± 6.0 particles per L (mean, SD)	(Naidoo *et al*., 2015)

	iLovu Estuary	Conical zooplankton net, filtration and identification	10.2 ± 11.3 particles per L (mean, SD)	(Naidoo *et al*., 2015)
	Durban Harbour	Conical zooplankton net, filtration and identification	70.3 ± 119. 3 particles per L (mean, SD)	(Naidoo *et al.*, 2015)
	uMdloti Estuary	Conical zooplankton net, filtration and identification	11.0 ± 11.5 particles per L (mean, SD)	(Naidoo *et al.*, 2015)
	St. Lucia Estuary	Conical plankton net, filtration, and identification	11.9 ± 11.2 particles per L (mean, SD)	(Govender *et al.,* 2020)
Sediment	Durban Bay	Gravity corer, density separation and identification	1750 pieces/kg-dry	(Matsuguma *et al*., 2017)
	Western Cape	Van Veen grab, density separation and identification	21-30 fibres per 250 ml of sediment	(Browne *et al*., 2011)
	South-eastern coastline of South Africa	Filtration, density separation and identification	688.9 ± 348.2 – 3308 ± 1449 particles.m^{-2} (mean, SE)	(Nel and Froneman, 2015)
	Isipingo Estuary	Corer, filtration, density separation and identification	143.5 ± 93.0 particles per 500 g of sediment (mean, SD)	(Govender *et al.*, 2020)
	Isipingo Estuary	Corer, density separation, filtration and identification	47.6 ± 22.8 (mean, SD)	(Naidoo *et al.*, 2015)
	uMgeni River	Corer, density separation, filtration and identification	41.7 ± 23.0 particles per 500 ml (mean, SD)	(Naidoo *et al*., 2015)
	iLovu Estuary	Corer, density separation, filtration and identification	13.7 ± 5.6 particles per 500 ml (mean, SD)	(Naidoo *et al*., 2015)
	uMdloti Estuary	Corer, density separation, filtration and identification	19.9 ± 16.2 particles per 500 ml (mean, SD)	(Naidoo *et al*., 2015)

	Durban Harbour	Corer, density separation, filtration and identification	159.9 ± 271.2 particles per 500 ml (mean, SD)	(Naidoo *et al.*, 2015)
	St. Lucia Estuary	Metal corer, filtration, density separation and identification	18.5 ± 34.4 per 500 g (mean, SD)	(Govender *et al*., 2020)
Biota				
Mussel species	Coast of Cape Town	Digestion, filtration and identification	2.33 ± 0.2 particles per g (mean, SE)	(Sparks and Immelman, 2020)
Waterbirds species	Barberspan Nature Reserve, North-West Province	Filtration and identification	1.44 ± 0.72 particles in feather brushing 1.53 ± 0.64 particles in faecal samples (mean, SE)	(Reynolds and Ryan, 2018)
Mullet (*Mugil cephalus*)	Bayhead mangroves of Durban harbour	Dissection and identification	3.0 ± 4.7 particles per fish (mean, SD)	(Naidoo *et al*., 2016)

MP (microplastic); FTIR (Fourier Transform-infrared spectroscopy); SE (standard error); SD (standard deviation); ml (millilitre); g (gram); L (litre).

2.3.3. Ecotoxicological effects of microplastics on aquatic organisms

The low biodegradability and half-life properties of microplastics in the environment increases their potential for ingestion by aquatic biota. Microplastics have the potential to act as a vector for the transfer and exposure of other contaminants such as persistent organic pollutants (POPs) to organisms (Pereao *et al*., 2020), enabling adsorbed pollutants to be transferred to different trophic levels (Ng and Obbard, 2006, Peng *et al*., 2017, Auta *et al*., 2017, Peng *et al*., 2020). Microplastics can induce physical, biochemical, and biological effects on organisms. Physical impacts could be entanglement or ingestion of microplastics by organisms. Due to the small size of microplastics, they can be mistaken for food (Li *et al.*, 2018, Pereao *et al*., 2020). The ingestion of microplastics can also induce delayed ovulation, decreased steroid hormone levels, reduced growth rate, reproductive failure, interference with enzyme production, gene exchange and even mortality (Pereao *et al.,* 2020, Peng *et al*., 2020). In addition, the adsorption of microbiota can influence the spatial movement of organisms as an avoidance mechanism (Peng *et al.,* 2020). The ability of microplastics to induce lethal and sublethal effects can be influenced by factors such as exposure duration, sensitivity and type of organism, microplastics' retention time in the organism's body, type, and shape of ingested microplastics as well as the organism's excretion rate (Pereao *et al*., 2020).

2.4. Engineered nanomaterials (ENMs)

Engineered nanomaterials **(**ENMs) are receiving a lot of attention due to their extensive production and applications in industry. However, these applications raise concerns about the potential human and environmental health risks related to ENMs. These concerns are being addressed at a slow pace. There is still a lack of information regarding sources and exposure of ENMs in the environment. The paucity of data, particularly regarding water resources, serves as motivation for this study to focus on the sources, distribution, occurrence, and bioaccumulation of ENMs. This section focusses on ENMs, their sources, distribution, and extent of occurrence, as well as their bioaccumulation in aquatic environments.

2.4.1. Sources and pathways of engineered nanomaterials (ENMs)

Ever-increasing applications and production of ENMs have inevitably increased their likelihood of intentional (e.g., incorporation of ENMs in products used as environmental remediation) and unintentional (e.g., ENMs in wastewater) release into the environment, either as original ENMs, transformed ENMs or product released (Selck *et al*., 2016, Lehutso *et al*., 2020). Pathways of ENMs to the environment can be through accidental spills during their manufacture, during product use, and end-of-life disposal. In particular, ENMs derived from domestic and industrial products are likely to be major sources, via disposal of sewage sludge as part of WWTWs (Fabrega *et al*., 2011, Wepener *et al*., 2013, Nthwane *et al*., 2019). The generalised environmental pathways for ENMs are illustrated in Figure 2.7.

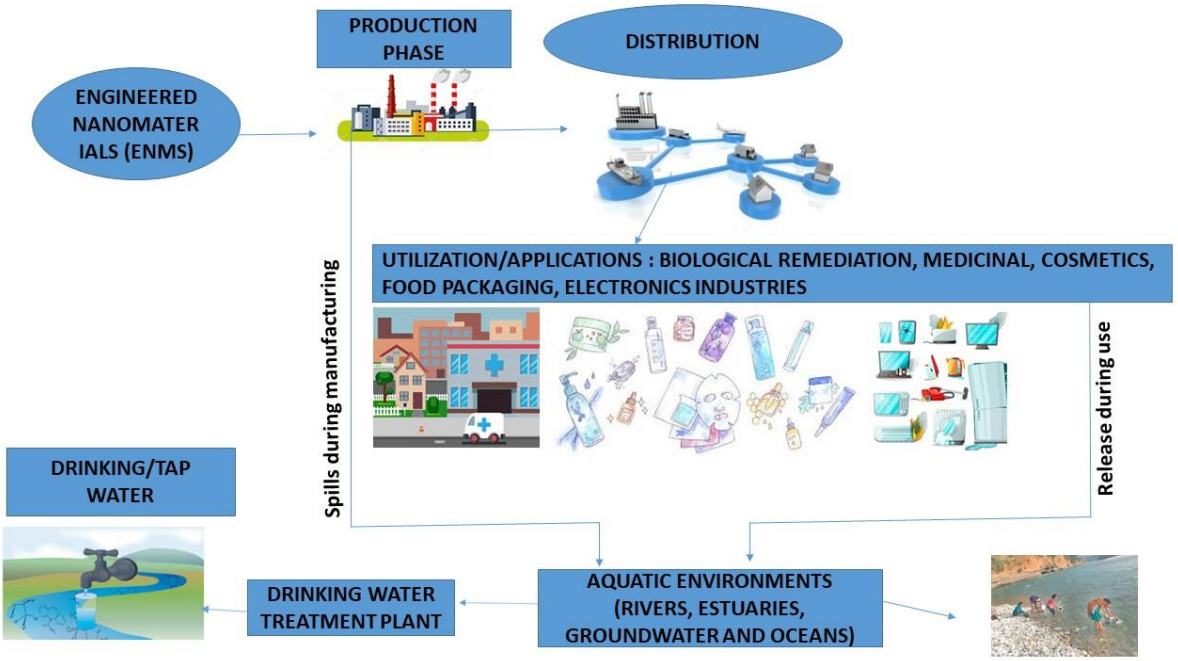


Figure 2.4: Representative sources and routes of ENMs and their pathway to the environment

2.4.2. Environmental distribution and occurrence of engineered nanomaterials (ENMs)

In past studies, aquatic compartments (water, sediments, and organisms) have been investigated for metal contamination and accumulation (Mzimela *et al*., 2003, Mzimela *et al*., 2014, Adeleke *et al*., 2020, Vetrimurugan *et al*., 2019, Edokpayi *et al.,* 2016, Majola *et al*., 2020, Izegaegbe *et al*., 2021, Mzimela and Izegaegbe, 2021). Recently, metals in nano form (nanoparticles) have been researched. Nanotechnology has reached consumer markets, and ENMs have been extensively applied in nano-enabled products (NEPs). Their wide distribution has been predominantly driven by their novel physicochemical properties. Therefore, wide applications of these NPs has increased their potential to reach the environment through different aforesaid sources and become released into natural systems, eventually reaching WWTPs, rivers, estuaries, and oceans (Kiser *et al*., 2009).

Few studies have been conducted on African water bodies regarding the environmental occurrence of ENMs and their toxicity potential (Moeta *et al.,* 2019). Large data sets are generated predominantly from international studies (e.g., Asia, South and North America, Europe, Singapore; Borm *et al*., 2006, Griffitt *et al*., 2008,

Navarro *et al*., 2008, Zhang *et al.*, 2015). Therefore, these data do not compare well to South African scenarios due to differences in socio-economic factors, disposal patterns, and waste management practises (Moeta *et al*., 2019). There are few studies existing in South Africa (Musee *et al*., 2010, Wepener *et al*., 2013, Moeta *et al*., 2019, Nthwane *et al*., 2019). For example, Nthwane *et al*., (2019) recorded TiO_2NPs in deionized wastewater and tap water, at concentrations of 0.12–1.16 µg/L and 0.16–0.17 µg/L, respectively. Once nanoparticles are introduced into aquatic environments, they tend to be distributed into water, suspended solids and sediments, and eventually accumulated by organisms through ingestion and absorption. Therefore, bioaccumulation of ENMs is another focus of the present study.

2.4.3. Bioaccumulation of engineered nanomaterials (ENMs)

This section discusses the bioaccumulation of ENMs (silver (Ag), titanium dioxide (TiO_2), aluminium oxide (Al_2O_3), iron oxide (Fe_2O_3) and copper oxide (CuO)) to aquatic organisms, further summarised in Table 2.6.

A dose-dependent accumulation pattern in tissues (gills > hepatopancreas > green glands > muscles) was reported after 23 days of exposure of *Procambarus clarkia* to 25, 125 and 250 mg/l of TiO_2NPs (Abd El-Atti *et al*., 2019). Accumulation of Ti in hepatopancreas due to its crucial role in pollutant storage, redistribution and detoxification processes was noted in the study. The authors also observed accumulation of Ti in gills, and this was associated with the large surface area of the epithelium and direct contact with water, which renders them more predisposed to the uptake of aqueous chemical pollutants. A low amount of Ti accumulated in muscles and green glands was ascribed to the fact that they are inactive organs in accumulating metals and not in direct contact with pollutants, therefore, the variation in tissue accumulation observed was attributed to the dissimilarities in the physiological role of each organ (Abd El-Atti *et al*., 2019). Ates *et al*., (2013) observed no significant accumulation of Ti in the muscles and brain of *Carassius auratus* exposed to 10 and 100 mg/L of TiO_2NPs for 5 days. However, accumulation of Ti in gills and intestines showed concentration dependency, increasing from 42.71 to 110.69 ppb in the intestine and from 4.10 to 9.86 ppb in the gills of fish, with increasing exposure doses from 10 to 100 mg/L. D'Agata *et al*., (2014) reported differential accumulation of

metallic contaminants in mussel tissues, specifically in their digestive glands, which had a high accumulation rate relative to the gills (Ti accumulated in gills was 10 times lower than in the digestive glands). This was attributed to the nature of gills to excrete larger particles as pseudofaeces, while smaller particles are transported to the digestive glands. *Danio rerio* (*D. rerio*) and *Elodea Canadensis* (*E. canandesis*) exposed to TiO_2NPs for 2 weeks showed a high accumulation of Ti compared to the control samples (Asztemborska *et al.*, 2018). This was ascribed to the NPs present in the sediment. Asztemborska *et al*., (2018) also exposed *D. rerio* to Ti contaminated *E. canandesis* and showed the accumulation of Ti in *D. rerio*. They concluded that *D. rerio* feeding on the contaminated plants was the main cause of Ti bioaccumulation. These results concurred with the Ag bioaccumulation of *D. rerio* which were fed with contaminated *Chironomus* larvae, revealing the ability of ENMs to biomagnify through the food chain (Asztemborska *et al*., 2014).

A dose-and time-dependent Ag bioaccumulation in the gills of juvenile *Prochilodus lineatus* exposed to 2.5 and 25 µg/L of AgNPs for 5 days and 15 days was reported by Ale *et al.*, (2018). Because gills are known to be a tissue with high blood flow, they are particularly affected by toxins (Ale *et al.*, 2018). Afifi *et al*., (2016) exposed *Oreochromis niloticus* (*O. niloticus*) and *Tilapia zillii* (*T. zillii*) to a concentration of 2 mg/L and 4 mg/L for 15 days to check the accumulation of Ag in brain tissues. Results revealed penetration of AgNPs in brain cells of *O. niloticus* and *T. zillii*, which speaks to the fact that particles are carried through the body of fish by blood circulation, subsequently accumulating in different important organs (Afifi *et al*., 2016). Asztemborska *et al*., (2014) conducted a study on the accumulation of AgNPs in *Chironomus* larvae and the fish *D. rerio* and it was observed that both species accumulated AgNPs. Results revealed active accumulation of AgNPs in *Chironomus* larvae within 30 hours, which decreased thereafter. This subsequent decrease of AgNPs indicates possible excretion of Ag as NPs or as ions. It was observed that the accumulation of Ag in *D. rerio* fed with contaminated *Chironomus* larvae was 110-fold higher than in organisms living in the water. Therefore, it can be concluded that contamination of NPs can accumulate from lower trophic to higher trophic levels.

Kaya *et al*., (2015) reported that colloidal suspensions of small ZnONPs (10–30 nm) modulate high oxidative stress and toxic effects on *Oreochromis niloticus* compared to the larger NPs (100 nm). Gurkan *et al*., (2019) reported that NPs of Al_2O_3, Fe_2O_3

and CuO accumulate in crabs in a tissue-specific manner and negatively affect immunity and other biomarkers. Gurkan *et al*., (2019) revealed that the effect, fate, and toxicity of NPs in the environment is governed by particle surface area, charge, dimension, shape, functioning and coating type.

Comparison of different studies showed accumulation in different tissues of organisms, notably, because bioaccumulation is directly associated with exposure conditions. However, other studies showed no, or low amounts of NPs accumulated due to dissimilarities in organisms' excretion or regulation rate. Moreover, size, surface chemistry, concentrations, exposure duration and other related characteristics may influence the toxicity of these NPs.

Table 2.6: Bioaccumulation of ENMs in aquatic organisms. *P. clarkii* (*Procambarus clarkia); C. auratus (Carassius auratus); P. lineatus (Prochilodus lineatus), O. niloticus (Oreochromis niloticus); T. zillii (Tilapia zillii); V. faba (Vicia faba); D. rerio (Danio rerio); E. canadensis (Elodea canadensis); A. baeerii (Acipenser baerii); O. mykiss (Oncorhynchus mykiss), O. mossambicuss (Oreochromis mossambicuss); C. aestuarii (Carcinus aestuarii); M. galloprovincialis (Mytilus galloprovincialis)*

ENPs Type	Uptake detection method	ENPs characteristics	Exposure media; water, concentration, and duration	Aquatic organisms	Results observed	Reference
TiO_2NPs	ICP-OES	55 ± 8 nm; crystal form	Aerated tap water; 0, 25, 125, 250 mg/L; 28 days.	*P. clarkii*	The level of tissue accumulation sequence: Gills > hepatopancreas > green glands > muscles.	(Abd El-Atti *et al*., 2019)
	ICP- OES and ICP - MS	21 nm	Seawater; 10 mg/L; 4 days	*M. galloprovincialis*	Ti accumulated in muscles in a tissue-specific manner.	(D'Agata *et al*., 2014)
	ICP- MS	10–30 nm, spherical	Dechlorinated tap water; 10 and 100 mg/L, 5 days	*C. auratus*	High accumulation was observed in the gills and intestine, while muscle and brain showed a small amount Ti accumulation.	(Ates *et al.*, 2013)
	ICP-MS	< 100 nm, spherical	Freshwater; 10 mg/L; 2 Weeks	*D. rerio*	Bioaccumulation of TiO_2NPs or ions was observed.	(Asztemborska *et al*., 2018)
	ICP -MS	10–50 nm, Al coated	Deionized water; 5, 25 and 50 mg/L; 48 hours	*V. faba*	A high concentration of Ti was recorded in roots	(Foltête *et al*., 2011)

	ICP-MS	< 100 nm		*E. canadensis*	Bioaccumulation of TiO_2NPs or ions was observed.	(Asztemborska *et al*., 2018)
AgNPs	Gamma spectrometer with an HPGe detector	< 100 nm	Freshwater; 0.1, 1 and 10 mg/L; 48 hours	Chironomid	AgNPs accumulated in Chironomid larvae for the first 30 hours of exposition and later decreased.	(Asztemborska *et al*., 2014)
	GF AAS	20–40 nm	Aerated dechlorinated water; 0, 2.5 and 25.0 µg//L; 5 and 15 days	*P. lineatus*	Dose and time-dependent Ag accumulation in the gills.	(Ale e*t al.,* 2018)
	ICP- AES with ULTIMA 2 apparatus	< 100nm	Deionized water; 2 and 4 mg/L; 15 days	*O. niloticus*	Ag accumulated in brain tissue in both concentrations.	(Afifi *et al*., 2016)
	ICP- AES with ULTIMA 2 apparatus	< 100nm	Deionized water; 2 and 4 mg/L; 15 days	*T. zillii*	Ag Accumulated in brain tissue in both concentrations.	(Afifi *et al*., 2016)
	Gamma spectrometer with an HPGe detector	< 100 nm	Freshwater; 0.05 mg/L; 8 days	*D. rerio*	Ag did not accumulate in the body of fish only remained in the digestive system of fish	(Asztemborska *et al*., 2014)
CuONPs	ICP-MS	40 nm	Brackish water; 1 mg/L; 14 days	*C. aestuarii*	CuONPs accumulated in order of hepatopancreas > haemolymph > gill > muscle.	(Gürkan, 2018)

	GF AAS	<50 nm	Seawater: 10 µg/L; 15 days	*M. galloprovincialis*	CuONPs were accumulated in gills and further induction of AChE and metallothioneins.	(Gomes *et al*., 2011)
Al_2O_3 NPs	ICP-MS	40 nm	Brackish water; 1 mg/L; 14 days	*C. aestuarii*	Tissue distribution of Al_2O_3 NPs was gill > muscle > haemolymph>hepatopancreas.	(Gürkan, 2018)
Fe_2O_3 NPs	ICP-MS	20–40 nm	Brackish water; 1 mg/L; 14 days	*C. aestuarii*	Fe_2O_3 NPs accumulated in the following sequential order: gill > hepatopancreas > muscle > haemolymph	(Gürkan, 2018)
ZnONPs	ICP-MS	10–30 and 100 nm	Freshwater; 1 and 10 mg/L; 14 days	*O. niloticus*	Accumulation for small NPs (10–30) was higher than for larger NPs.	(Kaya *et al*., 2015)
	ICP-MS	<100nm	Freshwater; 0, 0.5, 1.0 and 1.5 mg/L; 14 days	*O. mossambicus*	ZnONPs accumulated in liver at highest concentration ad further induced oxidative stress and histological changes.	(Shahzad *et al*., 2019)

ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometry), ICP-MS (Inductively coupled plasma mass spectrometry), ICP–AES (Inductively coupled plasma-atomic emission spectroscopy); GF AAS (Graphite furnace atomic absorption spectrophotometer); Al (Aluminium); Ti (Titanium); Ag (Silver).

2.5. Metals

Ecotoxicology is considered to be the best tool for the critical assessment of environmental quality, through the identification of disturbances and the impacts on the environment from toxic contaminants, using functions such as exposure or occurrence, and bioaccumulation and its effects (Mzimela and Izegaegbe, 2021). Bioaccumulation, bioconcentration and biomagnification are critical terms that are usually used as part of ecotoxicological evaluations. In this study, bioaccumulation is defined as a net accumulation of contaminants from the surrounding media by all possible routes including direct contact, ingestion, dietary exposures from any source such as water, food, and sediment (Newman *et al*., 2015, Guo *et al*., 2019). Generally, bioaccumulation occurs when the accumulation of contaminants is greater than the ability of an organism to egest or detoxify a contaminant (Newman *et al.*, 2015). In certain organisms a contaminant (e.g., metals) accumulates in higher amounts than in the ambient environment and this process is called bioconcentration (Necibi *et al.*, 2021). Bioaccumulation, coupled with the trophic transfer of chemicals via the food chain, results in a process called biomagnification (Zenker *et al.*, 2014). In the past few decades there was a rise in urbanisation and industrialisation, resulting from reckless exploitation of the Earth's natural resources. This has worsened the problem of environmental pollution worldwide. In particular, the aquatic environment has been polluted by numerous pollutants including metals, organics, microplastics, nanoparticles and others. Metals are discussed further in this section.

2.5.1 Sources and pathways of metals

Metals are of great concern as contaminants due to their persistence, toxicity and accumulation in ecological systems (Addo-Bediako *et al*., 2021). Metals reach the environments in dissolved or particulate form through natural pathways such as weathering processes, volcanic activity and forest fires (Majola *et al*., 2020). In contrast, human induced activities are a major source of metal pollution, given the extensive application of metals in various industries (Majola *et al.*, 2020, Mzimela and Izegaegbe, 2021). Metals reach various aquatic environments through the discharge of industrial, mining and domestic effluents, seepage of contaminated groundwater,

burning of fossil fuels, leaching of metals from landfill sites and the disposal of municipal wastes (Ateş *et al.*, 2020).

2.5.2. Environmental distribution and occurrence of metals

Once metals have been introduced into aquatic environments they tend to be distributed in water, sediments, and biota. Metal speciation in aquatic compartments is governed by various physical, chemical, and other related physicochemical properties of water. Metals in the water column are either diluted or removed by precipitation and deposited to the sediment. Because metals enter the aqueous phase in their inorganic form or hydrated ionic state, they are easily adsorbed to sediment particles through chemical bonds. Thus, they are mostly found in sediment rather than in the water column. Sediment becomes the primary repository of metal contamination, as organisms accumulate bioavailable metals in sediments, and in particular, sediment-dwelling organisms bioaccumulate metals through ingestion of contaminated food particles.

Metal contamination in aquatic compartments (water, sediments, and organisms) has been investigated globally (Uaboi-Egbenni *et al*., 2010, Araujo *et al*., 2020, Ateş *et al*., 2020, Apau *et al*., 2022) and in South Africa (Greenfield *et al*., 2011, Mzimela *et al*., 2003, Mzimela *et al*., 2014, Edokpayi *et al*., 2017, Vetrimurugan *et al*., 2019, Adeleke *et al*., 2020, Izegaegbe *et al*., 2021, Majola *et al*., 2020, Mzimela and Izegaegbe, 2021). In South Africa, metal contamination studies have been conducted in various regions and different compartments. Patience *et al*., (2021) conducted a study in the intensively irrigated region of Luvuvhu catchment in Limpopo province to evaluate the occurrence and distribution of nutrients and trace metals in the groundwater and their suitability for drinking. Among the five metals analysed (chromium (Cr), zinc (Zn), lead (Pb), boron (B) and silicon (Si), only Pb exceeded WHO acceptable guidelines for drinking water. Edokpayi *et al*., (2017) assessed the impact of seasonal variation on the contamination level of metals in the Nzhelele River, also in the Limpopo province. Results showed that metals such as aluminium (Al) and iron (Fe) exceeded DWAF threshold values, which signifies a risk to human health. Lead and mercury were above the DWAF threshold value for human consumption and sustainable aquatic life in a study conducted by Olujimi *et al*., (2015) in Cape Town. In addition, Mzimela *et al*.,

(2014) reported spatial and temporal variations of metal concentrations in water samples from the uMhlathuze Estuary, with notably elevated levels of Al, Cr, Fe, manganese (Mn) and Zn measured during summer.

Mzimela *et al*., (2014) also demonstrated spatial differences in muddy, high organic areas of the uMhlathuze Estuary which had the highest metal concentration compared to other sites. Izegaegbe *et al*., (2020) reported higher metal concentrations in the subtidal mudflats than in the marine sand at the mouth of the uMhlathuze Estuary and Richards Bay Harbour, respectively. Nickel (Ni) and Cr levels recorded in uMhlathuze Estuary sediments exceeded sediment quality guidelines values of Threshold Effect Level (TEL) and Effect Range Low (ERL) (adopted from Australia, New Zealand, and United State of America (USA)), indicating its potential toxicity to biota. Addo-bdlako *et al*. (2021) recommended that urgent action be taken to control effluents derived from anthropogenic activities in the rivers, in particular the Spekboom River, Limpopo, where studies have revealed deterioration of sediment quality with elevated levels of Cr and Ni in all sites studied, signifying the high impact of anthropogenic activities surrounding this region. Metal accumulation in sediment depends on the type of sediment and organic matter content (Adeyeke *et al*., 2020). In addition, the partitioning of metals may be influenced by environmental factors, such as an increase in metals with increasing salinity and/or turbidity (Mzimela *et al.,* 2014). The spatial and temporal discrepancies in metal concentration in various media of different regions have been linked with anthropic factors (e.g., effluent from agricultural, domestic, and industrial activities) and hydrological factors (e.g., dilution and evaporation).

2.5.3. Bioaccumulation of metals on aquatic organisms

Organisms, mainly crustaceans, exposed to contaminants in sediments and water generally accumulate metals through ingestion and absorption. In particular, crustaceans accumulate metals from surrounding media depending on metal speciation and bioavailability (Adeleke *et al*., 2020). Literature reports varying concentrations of metals in tissues, organs, and bodies, which are governed by membrane permeability and enzymatic processes in different organisms. Izegaegbe *et al*., (2020) investigated the bioaccumulation of trace metals in the burrowing crab

Paratylodiplax blephariskios from Richards Bay Harbour. Findings showed that the highest tissue concentrations of Co, copper (Cu), Mn, Pb, and Zn were recorded in crabs from Bhizolo Canal compared to other sites, and high Biota Sediment Accumulation Factor (BSAF) values of Co, Cu, Mn and Zn in all sites confirmed that crabs bioaccumulated these metals. Adeleke *et al*., (2020) reported variation in tissue accumulation of metals in the sand bubbler crabs *Dotilla fenestrata* collected from Durban, Richards Bay Harbour and the uMlalazi Estuary. A similar trend was observed in all systems studied, where tissue metals concentration was in the order exoskeleton > gills > digestive glands. Copper was the highest accumulated metal followed by Zn, Pb and cadmium (Cd). Elevated levels of metals were measured in the gills (silver (Ag), Cr, Cd, Co, and Zn) and digestive glands (Al, Ni, Fe, Mn and Pb) of the mud crab *Chiromantes eulimene* from the uMhlathuze Estuary (Majola *et al*., 2020). Environmental conditions may influence the uptake and accumulation of metal by crabs, as an increase in bioavailable metal levels results in higher levels of metal accumulation. Various aquatic species of crustaceans regulate essential metals (e.g., Zn, copper (Cu) and Mn, which are important for metabolic processes) in their bodies, even though these metals become toxic at higher concentrations. On the other hand, they tend to accumulate non-essential metals in their bodies (Cd, Pb and Ag), which are reported to be toxic even at trace concentrations (Chan *et al*., 2021). The levels of metal concentrations in crabs cannot be accurately measured, as the accumulation significance depends on the type of tissue and type of species involved. It is not possible to definitively conclude that the accumulation of metals has some implications on the crab's health status until certain biochemical, physiological, and behavioural responses are borrowed from other fields (such as biomarker studies) to assess both exposure and effects of organisms to contaminants.

2.6. Biomarker response of organisms from contaminants in aquatic environments

The input of anthropogenic contaminants such as microplastics, pharmaceuticals, pesticides and metals into water resources has the potential to affect the health of aquatic ecosystems. These contaminants reach the waterways through different point and non-point sources. Exposure to these contaminants poses various risks to aquatic animals via numerous mechanisms such as direct toxicity of both acute and chronic

exposures (Hook *et al.*, 2014). Ecotoxicologists have adopted physiological, behavioural, and biochemical approaches to better understand the effects of such contaminants. Biomarkers, borrowed from the field of medicine, can be used to measure ecosystem health and function. Biomarkers are generally explained as detectable biochemical and tissue level changes that indicate changes in physiology. This includes measurements of biology and physiology parameters to assess the health of indicator species within aquatic environments. The biomarker response of an organism to chemical contaminants is necessary to efficiently measure the degree of pollutant exposure and biological effects. Biomarkers integrating both exposure and biological effects, such as acetylcholinesterase (AChE), are particularly useful (Dalzochio *et al*., 2016). Acetylcholine (ACh) is an excitatory cholinergic neurotransmitter which primarily regulates neuromuscular transmission in both vertebrates and invertebrates. It is synthesized by the choline acetyltransferase enzyme from acetyl coenzyme A and choline in the nerve terminals. The neuromuscular transmission is both glutamatergic and cholinergic in invertebrates. Cholinesterases (ChEs) are further classified into two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Deidda *et al.,* 2021).

Acetylcholinesterase is the essential enzyme which breaks down the neurotransmitter acetylcholine and facilitates its removal from the synaptic cleft or blood plasma, via the process of hydrolysis (Fulton and Key, 2001, Narra *et al.,* 2012). Various organic and inorganic contaminants are reported to be potent inhibitors of AChE activity. The inhibition of AChE results in ACh excessive accumulation in synapses and continuous stimulation of cholinergic receptors, resulting in changes to the neurotransmission, and leading to paralysis (Narra *et al.*, 2012, Deidda *et al.,* 2021). Acetylcholinesterase activity has been widely used as a biomarker for invertebrates such as molluscs and crustaceans (Deidda *et al*., 2021). The use of haemolymph, nerve ganglion, muscle, gills, mantle, and whole-body tissues of invertebrates has been the primary means to evaluate the effects of contaminants on AChE activity (Fulton and Key, 2001). Inactivation of AChE is believed to be caused by organophosphate, carbamates, and heavy metals (Tu *et al*., 2009, Narra *et al.*, 2012). However, some studies have revealed the sensitivity of AChE to other CECs. For example, Juhel *et al*., (2017) showed that AChE activity was strongly inhibited by carbamazepine and atrazine in *Perna viridis*. Yu *et al*., (2018) exposed the crab *Eriocheir sinensis* to different

concentrations of microplastic particles of polystyrene for 7 days to assess their effects on oxidative stress in the liver. Their results showed the inhibited AChE activity, these findings demonstrate that exposure of organisms to CECs may lead to alterations in the feeding behaviour, growth and survival of these organisms. However, few studies of this kind have been conducted in South Africa, and in particular, in the northern KZN. Thus, this study adopted the approach of using AChE as a biomarker of exposure and effects to contaminants in the mud crabs *Chiromantes eulimene* inhabiting the uMhlathuze and uThukela River Estuaries.

2.7. Conclusion

CECs are widely distributed in South African waterways and can bioaccumulate and biomagnify along trophic levels. Their occurrence has been attributed to poor solid waste disposal and the inefficiency of WWTWs. Therefore, research based on these contaminants is imperative, and major improvements in the efficiency of WWTPs are needed.

A rapid increase in the production and applications of microplastics, owing to their desirable properties, increases the potential for environmental exposure, leading to concerns regarding their possible risks on the environment and human health. Some laboratory and field studies have already reported sources and occurrence, distribution, and the negative impact of microplastics.

Extensive global usage increases the potential for organics to reach different aquatic compartments, and to become available at trace concentrations which can be environmentally hazardous. Most of these compounds are indispensable to humans. For example, pharmaceuticals are important for sustaining our human health and for veterinary purposes, and pesticides are key to maintaining agricultural production and therefore, indirectly, human health. Table 2.5 demonstrates that these CECs are widely distributed in SA waterways and can have adverse biological effects.

Engineered nanomaterials, environmental release and its associated effects are addressed at a slow pace relative to their production rate. However, studies on their distribution and toxicity effects are increasing, and many local studies have emerged from international studies or on pristine ENMs.

Metal contamination has been reported in various aquatic compartments, and different aquatic species have been shown to accumulate these bioavailable metals in increasingly higher concentrations. However, it is not easy to conclude that the accumulation of metals has a negative impact on the crab's health status through bioaccumulation studies alone, and so studies incorporating certain biochemical, physiological, and behavioural responses are needed to assess both exposure to and the effects of contaminants to organisms.

The effects of CEC pollution on the aquatic environment in South Africa are still largely speculative. Therefore, researchers should conduct more research on the sources, distribution, and abundance of these contaminants, to carefully and comprehensively assess risk related to CECs.

In addition, it is important to identify areas of concern for future consideration, so that South Africa's limited water resources and their ecosystems are safeguarded for future generations.

CHAPTER 3:

STUDY AREA, MATERIALS, METHODS AND DATA ANALYSIS

3.1. STUDY AREAS

3.1.1. UMhlathuze River-Estuary

UMhlathuze River (Figure 3.1) is one of the largest rivers in KwaZulu Natal (KZN), which originates from the Babanango hills. It has a catchment area of 4209 km^2 which falls within the Usuthu to Mhlathuze Water Management Area (WMA) and consists of minor tributaries such as Nseleni, Empangeni and Mfule Rivers. It flows to the southwest of Empangeni, Ngwelezane, and to the south of Felixton, and discharges into the Indian Ocean via the uMhlathuze Estuary just south of Richards Bay Harbour (DWAF, 2004a). This river system drains a catchment that is categorised as highly rural but is intensively urbanised and industrialised towards the coastal reaches (Lin *et al*., 2004). UMhlathuze River is heavily impacted by activities such as water abstraction for domestic use, various industries, agricultural activities, sand mining and recreation (DWAF, 2004a).

Rainfall distribution within the catchment is associated with the topographic features and is estimated at approximately 583 million m^3/annum. Primary land uses which require water resources are plantations of wattle, pine and *Eucalyptus*, situated in the upper reaches. In the coastal and some inland reaches, plantations of sugarcane, citrus, vegetables, and maize are prevalent (DWAF, 2004a). In addition, the largest coal export facility in Africa is found in the area. Other industries in the vicinity of the estuary include an aluminium smelter, fertilizer plants, and mining activities of iron ore, titanium, and zircon (Elumalai *et al*., 2017).

3.1.2. UThukela River-Estuary

UThukela River (Figure 3.1) is the second largest river in the country and is located along the coast of KwaZulu-Natal (KZN), about 102 km north of Durban. The total catchment area of the uThukela River is approximately 29 036 km^2. The river meanders through central KZN and discharges through the uThukela Estuary into the Indian Ocean. It includes major tributaries such as the Little uThukela, Klip, Bloukrans,

Bushmans, Sundays, Mooi, Buffalo rivers, as well as a minor tributary, Mandeni River (DWAF, 2004b). It is important for water supply in various industrial, domestic, and agricultural sectors. The extensive utilization of the water services in this river may negatively impact the water quality, quantity, and the entire ecological aspect of the system (Vetrimurugan *et al.,* 2019, Wade *et al*., 2021).

Rainfall distribution is estimated at 3 900 x 10^6 m^3 (Ntanganedzeni *et al*., 2018). The uThukela River catchment supports various land use types such as human settlements, extensive sugarcane plantations, dry land maize cultivations and processing factories, as well as diverse industrial activities in the Mandeni-Sundumbili industrial complex, Thukela Rail and Sappi Thukela paper mill (Stryftombolas, 2008).

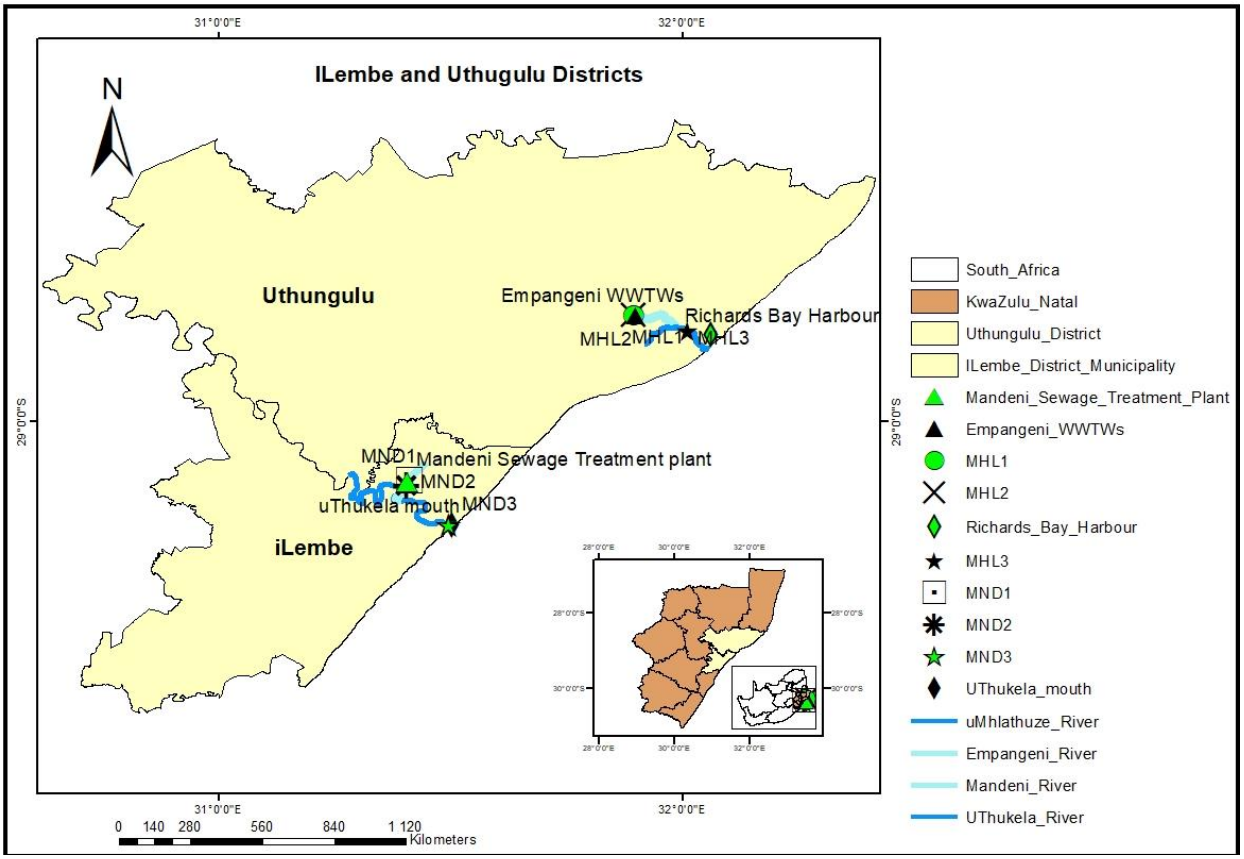


Figure 3.1: Map of uMhlathuze and uThukela systems showing sampling sites, Mhl (uMhlathuze River-Estuary) and Mnd (uThukela River-Estuary). The sampling sites

were chosen based on surrounding anthropogenic activities. Each site was comprised of three sampling sites, listed in Table 3.1, and shown in Figure 3.1.

Table 3.1: Sampling sites in the uMhlathuze and uThukela River-Estuary systems

SITE NAME	GPS CO-ORDINATES	SITE DESCRIPTION
Mhl 1	28˚ 46' 39. 59" S 31˚ 52 '57.37" E	Located in Empangeni River, a tributary of the uMhlathuze River. It is above the discharge point of a WWTW at Empangeni Town.
Mhl 2	28˚ 46' 39. 75" S 31˚ 52' 57. 14" E	Also located in Empangeni River, however, this site is below the effluent discharge point of WWTW at Empangeni Town.
Mhl 3	28˚ 48' 15. 81" S 32˚ 0' 41. 90" E	Located in uMhlathuze Estuary, near Richards Bay Harbour. It is also nearby commercial and subsistence agricultural activities.
Mnd 1	29˚ 7' 36. 00" S 31˚ 24' 40 50" E	Located in Mandeni River, a tributary of the uThukela River. This site is situated upstream of the sewage plant in the Mandeni Town.
Mnd 2	29˚ 8' 13. 30" S 31˚ 24' 22. 69" E	Also located in Mandeni River but downstream of the sewage plant at Mandeni Town.
Mnd 3	29˚ 13' 19. 55" S 31˚ 29' 50. 65" E	Located in uThukela Estuary, at the mouth.

3.2. MATERIALS AND METHODS

3.2.1. Fieldwork

3.2.1.1. Water sampling

Surface water samples were collected using an HDPE plastic bucket that was rinsed with site water first before the sample was collected. A total of 60 sub-surface water

samples were collected for the determination of organic, microplastic and inorganic/ENMs parameters (Table 3.2). Cleaning of 1 L Schott® bottles involved scrubbing them with a phosphate free soap, rinsing with distilled water, and then soaking them in an acid bath (10% nitric acid, Merck, South Africa) for 24 hours before rinsing with distilled water and air drying. Samples for analysis of organics (e.g., pharmaceuticals, pesticides) and microplastics were collected with 1 L Schott® bottles during low flow conditions (June 2019) (Table 3.2). Samples for inorganic elemental analysis and ENMs characterisation were collected with 1 L plastic bottles during low flow (August 2020) and high flow (October and February 2020) conditions (Table 3.2). Sampling for microplastics was in triplicate. The lids of all sampling bottles were lined with aluminium foil to avoid any possible contamination before they were sealed. Water samples were stored in a cooler box and transported to the laboratory where they were stored at 4°C pending the analyses.

In situ turbidity (NTU), dissolved oxygen (mg/L), temperature (°C), pH and salinity (ppt) were measured using an Orion Star A329 multi-meter (ThermoScientific). The multi-meter was calibrated in the laboratory using standard solutions prior to use in the field.

Table 3.2: Number of surface water samples collected at each sampling site per determinants

	June 2019	August 2019	February 2020		October 2020	
	Organics	Elemental and ENMs	Microplastics	Elemental and ENMs	Microplastics	Elemental and ENMs
Mhl 1	1	1	3	1	3	1
Mhl 2	1	1	3	1	3	1
Mhl 3	1	1	3	1	3	1
Mnd 1	1	1	3	1	3	1
Mnd 2	1	1	3	1	3	1
Mnd 3	1	1	3	1	3	1

NB: The initial trip was for the screening of organics only, while latter trips focussed on microplastics, elemental and ENMs analyses.

3.2.1.2. Collection of crabs

Hand nets were used to collect females (n=10) and male (n=19) crabs, *Chiromantes eulimene* from the mudflat areas of uMhlathuze (Mhl 3) and uThukela estuaries (Mnd 3) during high (February 2021) and low flow (July 2021). Overall, 58 female and male crabs for both flows were collected for metal analyses, including 18 male crabs for biochemical analyses. Crabs were rinsed to remove debris and placed in an 80 L tank containing seawater to minimise handling stress and were immediately transported to the ecotoxicology laboratory where they were stored in 1 L plastic honey jars and snap frozen at -20°C until inorganics and biochemical analyses were conducted.

3.2.3. Sample preparation and analysis for organics

Water samples (*n*=1l) were analysed for organics using gas chromatography-mass spectrophotometry (GC-MS) at the Analytical Research Facility of the University of the Free State. The respective samples in foil covered Schott glass containers were kept cool in ice during transportation.

3.2.2. Sample preparation and analysis for microplastics

The method for quantifying microplastics was adapted from the National Oceanic and Atmospheric Administration (NOAA) standard protocol (Masura *et al*., 2015). Briefly, each sample was sieved with a sieve stack of decreasing stainless steel mesh, size of 5 mm at the top and 63 µm at the bottom. Schott® bottles were rinsed with distilled water from a squirt bottle to remove all remaining visible debris. The materials retained by the 63 µm mesh were decanted into a glass beaker. Glass beakers containing samples were then loosely covered with aluminium foil and placed in a drying oven at 50°C for 24 h to induce evaporation. The remaining samples were then cleaned for any biological material through digestion as described by Masura *et al*., (2015). Briefly, 20 mL of 30% hydrogen peroxide (Merck, South Africa) was added to the sample in the presence of a 20 mL iron solution. The iron solution was prepared by the addition of 3 mL of sulphuric acid (30%, Merck, South Africa) and 7.5 g of iron sulphate (Merck, South Africa) in 500 mL of distilled water. The mixture was allowed to stand for 5 min and thereafter, the mixture was heated on a hotplate (75°C) for 30 min, using a stirrer bar. During this process the samples were covered with a watch glass. Samples with

too much sediment were subjected to density separation, which comprised adding 6 g of sodium chloride (NaCl) (Merck, South Africa) to the sample and allowing it to boil until the NaCl dissolved. The sample was then transferred to the density separator, which was loosely covered with aluminium foil. The sample was allowed to settle for 12 hours until complete settling of the solid was observed. Microplastics were expected to float on the top. The sample was filtered through a Whatman filter paper (0,45 µm) (Sigma, South Africa) using a filtering pump (Labotec, France), and filters were placed in a clean glass petri dish covered with aluminium foil prior to microscopic identification.

Filters in the petri dish were examined, followed by identification and characterisation of microplastics using a dissection microscope (Lesia ES2, China), based on morphology and colour using typologies (Hidalgo-Ruz *et al.*, 2012). Briefly, a particle was considered to be a microplastic if there were no cellular or organic structures visible. Particles were expected to exhibit homogenous colour throughout. However, exceptions were made where a process such as biofouling could have potentially disguised the colour. Fibres were expected to be equally thick throughout their entire length. Microplastics were expected not to break when prodding, as microplastics are known for their flexibility and strength.

Acetone (65%, Merck, Johannesburg, South Africa) was also used to ensure whether a particle was a microplastic or not, particularly for foam and film microplastics. Particles were nudged in a puddle of acetone and if they became sticky or dissolved within a few seconds, the particle was recorded as a microplastic. The hot needle test was also performed. The particle was removed from the filter to avoid destroying the sample. A particle was considered a microplastic if it shrank away, melted, or burnt black. If a particle did not react in any of these ways, or if there were any doubt during identification due to size being smaller, it was not counted in order to establish a conservative number of the microplastics found in the sample.

FTIR Spectrometer Spectrum Two Universal attenuated total reflectance (ATR Plate) (Perkin Elmer, UK) was used for polymer identification in a spectral range of 400–4000 cm^{-1}, at a resolution of 4 cm^{-1}. The ATR diamond crystal was cleaned with ethanol and a background scan was performed between samples. Due to the need for manual transfer of particles onto the crystal of this instrument, only 30% of particles, mostly

those with larger size, were analysed for polymer identification. Each particle was compressed against the diamond with a force of at least 99 N to ensure good contact between the samples and ATR crystal. The FTIR spectra of the analysed particle were matched and identified through the available polymer library (Asensio *et al.*, 2009, Jung *et al.*, 2018). The spectral matches were identified based on the peak position, where the base was corrected, and noises were eliminated to avoid any misinterpretation. The reproducibility of counting microplastics was tested by analysing a sample from the same site in duplicate. For a polymer to be accepted, a minimum of four bands were required to directly match or be within those in the previously mentioned libraries (Asensio *et al*., 2009, Jung *et al*., 2018).

Three blanks consisting of distilled water were performed alongside each sample batch to account for possible background contamination during counting and filtration where samples were not covered, as analyses were not performed under laminar flow/fume hood. Where microplastics were identified in all three filters of the blanks, the average was calculated, and they were subsequently removed from each sample in that batch. Sample processing was done in a laboratory with closed windows, the minimum number of people present (≤ 3 individuals) and disabled air conditioning, to reduce any possible airborne microplastic contamination. All glassware, laboratory surfaces and filtration equipment were thoroughly rinsed with 30% ethanol and distilled water prior to and after use. Petri dishes and metal forceps were routinely inspected under a microscope to check for microplastic contamination. Glass or metal equipment were used as much as possible. Samples were covered with a watch glass and/or aluminium foil when not handled.

3.2.4. Sample preparation and analysis for engineered nanomaterials (ENMs)

Nanoparticle tracking analysis (NTA; NS500, NanoSight, United Kingdom) was used to determine particle size (nm) and concentration (particles/mL) in the samples collected. The water samples were prefiltered with a 0.45 µm syringe filter in order to remove larger particulates that could cause blockages in the piping of the equipment. The polystyrene latex 100 nm standard was used to verify the accuracy of the NTA prior to sample analysis. All analyses were done in triplicate, where 30 s videos each were recorded by setting the camera level between 4 to 15. De-ionised water was

used as a diluent in instances where dilution was required, with an ideal analysis concentration between 10^7 and 10^9 total particles per ml.

In preparation for transmission electron microscopy (TEM) characterisation, each 1 L sample was homogenized by shaking for 1 min. To improve sample visibility during electron microscopy analysis, the 1 L sample was preconcentrated by transferring into 50 mL centrifuge tubes and centrifuged at 10 000 rpm for 45 min. The supernatants were discarded, and the centrifugation procedure was repeated until the entire 1 L sample was concentrated. The resultant pellet was dispersed in 1 mL of deionised water and sonicated in a water bath (Ultrasonic bath RK 514 BH cap, Labotec, South Africa) for 5 min. The 1 mL concentrated sample was vortex for 1 min, and a Cu grid with a holey carbon film was dipped in the sample solution and air-dried for 12 hr, followed by TEM-EDX analysis.

Images were obtained using a JEOL-JEM 2100 high-resolution transmission electron microscope coupled to energy-dispersive X-ray spectroscopy (HRTEM-EDX; Tokyo, Japan), fitted with a LaB6 filament operated at 200 kV. Sizes of the engineered nanomaterials were measured using the ImageJ software.

3.2.5. Sample preparation for elemental analysis

3.2.5.1. Water samples

Elemental analysis aluminium (Al), titanium (Ti), zinc (Zn), silicon (Si), chromium (Cr), manganese (Mn), iron (Fe), copper (Cu), nickel (Ni) and lead (Pb) was conducted with Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES, Perkin Elmer Optima, 2100DV, Germany). Water samples (1 L) were pre-digested by adding 5 mL of nitric acid (HNO_3) (70%, Merck, South Africa) into each digestion vessel containing water samples, for approximately 10 minutes. Samples were further digested using microwave digestion. All sample digests were filtered using a 0,45 µm filters syringe (Merck, South Africa) and prepared for ICP-OES analysis.

3.2.5.2. Crab samples

Prior to oven drying, the weight, carapace length and width of the crabs were measured. Sex in crabs was differentiated based on that females have spherical

underside while males have narrow candled-shaped underside. Moreover, male crabs were larger than females. Genders were processed separately; however, they were not differentiated according to size. The whole crab samples were then placed overnight in an oven (Prolab PL100, South Africa) at 60°C to dehydrate. The dried samples were ground to powder using pestle and mortar, to guarantee homogeneity. Ground samples were then acid digested. Briefly, 10 mL of nitric acid (65%, Merck, South Africa) and 1 mL of hydrogen peroxide (30 % Merck, South Africa) were added to the sample and the sample was digested on a hotplate or in a microwave digester (Ethos Easy Advanced Microwave Digestion System, Italy). After complete digestion, the samples were filtered using 45 mm Whatman filter paper (Sigma, South Africa) and diluted up to 40 ml with distilled water and stored in glass bottles. The samples were then transported to the University of KwaZulu-Natal, Chemistry Department for metal analysis using an ICP-OES.

3.2.6 Sample preparation and analysis for Acetylcholinesterase enzyme (AChE)

Extraction was performed on partially frozen male crabs only collected in different seasons from the uMhlathuze and uThukela Estuaries. Gills were extracted on ice for assay and 9 individual crabs were pooled to make one sample. The Ultra-Turrax homogeniser (IKA T10, Germany) was employed for homogenization, where 5 mL of ice-cooled saline phosphate buffer (0,1 Mm, pH. 7,5) and tissue samples were added together in 14 mL Eppendorf tubes (Merck, South Africa) and homogenized while on ice. The phosphate buffer was briefly prepared by dissolving 4,5 g sodium chloride (Merck, South Africa), 6 g of potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany) and 7.1 g of potassium hydrogen phosphate (K_2HPO_4) (Merck, Germany) in 2 L of distilled water. Sodium hydroxide (NaOH) and hydrochloric acid (HCL) (both from Merck, South Africa) were used to adjust the pH to 7.0, measured using a laboratory scale digital pH meter. The homogenate was centrifuged at 14 000 rpm for 10 min and the clear supernatant was transferred into the new 3 mL microtube and immediately frozen at −80°C, and further analysis of AChE activity was undertaken within 24 hours using an AChE kit, MAK 119 (Sigma, South Africa).

For AChE activity, the working reagent was prepared by the addition of reagent and assay buffer, and the reagent was used within 30 minutes followed by the addition of

200 µL of water (assay blank) and 200 µL of calibrator into separate wells of a 96-well plate. Five sample replicates of 10 µL were pipetted into separate wells of the 96-well plate. A total of 190 µL of working reagent was transferred to all sample wells and mixed. Samples were incubated at room temperature prior to the absorbance measurement using a microplate reader (Analytical and diagnostic products, South Africa). After 2 min, the initial absorbance measurement was taken at a wavelength of 412 nm $(A_{412})_{initial}$. The samples were further incubated and after 10 min the final measurement was taken at the absorbance of 412 $(A_{412})_{final}$.

AChE activity was calculated according to Equation 1:

AChE activity (units/L) = $\frac{((A412)final - A(412)initial)}{(A(412)calibrator - A(412)blank)}$ *n* 200…….....Equation 1

200 = Equivalent activity (units/L) of the Calibrator when the assayed was read at 2 minutes and 10 minutes.
n = Dilution factor (* dilution was not considered).
$(A_{412})_{calibrator}$ = Absorbance of the calibrator at 10 min.
$(A_{412})_{blank}$ = Absorbance of the blank at 10 min.

3.3. DATA ANALYSIS

Data were expressed as mean values ± SE of the mean, and statistical analysis was performed using SPSS version 25. Non- parametric tests were performed using Plymouth Routines In Multivariate Ecological Research (PRIMER), version 6 software. A Student's T-test was performed to compare seasonal microplastics abundance. One-way ANOVA was used to compare the significant difference in microplastics abundance of the mean between sites. Results for ENMs were subjected to Image J in order to measure particle sizes. One-way ANOVA was used to compare metal concentrations in water and crabs between sites. The principal component analysis (PCA) was employed to explore the relationship between metal concentration in water, the physicochemical parameters and in the crabs. Significant differences between AChE activity in the gills of crabs collected seasonally were explored using One-way ANOVA. Values of $p < 0.05$ were considered to be the level of statistically significant difference. Microplastics and metals figures for the two seasons were pooled (referred

to as combined, henceforth) only in cases where there were no significant inter-seasonal differences (ANOVA).

CHAPTER 4:

EXPOSURE ASSESSMENT OF CONTAMINANTS OF EMERGING CONCERN IN UMHLATHUZE AND UTHUKELA RIVER-ESTUARIES, KWAZULU-NATAL (KZN)

4.1. INTRODUCTION

New chemicals continue to be produced, and together with previously unrecognised chemicals, their disposal can pose a risk to the environment. This collection of chemicals is termed contaminants of emerging concern, or CECs (Kroon *et al*., 2020). CECs include, but are not limited to, pharmaceuticals, lifestyle drugs, pesticides, polycyclic aromatic hydrocarbons (PAHs), microplastics and engineered nanomaterials, or ENMs, (La Farre *et al*., 2008, Kroon *et al*., 2020). Such chemicals raise concerns with regards to aquatic health as some, predominantly organics, are known to be persistent, carcinogenic, as well as able to induce undesirable effects on water resources even at minute concentrations (Tijani *et al*., 2013, Newman *et al*., 2015). Engineered nanomaterials (ENMs) are suspected for enhanced reactivity (Yokel and MacPhail, 2011), and microplastics are poorly degradable and exhibit high uptake potentials (Pereao *et al*., 2020). In developing countries such as South Africa (SA) the presence of CECs in water resources is largely unknown as they are not included in routine monitoring programmes (e.g., the NTMP). However, some legacy persistent organic pollutants (POPs) are regulated under the Stockholm convention (Bouwman, 2004), and other CECs are included in the National Toxicity Monitoring Programme (NTMP) of the Department of Water and Sanitation (DWS) (Department of Water and Sanitation, 2012). In South Africa, little new information regarding the potential environmental implications of CECs had been generated so far. Therefore, studies focusing on the sources, exposure and effects of CECs are necessary.

This study selected categories of CECs (microplastics, pharmaceuticals and lifestyle drugs, pesticides, and engineered nanomaterials (ENMs) to be studied in samples taken from the uThukela and uMhlathuze River-Estuary systems, based on their production and consumption rate, presence and potential implications in water resources. UMhlathuze and uThukela River-Estuary systems were selected as study areas because previous research on the occurrence of CECs in the KwaZulu-Natal river systems has focused only on southern KZN systems (e.g., Durban Harbour, uMgeni and Isipingo). These rivers flow through municipalities (uMhlathuze

municipality and uThukela district municipality) which are significant economic hubs for the KwaZulu-Natal province, as they support an array of industrial and agricultural activities, in addition to a rising number of human settlements (KwaZulu-Natal Department of Economic Development and Affairs, 2017). This chapter examines the extent of occurrence for CECs in the two systems. A qualitative study was conducted in order to identify pharmaceuticals, lifestyle drugs and pesticides dissolved in surface water in the two systems. In addition, microplastics and engineered nanomaterials were characterised. Samples were collected seasonally from both systems.

4.2. RESULTS

4.2.1. Physico-chemical characterization of surface water samples

4.2.1.1. UMhlathuze River-Estuary

Physico-chemical parameters measured during August 2019 and October 2020 (low flow) and February 2020 (high flow) at three sites in uMhlathuze River-Estuary are provided in Table 4.1. Temperature ranged from 23.7−26.4°C and 19.9−22.0 °C during high flow and low flow, respectively. Salinity was markedly and consistently higher in Mhl 3 (estuarine site). Dissolved oxygen also tended to increase towards the estuarine site. Dissolved oxygen was consistently low at Mhl 2, across seasons. This site is located below the effluent discharge point.

Table 4.1: Physico-chemical parameters measured at uMhlathuze River-Estuary

SAMPLING DATE	SITE	TEMPERATURE (°C)	DISSOLVED OXYGEN (mg/L)	SALINITY (ppt)	TURBIDITY (ntu)	pH
August 2019	Mhl 1	19.6	7.64	0.26	21.7	7.55
	Mhl 2	20.4	7.01	0.30	23.6	7.72
	Mhl 3	22.0	8.43	7.4	4.6	7.38
February 2020	Mhl 1	23.7	9.56	0.77	nm	7.18
	Mhl 2	25.4	8.54	0.59	nm	7.25
	Mhl 3	26.4	9.0	7.6	nm	7.40
October 2020	Mhl 1	18.5	7.45	0.3	6.1	7.75
	Mhl 2	19.8	6.32	0.43	7.7	7.69
	Mhl 3	20.9	10.23	4.045	24.1	7.02

Mhl (uMhlathuze River-Estuary): nm (not measured)

4.2.1.2. UThukela River-Estuary

Physico-chemical parameters measured during August 2019, October 2020 (low flow) and February 2020 (high flow) at three sites in uThukela River-Estuary are provided in Table 4.2. Temperatures ranged from 18.1–20.4°C during low flow and from 25.7–28.0°C during high flow conditions. Salinity was consistently low (< 1) throughout sampling. Overall, pH values ranged from 7.08–8.13, with high values recorded in October during low flow. Dissolved oxygen values displayed an increasing trend towards the estuarine site (Mnd 3).

Table 4.2: Physico-chemical parameters measured at uThukela River-Estuary

SAMPLING DATE	SITE	TEMPERATURE (°C)	DISSOLVED OXYGEN (mg/L)	SALINITY (ppt)	TURBIDITY (ntu)	pH
August 2019	Mnd 1	18.1	3.88	0.62	29.9	7.70
	Mnd 2	19.0	4.0	0.92	17.3	7.84
	Mnd 3	20.4	5.31	0.69	5.1	7.42
February 2020	Mnd 1	25.7	9.68	0.333	nm	7.20
	Mnd 2	26.1	7.31	0.500	nm	7.45
	Mnd 3	28.0	9.77	0.279	nm	7.08
October 2020	Mnd 1	18.8	6.12	0.33	29.5	8.06
	Mnd 2	19.2	4.08	0.56	35.8	8.03
	Mnd 3	21.7	6.09	0.47	20.9	8.13

Mnd (uThukela River-Estuary) and nm (not measured).

4.2.2. Occurrence of pharmaceuticals and lifestyle drugs in surface water samples

4.2.2.1. UMhlathuze River-Estuary

Surface water samples collected from three sites at uMhlathuze River-Estuary were screened for the occurrence of various classes of pharmaceuticals and lifestyle drugs. A total of 38 compounds were recorded, belonging to 14 therapeutic classes (Appendix A4). The class detection profile was Mhl 2 (12 classes) > Mhl 3 (6 classes) > Mhl 1 (4 classes). All compounds except one (antihelmintics) were recorded in Mhl

2 (Figure 4.1a). Classes frequently detected were psychoanaleptics, antifungals, antipsychotics and antiepileptics. Notable compounds such as carbamazepine, fluconazole, hydroxybupropion and nicotine were also detected at all sampling sites. Some compounds had limited occurrence (i.e., present only in one site) (Figure 4.1b). For example, cocaine and methamphetamine were only recorded in Mhl 1. Flubendazole and erythroaminobupropion were also only recorded in Mhl 3.

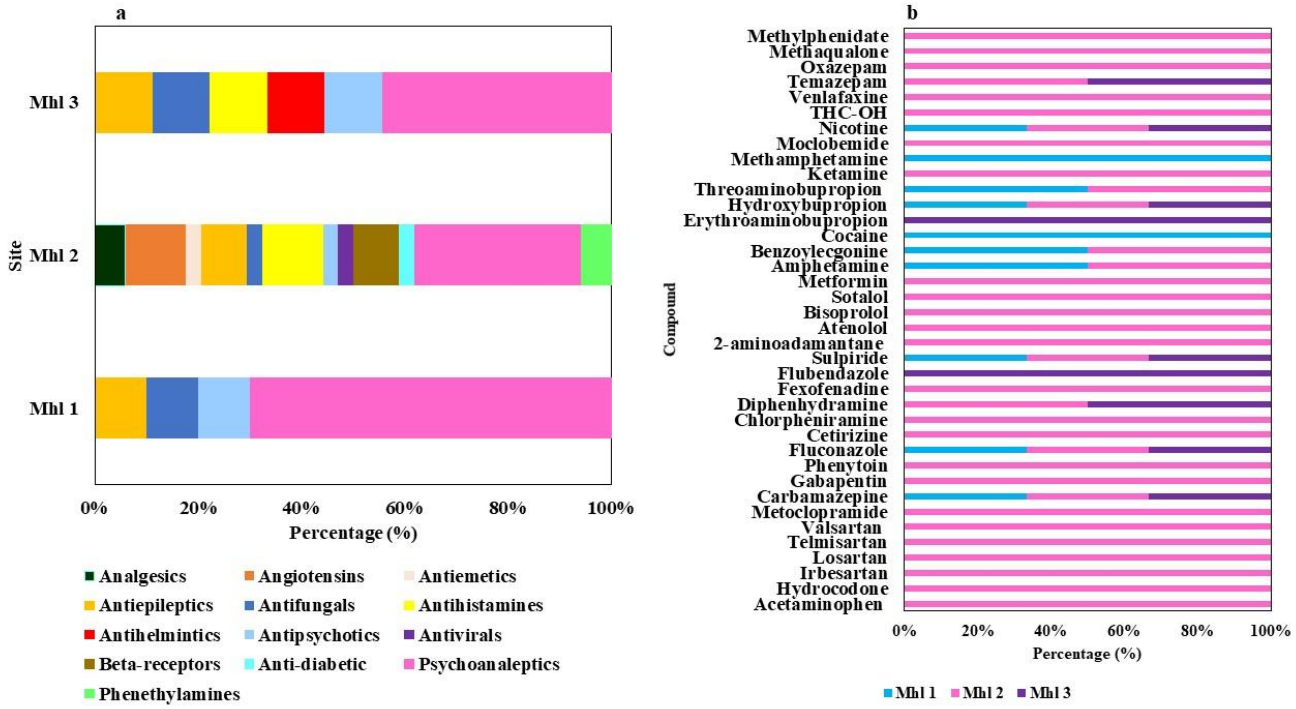


Figure 4.1: Frequency of detection of pharmaceuticals and lifestyle drugs at uMhlathuze River-Estuary. Summary of the occurrence of therapeutic classes are presented in figure (a) whilst a detailed account on the occurrence of compounds per site are presented in figure (b)

4.2.2.2. uThukela River-Estuary

At the uThukela River-Estuary, 20 compounds were detected belonging to 13 therapeutic classes (Appendix A4). Detected compounds (classified according to their classes) showed the order of occurrence, Mnd 2 (9) > Mnd 3 (7) = Mnd 1 (7) (Figure 4.2a). Classes that were frequently detected were psychoanaleptics, antiepileptics, antibiotics and antifungals. Carbamazepine, sulfamethazine and fluconazole were

detected in all sampling sites. Nicotine was recorded in Mnd 1 and Mnd 3 (Figure 4.2b). Some compounds had limited distribution. For example, chlorothiazide and acebutalol were recorded at Mnd 1 only. Tramadol, irbesartan, nevirapine and atenolol were recorded at Mnd 3. Disopyramide, convallatoxin, amphetamine, hydroxybupropion, threoaminobupropion, testosterone and mirtazapine were recorded at Mnd 2 (Figure 4.2b).

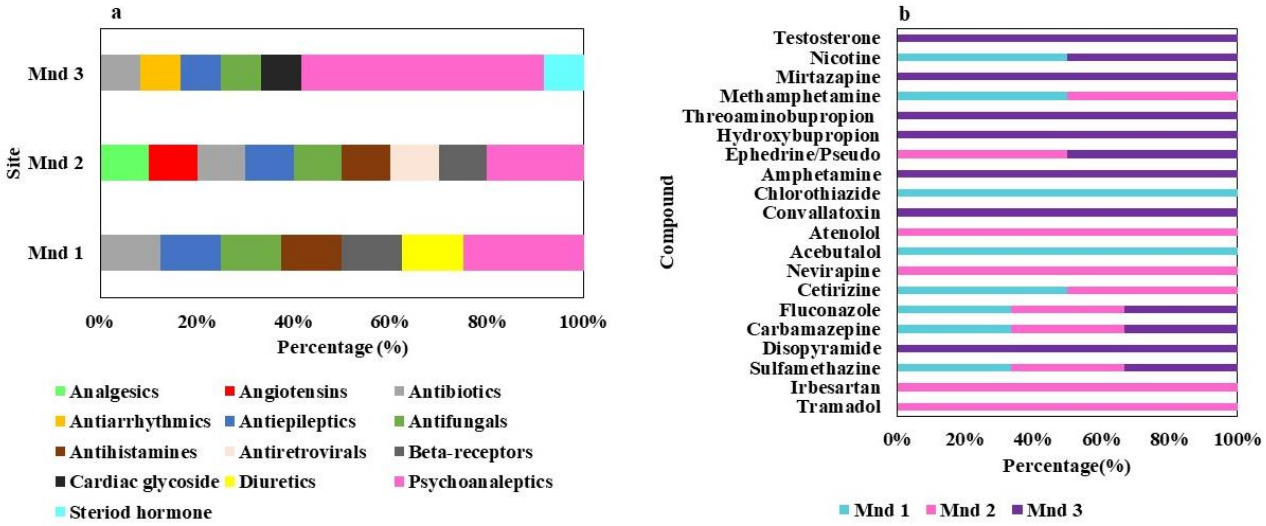


Figure 4.2: Frequency of detection of pharmaceuticals and lifestyle drugs at uThukela River-Estuary. Summary of the occurrence of therapeutic classes are presented in figure (a) whilst a detailed account on the occurrence of compounds per site are presented in figure (b)

4.2.3. Occurrence of pesticides in surface water samples

4.2.3.1. UMhlathuze River-Estuary

A total of 13 compounds were recorded, and these belonged to the classes of herbicides, fungicides and insecticides (Appendix A5). Herbicide compounds were frequently detected, while insecticides were detected at only two sites (Mhl 1 and Mhl 2). Although fungicides were detected at all sampling sites, they were less frequently detected than herbicides (Figure 4.3a). Only three compounds were detected in all sites (Figure 4.3b).

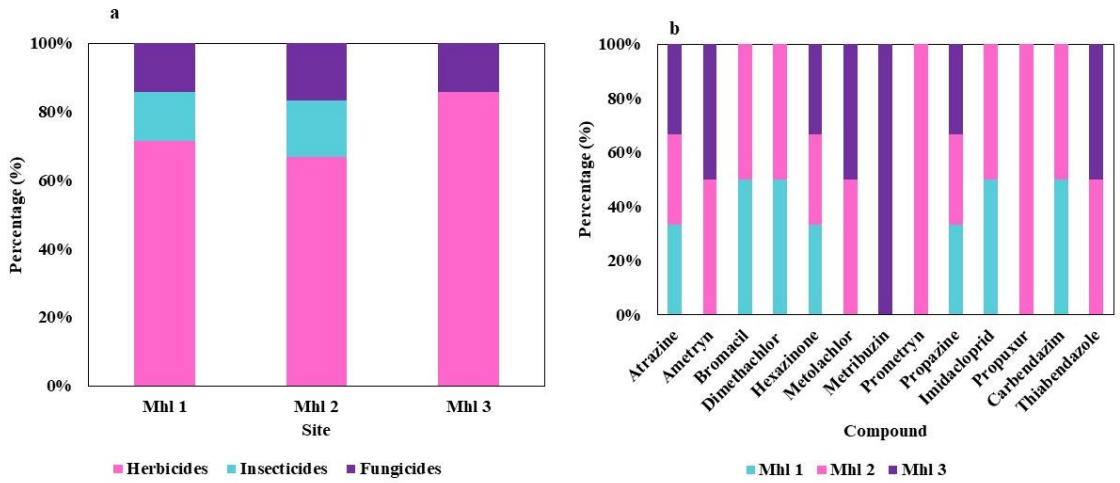


Figure 4.3: Frequency of detection of pesticides at uMhlathuze River-Estuary. Summary of the occurrence of pesticide classes are presented in figure (a) whilst a detailed account on the occurrence of compounds per site are presented in figure (b)

4.2.3.2. UThukela River-Estuary

A total of 14 pesticides were detected, belonging to the classes of herbicides, fungicides, and insecticides (Appendix A5). Herbicides were among the most frequently detected, whilst fungicides were not detected at the estuarine site, Mnd 3 (Figure 4.4a). Only four compounds were detected from all sampling sites. Other compounds had a limited occurrence in the system (Figure 4.4b).

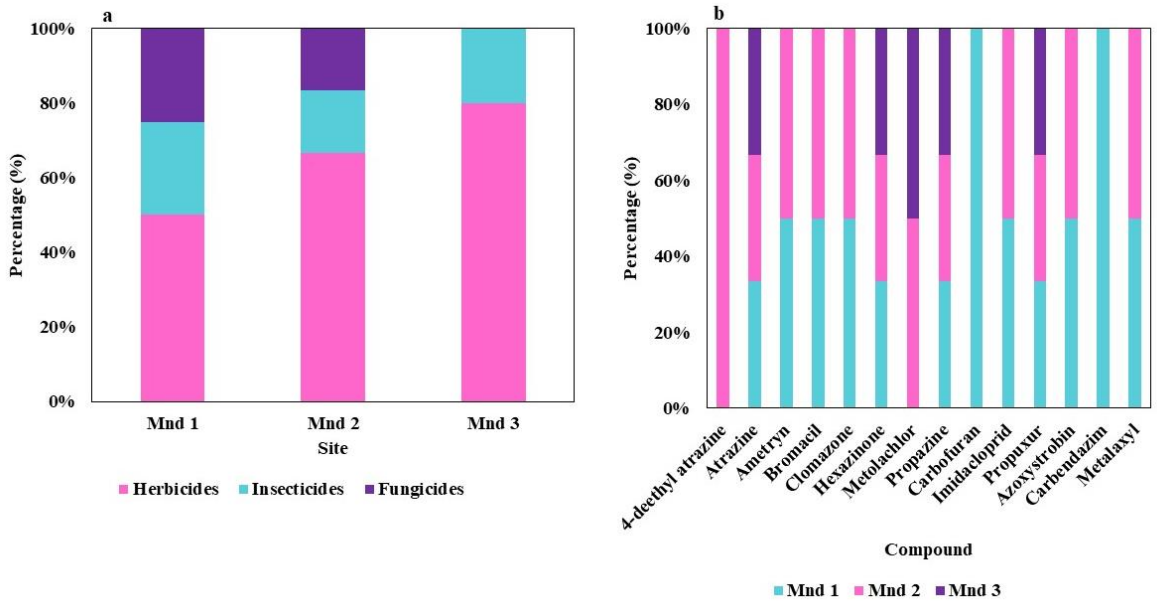


Figure 4.4: Frequency of detection of pesticides at uThukela River-Estuary. Summary of the occurrence of pesticide classes are presented in figure (a) whilst a detailed account on the occurrence of compounds per site are presented in figure (b)

4.2.4. Occurrence of microplastics in surface water samples

4.2.4.1. uMhlathuze River-Estuary

Microplastic particles were found in all collected samples. The number of microplastics collected at uMhlathuze River-Estuary did not differ significantly between sites (df = 2, F = 1.16, p > 0.05) or between seasons (df = 1, F = 3.29, p > 0.05). The number of microplastics was generally higher during high flow season (February) than at low flow season (October, Figure 4.5). Pooled data shows a trend of decreasing microplastic abundance towards the estuarine site Mhl 3. Microplastics were dominated by fibres, and white microplastics were the most dominant at all sampling sites (Table 4.3).

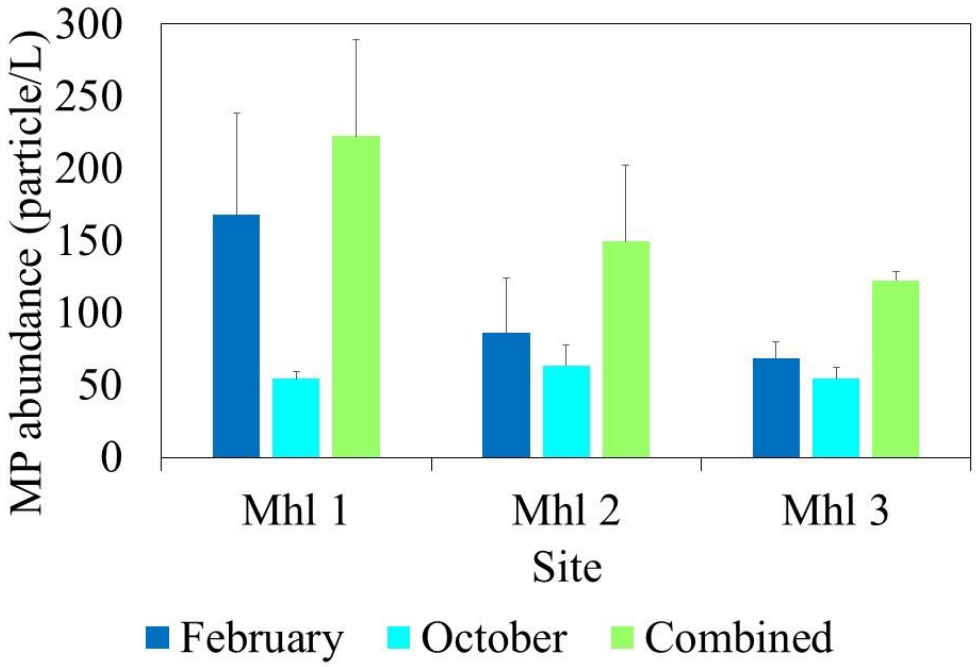


Figure 4.5: Microplastic abundance (mean ± SE) in surface water of uMhlathuze (Mhl) River-Estuary during February (high flow) and October (low flow)

Table 4.3: Abundance (n = 6) of microplastics in uMhlathuze River-Estuary, seasons combined

Site name	Abundance (particle/L; mean ± SE)	Morphology and polymer type	Abundance of fibre
Mhl 1	222.33 ± 66.88	Fibres, films	White > other > blue > red
Mhl 2	149.67 ± 52.45	Fibres, films:	White > other >blue >red
Mhl 3	122.67 ± 6.17	Fibres, films	White > blue >red >other

Fibres accounted for 97% of the microplastics that were recovered, whereas films were the least common type across all sites, accounting for 3% (Figure 4.6a). Microplastics encountered in surface water samples comprised various polymers, including poly(propylene) (PP), polycarbonate (PC), polystyrene (PS), nylons (NY), low-density poly(ethylene) (LDPE), high-density poly(ethylene) (HDPE), poly (ethylene terephthalate) (PETE), polyvinyl chloride (PVC), acrylonitrile butadiene styrene (ABS), latex (LX), nitrile (NT), poly(ethylene vinyl acetate) (EVA), poly(ethylene vinyl acetate) + poly (cyclohexanone) (EVA + poly(cyclohexanone)),

poly (butyl acrylate) (PBA), polyurethane (PU) and polyethylene (PE). The most abundant polymer types encountered in uMhlathuze River-Estuary were PETE and NY (71%), PP and EVA + poly(cyclohexanone) (64%), PC (57%), PBA (50%), EVA and LDPE (29%) (Figure 4.6b). The important absorption bands and graphs for identified polymers are summarised in Appendices A1 and A2.

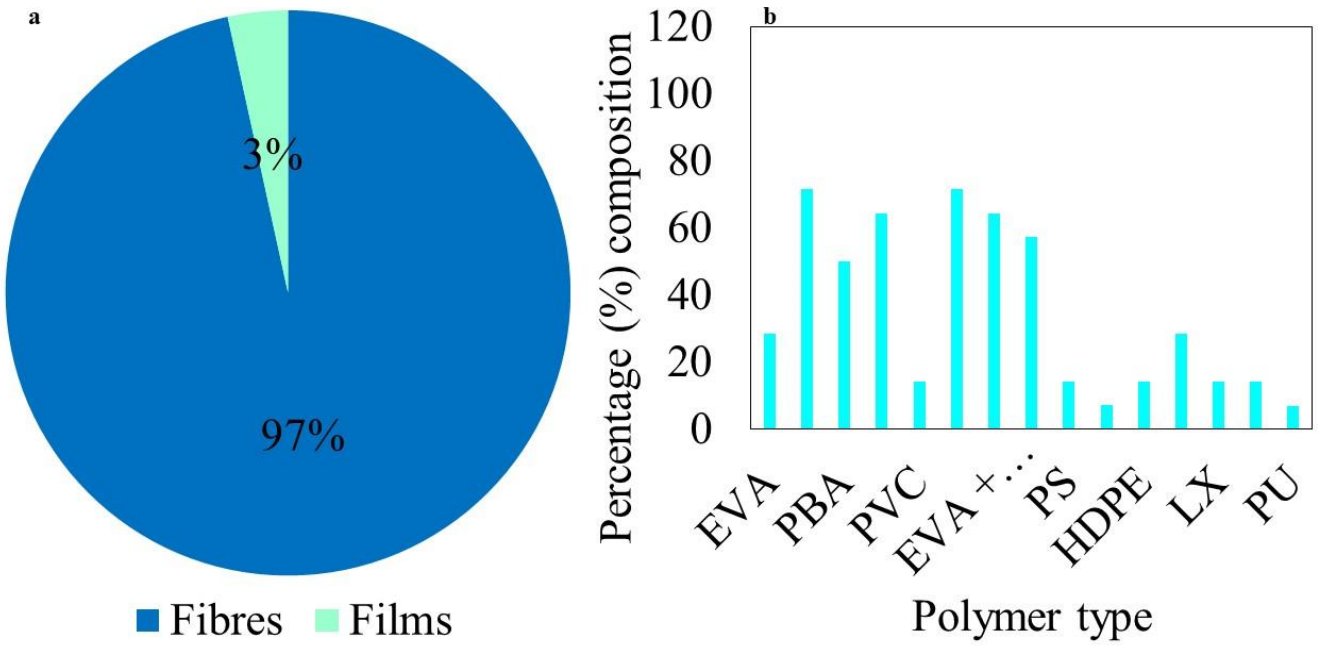


Figure 4.6: Percentage composition (%) of microplastics typology (a) and polymers (b) from surface water collected from uMhlathuze River-Estuary

Identified colours for combined data indicated the following sequence in the uMhlathuze River-Estuary, white > red > other (green, purple, and black) > blue (Figure 4.7).

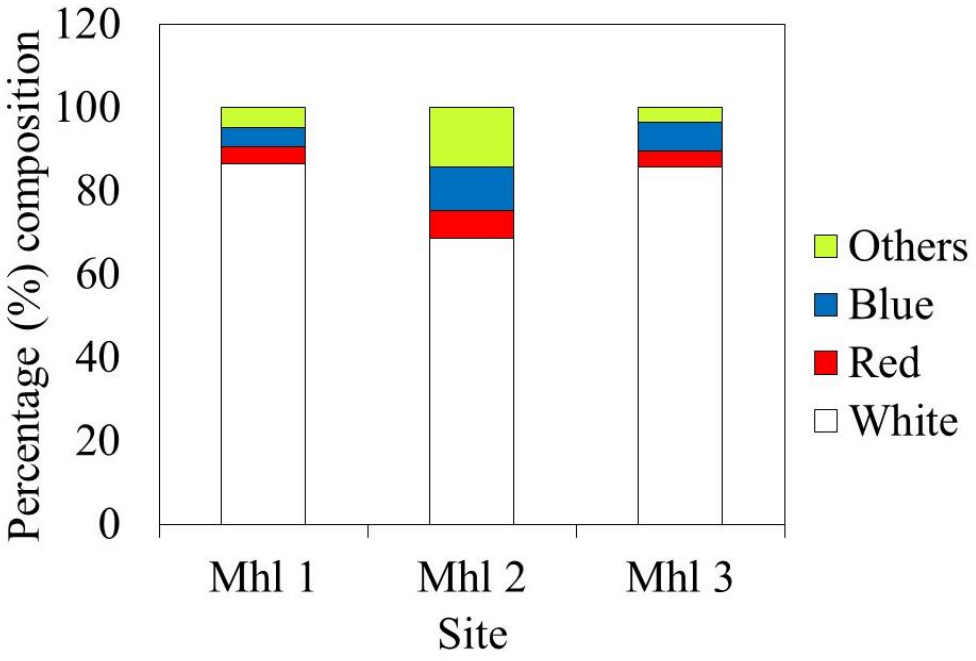


Figure 4.7: Percentage (%) composition of microplastics colours from surface water samples collected in the uMhlathuze River Estuary.

4.2.4.2. uThukela River-Estuary

Microplastic abundance in uThukela River-Estuary showed no significant difference between sites (df = 2, F = 0.24, p > 0.05) or between seasons (df = 1, F = 0.27, p > 0.05). Microplastic abundance was low at Mnd 1 (upstream of the sewage plant) compared to other sites during high flow (February). In October (during low flow), the trend was reversed, with microplastic abundance decreasing from Mnd 1 to Mnd 3 (Figure 4.8). Pooled data showed a similar trend to that observed during low flow (Figure 4.8, Table 4.4).

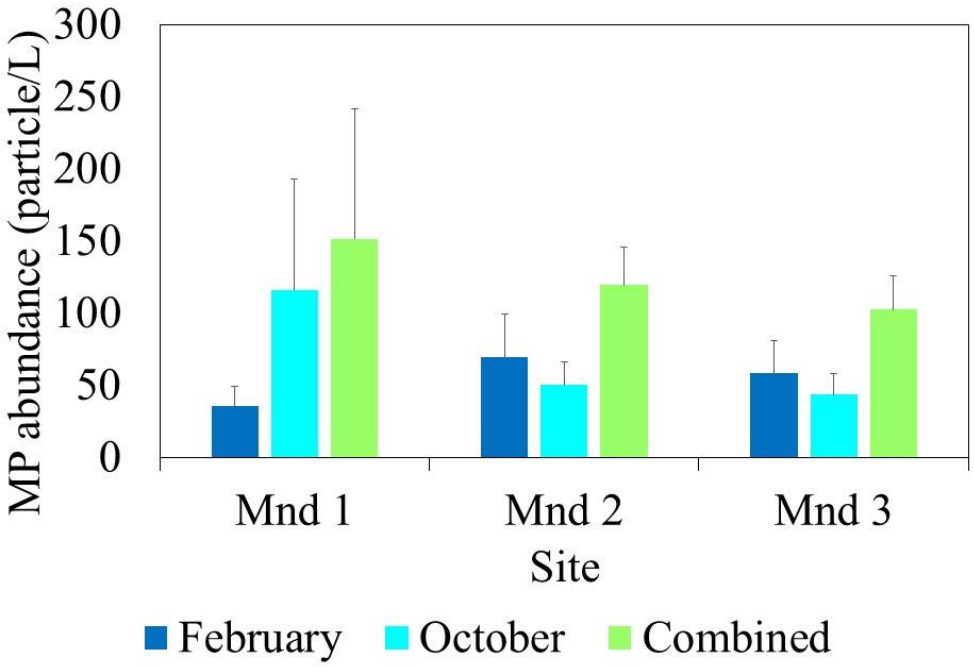


Figure 4.8: Microplastic abundance (mean ± SE) in surface water of the uThukela River-Estuary during February (high flow) and October (low flow).

Table 4.4: Mean abundance (n = 6) of microplastics in the uThukela River-Estuary for the combined seasons.

Site name	Abundance (particle/L; mean ± SE)	Morphology and polymer type	Abundance of fibre
Mnd 1	151.33 ± 90.40	Fibres, films	White > red >blue >other
Mnd 2	119.33 ± 26.33	Fibres, films	White > red > other > blue
Mnd 3	102.33 ± 23.67	Fibres, films.	White > red > blue > other

The most common type of microplastic particles found in uThukela River-Estuary were fibres (accounting for 99%), followed by films, accounting for 1%, (Figure 4.9a). Microplastics encountered in surface water samples comprised various polymers, including poly(propylene) (PP), polycarbonate (PC), polystyrene (PS), nylons (NY), low-density poly(ethylene) (LDPE), high-density poly(ethylene) (HDPE), poly (ethylene terephthalate) (PETE), polyvinyl chloride (PVC), acrylonitrile butadiene styrene (ABS), latex (LX), nitrile (NT), poly(ethylene vinyl acetate) (EVA), poly(ethylene vinyl acetate) + poly (cyclohexanone) (EVA + poly(cyclohexanone)),

poly (butyl acrylate) (PBA) and polyethylene (PE). In the uThukela River-Estuary PETE and NY (79%), PP (71%), PBA and PC (57%), LDPE (50%), EVA and EVA + poly(cyclohexanone) (43%) and ABS (29%) were dominant (Figure 4.9b). The important absorption bands and graphs for identified polymers are summarised in Appendices A1 and A3.

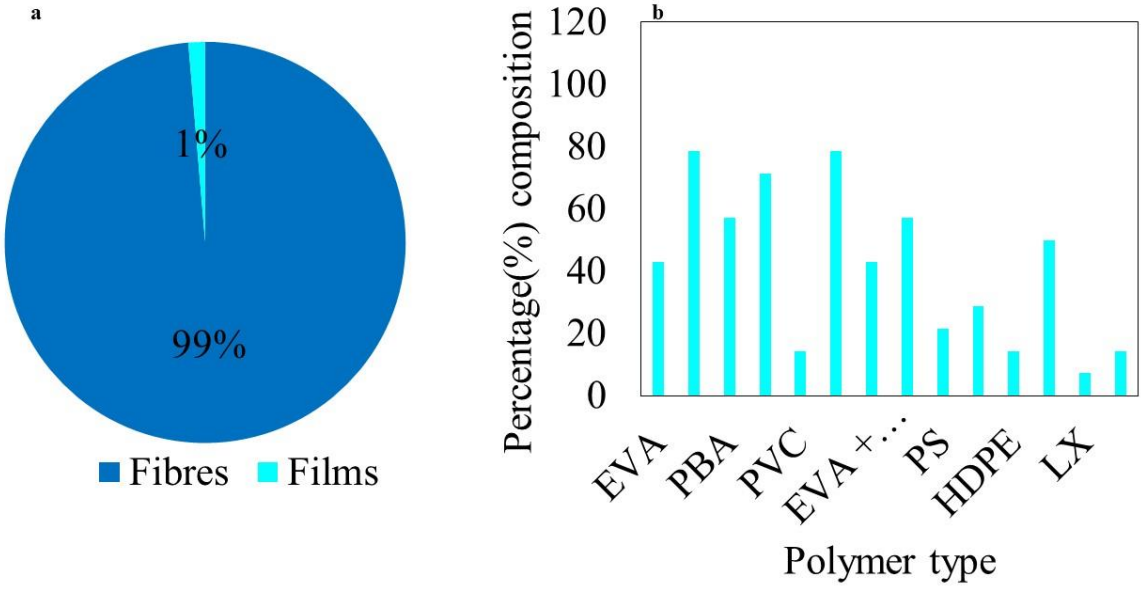


Figure 4.9: Percentage composition (%) of microplastics typology (a) and polymers (b) from surface water collected from the uThukela River-Estuary.

The colour profile in the uThukela River-Estuary was in the sequence white > blue > red > other (green, purple and black), shown in Figure 4.10.

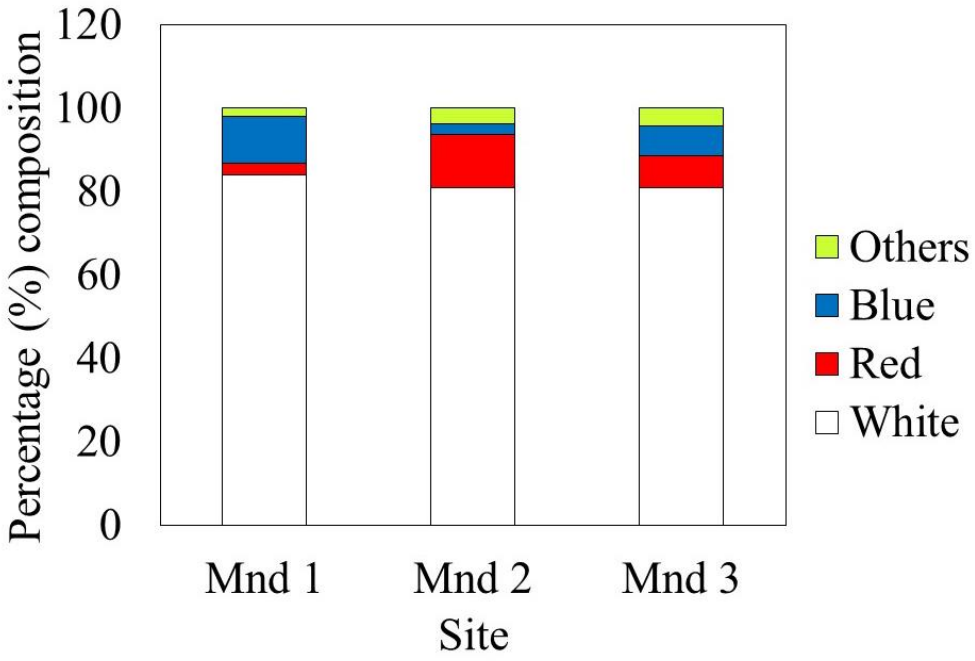


Figure 4.10: Percentage (%) composition of microplastics colours in surface water samples collected at the uThukela River-Estuary.

4.2.5. Occurrence of engineered nanomaterials (ENMs) in surface water samples

4.2.5.1. uMhlathuze River-Estuary

The modal size obtained from nanoparticle tracking analysis (NTA) ranged from 78.0–92.1 nm. The average values of particles obtained from transmission electron microscope (TEM) ranged from 106.6–117.6 nm (Figure 4.11). The results for other replicates are shown in Appendices A6 and A7. In the uMhlathuze River-Estuary, particles were polydisperse in shape and size. Samples collected during February and August showed the presence of irregular, sheet-like, hexagonal, and semi-spherical particles. Energy-dispersive X-ray (EDX) spectra showed the common presence of silicon (Si), iron (Fe), titanium (Ti), zinc (Zn), copper (Cu) and calcium (Ca), with Ti dominating (Figure 4.12).

Sample name	NTA (nm)	TEM (nm)
Mhl October	78.0-92.1	117.6
Mhl August	80.7-87.6	106.6

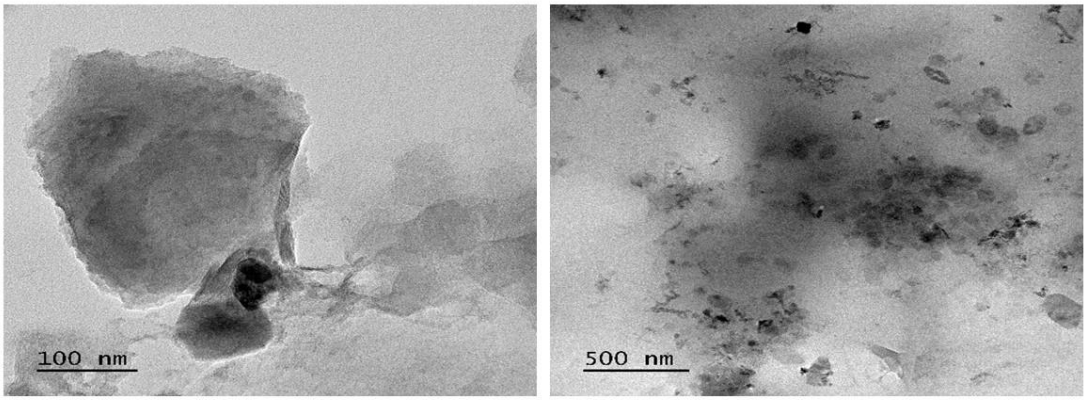


Figure 4.11: Particle sizes (nm) and images of extracted engineered nanomaterials (ENMs) determined by nanoparticle tracking analysis (NTA) and transmission electron microscope (TEM) in surface water samples from uMhlathuze River-Estuary.

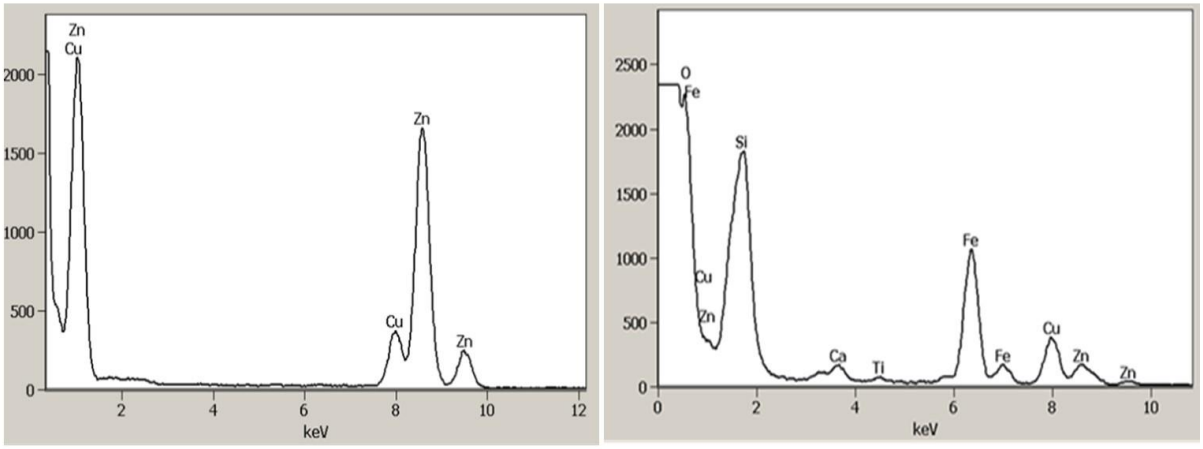


Figure 4.12: Energy-dispersive X-ray (EDX) of the surface water samples at the uMhlathuze River-Estuary.

4.2.5.2. uThukela River-Estuary

The modal size obtained from NTA ranged from 72.1–100.6 nm, and TEM obtained particles had a size ranging from 103.3–106.9 nm. The sizes of the particles are summarised in Figure 4.13. The results for other replicates are listed in Appendices A6 and A8. The particles in the uThukela River-Estuary (August and October) were predominantly hexagonal and spherical. Energy-dispersive X-ray (EDX) showed the presence of Zn, Cu, Si, Ti, Ca, and Fe (Figure 4.14).

Sample name	NTA (nm)	TEM (nm)
Mnd October	86.6-100.6	103.3
Mnd August	72.1-113.7	106.9

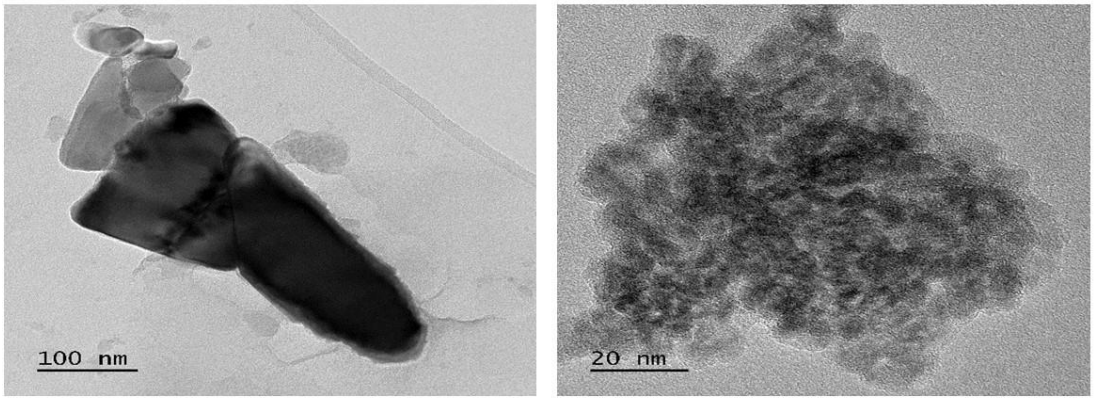


Figure 4.13: Particle sizes (nm) and images of extracted ENMs determined by nanoparticle tracking analysis (NTA) and transmission electron microscope (TEM) in surface water samples from uThukela River-Estuary.

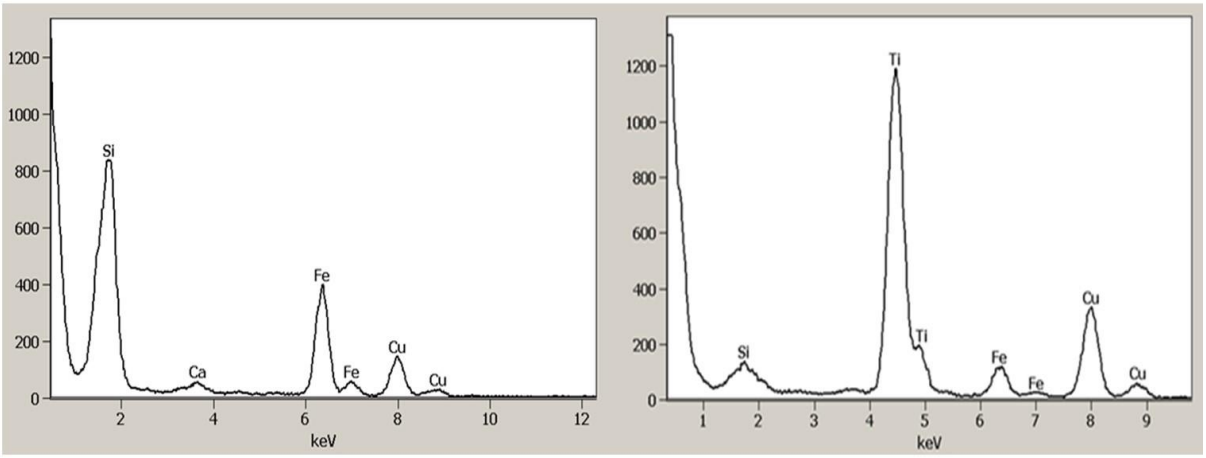


Figure 4.14: Energy-dispersive X-ray (EDX) of the surface water samples from the uThukela River-Estuary.

4.3. DISCUSSION

4.3.1. Physico-chemical characterization of surface water samples

4.3.1.1. uMhlathuze River-Estuary

Salinity increased from Mhl 1 to Mhl 3 as the conditions changed from freshwater to estuarine conditions. An inverse relationship in dissolved oxygen during low and high flow conditions was noted; dissolved oxygen increased from Mhl 1 to Mhl 3 during low flow conditions and vice versa. Activities that might affect the water quality at uMhlathuze River-Estuary include agriculture, industrial and domestic activities, as this system drains a catchment that is categorized as rural but becomes intensively urbanised and industrialized towards the coastal reaches.

4.3.1.2. uThukela River-Estuary

Values of salinity recorded from Mnd 1 to Mnd 3 showed that the uThukela River-Estuary is fresh water right up to the mouth. This is not surprising, as this system is known to be river dominated. Neutral pH levels were observed in all sampling season, except in October when an alkaline state was observed. Considerably lower levels of oxygen were noted at the Mandeni stream (Mnd 1 and 2) compared to at the uThukela mouth (Mnd 3), in all seasons. Reduced oxygen levels in these sites were possibly due to the poor water quality, which is influenced by activities such as the Isithebe industrial complex, municipal sewage effluent discharge and the Sappi mill (Wade *et al*., 2021). Prolonged exposure to these lower concentrations might have a negative impact on aquatic biota.

4.3.2. Occurrence of pharmaceuticals and lifestyle drugs in surface water samples

4.3.2.1. uMhlathuze River-Estuary

Psychoanaleptics were one of the most frequently detected compounds. The occurrence of lifestyle drugs (cocaine, benzoylecgonine and methamphetamine) at Mhl 1 and 2 implies high consumption or abuse of this drug class by people residing at Empangeni. Occurrence of cocaine both in form of parent and transformation products (benzoylecgonine), implies short residence times for cocaine, as it is present in the upstream of the river and absent in downstream. Transformation products of

bupropion (threoaminobupropion, hydroxybupropion and erythroaminobupropion) were present in all sites, while bupropion was not detected in all sites. This indicates that bupropion is easily metabolised and produces TPs compounds which are persistent, as they were detected even in the estuary. In addition, this can be attributed to incomplete removal and continuous discharge into the systems, and there is a possibility that these compounds accumulate constantly in the environmental media and therefore result in the pseudo-persistent metabolites. Further physical, chemical, and biological processes could be responsible for such results.

Antiepileptics such as carbamazepine, gabapentin and phenytoin were detected at uMhlathuze River-Estuary. Wide detection of carbamazepine indicates the possibility of a high degree of usage and or abuse of this drug, as well as indiscriminate disposal in Empangeni and Richards Bay area. The presence of carbamazepine in the present study may be due to excretion via urine, given its persistence coupled with its known low removal rate (10%) by the treatment plants (Vumazonke *et al*., 2020). Gabapentin and phenytoin were less frequently detected in uMhlathuze (detected in Mhl 2). These compounds are not frequently used in African countries (Madikizela *et al.,* 2017).

In addition, some of the detected compounds are endocrine disrupting chemicals (EDCs), for instance, carbamazepine and acetaminophen. Concentrations of carbamazepine were found to be lethal when exposed to *Daphnia magna (*Minguez *et al.,* 2016*).* Acetaminophen is known as an analgesic compound that is widely detected worldwide, regardless of its rapid degradation by micro-organisms (Bagnis *et al*., 2020). In this study it was present only in Mhl 2 (Empangeni River below the WWTWs discharge point). This implies that WWTWs might be the source of contamination. However, it was quickly removed from water samples collected away from the source. Acetaminophen has been found to be lethal in *Ceriodaphnia dubia* (Ferrari *et al*., 2003). Further information on the lethality of pharmaceutical compounds is summarised in Appendix A4. The wide occurrence of pharmaceuticals and lifestyle drugs in the uMhlathuze river-estuary signifies that further studies on exposure are needed to estimate environmental risk in the region.

4.3.2.2. uThukela River-Estuary

Psychoanaleptics, including a variety of antidepressants and stimulant drugs, were amongst the most frequently detected compounds in uThukela River-Estuary. This indicates the possibility of high consumption or abuse of this drug class. Certain psychoanaleptic drugs were recorded both in parent form and transformation products (TPs). These included threoaminobupropion and hydroxybupropion, which were only detected at Mnd 3 (estuarine site), while the parent compound (bupropion) was not detected. These results could indicate the short residence times of the parent compounds in water. This can be attributed to their incomplete removal and continuous discharge into water resources; there is a possibility that these compounds are constantly introduced into the environmental media.

Compounds such as sulfamethazine, carbamazepine and fluconazole were 100% present in all sites. This is a general indication of large consumption and possible abuse of these drugs in adjacent areas within the studied sites. The antibiotic sulfamethazine is widely used as a veterinary medicine (Bu *et al*., 2013, Xu *et al*., 2019), and thus livestock farming, aquaculture and poultry-fishery within the catchment are also potential sources. Carbamazepine is a common anti-seizure drug known as a useful marker for sewage contamination due to its persistence and high solubility. Wide detection of carbamazepine in this study indicates sewage contamination and a possibility of a higher degree of indiscriminate disposal, of usage and or abuse of this drug by people residing in the Mandeni area. The antifungal fluconazole is commonly given for human use as well as for horses, cats and dogs (Bagnis *et al.*, 2020), and so livestock farming and/or agricultural activities might be potential sources.

Detection of antiretroviral drugs is mainly ascribed to the high prevalence of HIV/AIDS in South Africa. Nevirapine is specifically used for the prevention of mother-to-child transmission (Wood *et al*., 2015). Its environmental persistence, coupled with the inefficiency of the sewage plant at Mandeni town, was considered to the probable source of this contamination (Ngumba *et al*., 2016). Testosterone is a natural steroid hormone mainly derived from urine and faeces excretion by humans, livestock, and aquaculture (Manickum and John, 2014). It was found at Mnd 3, and the mouth of the uThukela River a site for watering livestock. The presence of pharmaceutical and

lifestyle drugs requires further investigation, particularly regarding the potential environmental risk assessment.

4.3.2.3. Comparison with other studies

The pharmaceuticals reported in this study have been recorded elsewhere in water systems of KZN and globally (Bagnis *et al*., 2020, Vumazonke *et al.*, 2020). Their presence was mainly attributed to the direct discharge of domestic waste, municipal and hospital wastewater. These results show that Mhl 2 and Mnd 2 (after the WWTWs or sewage plant discharge point) had the highest number of compounds. Some of them were only present after effluent discharge, which pointed to human consumption rather than improper solid waste disposal. Furthermore, the findings indicate that the current WWTWs are unable to efficiently remove such types of pollutants. However, the contamination detected from Mhl 1, Mnd 1 (before WWTWs or after upstream of the sewage plant) and 3 (estuarine sites) of both systems can be attributed to the direct discharge of untreated sewage, inputs from informal settlements and agricultural activities, as well as a lack of proper strategy to collect unused/expired drugs. These results confirmed environmental pollution by pharmaceuticals and lifestyle drugs in uMhlathuze and uThukela River-Estuary.

4.3.3. Occurrence of pesticides in surface water samples

4.3.3.1. uMhlathuze River-Estuary

Herbicides were the most dominant class of pesticides found in this study. Herbicides, particularly atrazine, are among the most frequently sold and applied pesticides due to their effective pest control characteristics (mainly photosynthesis inhibition) on a wide array of crops (Dabrowski, 2014). Thus, the wide distribution of herbicides can be attributed to extensive usage and their relatively higher water solubility properties, compared to insecticides which are expected to be distributed in sediment due to their hydrophobicity properties (Stehle *et al*., 2019). Herbicides such as atrazine and metolachlor are widely used to control weeds along streets as well as for alien vegetation removal, and so surface runoff can be considered as a potential source.

The fungicide thiabendazole is also known as an antihelmintic drug that is used for the prevention of infections caused by worms. This fungicide had a limited distribution and

was only detected below the effluent discharge point (Mhl 2 and 3). This may be due to incomplete removal and surface runoff from recreational parks and golf courses in the Empangeni area (Blair *et al*., 2013). Its presence at the estuarine site (Mhl 3) could be attributed to agricultural activities. Both parent and transformation products (TPs) were detected for some compounds in this study. This includes the pair of terbuthylazine (Mhl 1,2 and 3) and sebuthylazine desethyl (Mhl 1 and 3). This finding highlights the residence time of pesticides in the studied water samples. UMhlathuze river-estuary was mainly dominated by parent compounds and fewer TPs, implying that pesticide contamination was mainly from recent applications and to a lesser extent lingering contamination. The relative dominance of recent application may be related to a higher flushing rate in the uMhlathuze area.

4.3.3.2. uThukela River-Estuary

Herbicides were also a dominant class in uThukela. Herbicides, mainly atrazine, were detected at all sites. Atrazine is applied for the post and pre-emergence control of broadleaf and grassy weeds. This contamination could be attributed to surface runoff from agricultural activities. Atrazine is known for its endocrine-disrupting effects to wildlife organisms, such as feminisation and reproductive dysfunction (Hayes *et al*., 2011). Parent and TP forms were also recovered in uThukela River-Estuary, including terbuthylazine desethyl and terbuthylazine, atrazine and 4-deethyl atrazine. The presence of parent compounds and TPs from upstream up to the mouth of the uThukela river-estuary indicates that contamination arises from both recently applied pesticides and historical use.

4.3.3.3. Comparison with other studies

Pesticides were detected more frequently in mixtures of two or more, including their metabolites. This occurrence has also been reported in international and local studies of marine to freshwater environments (Dabrowski, 2014, Newman *et al.*, 2015, Stehle *et al*., 2019, Curchod *et al.*, 2020). Notably, studies have recorded that surface waters have been exposed to pesticide contamination for years, and consequently, some pesticides have been banned or are routinely monitored in many countries. Atrazine, for example, is banned in some European countries, but it is still used in South Africa.

4.3.4. Occurrence of microplastics in surface water samples

4.3.4.1. uMhlathuze River-Estuary (Mhl)

The distribution profile of microplastics in the studied sites generally showed a decreasing concentration trend along the river, with high concentrations recorded at riverine sites. High microplastic concentrations at Mhl 1 showed that influent and effluent from the WWTW was not a primary source. High microplastic concentrations were recorded during high flow (February), probably due to the fact that rivers and estuaries receive large stormwater inputs which are suspected to contain various microplastics.

Generally, fibres in rivers and estuaries are often associated with the effluent from WWTWs, more particularly from fibres shed during washing of textiles and clothing (Govender *et al*., 2020). This was the likely source, given the high population density (410 465 in 2017) in uMhlathuze Municipality (KwaZulu-Natal Department of Economic Development and Affairs, 2017). This municipality also has tribal areas, whose people rely on the uMhlathuze River tributaries for their domestic and agricultural purposes. Industrial effluent might be another source of fibres found in this study, given that the predominant fibres found were mostly composed of PP, PETE and EVA, which are extensively used in the electronics, motive, footwear, medical and consumer industries. In addition, surface runoff from agriculture could have been possible source for the presence of microplastics contamination, due to various polymers detected which are widely used in agriculture. For example, PBA is used in agriculture for the encapsulation of herbicides (Duis and Coors, 2016), LDPE for increasing temperatures, protection of crops and the preservation of irrigation water in soil layers. Polystyrene is often used in horticulture for seed germination. Given the various industries and agriculture within uMhlathuze Municipality, an increase in such contamination is expected in the near future as the area is earmarked for new industrial and agricultural zones.

4.3.4.2. uThukela River-Estuary (Mnd)

A decreasing concentration trend towards the estuarine site was also observed at the uThukela River-Estuary when seasons were combined. Elevated microplastic

concentrations at Mnd 1 (upstream of the sewage plant) could be due to domestic and industrial effluent, given that this site only drains effluent from rural areas and dominating industries in the Mandeni area. High concentrations of microplastic observed at Mnd 3 (estuarine site) during February (high flow) could be attributed to diffuse sources rather than direct pollution, given that the Mnd 3 site drains the townships of both Mandeni and Thukela. In addition, the abundance of microplastics at the uThukela River-Estuary could be attributed to the direct disposal of products composed of microplastics into the river (personal observation).

Polymers identified at different sampling sites of the uThukela River-Estuary indicate that the types of microplastics identified were from various sources. The dominant polymers recorded were PETE, NY, PP, PBA, PC, LDPE, EVA, and EVA + poly(cyclohexanone), indicating a wide range of sources. PETE is mainly used in the manufacturing of clothing fabrics; thus, its high detection could be attributed to fabric manufacturing industries in the Mandeni area. Polystyrene is usually used in personal care products such skin products and the packaging of consumer products (Duis and Coors, 2016, Vetrimurugan *et al*., 2020). PBA and HDPE are mainly applied in consumer products, for example, the acrylic that is used for nails and painting (Duis and Coors, 2016). Thus, the abundance could be ascribed to domestic effluent and or to the direct disposal of products, as the study sites Mnd 1 and Mnd 2 were in close proximity to residential areas. Other polymers such as PVC, EVA, EVA+ poly(cyclohexanone), PC, NY, PS, ABS, LX and NT are commonly applied in a wide range of commercial sectors (e.g., construction, medical devices, automotive, electronics, laboratory equipment, household uses, aquaculture, air-blasting and packaging industries) (Duis and Coors, 2016, Andrady, 2017) and thus their presence could be ascribed to the effluent from industries and agricultural activities dominating the Mandeni area.

4.3.4.3. Comparison with other studies

The high microplastics abundance recorded in the uMhlathuze River-Estuary during high flow conditions is similar to the findings of Govender *et al*., (2020), who also recorded high concentrations during the wet season. Microplastics were dominated by fibres in this study, which is similar to the findings of Nel and Froneman (2015), and Govender *et al*., (2020). Films, fragments and nurdles, which are also of great concern

in the aquatic environment, did not appear to be significant sources of microplastic contamination in the surface water of uMhlathuze and uThukela River-Estuary in this study. Films were recorded in low proportion at the uMhlathuze and uThukela systems, similar to the findings by Govender *et al*., (2020) who studied water and sediments in St. Lucia, Durban harbour, uMgeni and Isipingo estuaries. Breakdown of larger particles, surface runoff, synthetic textiles or effluent from household sewages or industries have been found to be potential sources of microplastics contamination (Wang *et al*., 2017, Govender *et al*., 2020).

Various colours of microplastics were detected in this study, which is similar to other local studies (Govender *et al*., 2020, Vetrimurugan *et al.,* 2020) and international studies (Andrady, 2017, Wang *et al*., 2017). Wang *et al*., (2017) associated the presence of white fibres with ropes from recreational fishing and washing of textiles/clothing. The presence of black and white fibres generally indicates the usage of cosmetic products, commercial fishing ropes and other commercially dyed packaging materials (Andrady, 2017, Vetrimurugan *et al*., 2020). Vetrimurugan *et al*., (2020) ascribed the presence of blue fibres to the usage of exfoliating scrubs and cosmetic products. Microplastics colour may be considered to be an important factor in the ingestion of microplastics by different species. For example, pelagic fish such as *Decapterus muroadsi* (Ory *et al*., 2018) and juveniles of *Oreochromis mossambicus*, *Terapon jarbua, Ambassis dussumieri* and *Mugil* species (Naidoo *et al*., 2020) have been reported to ingest blue coloured microplastics over other colours, due to their resemblance with the blue copepods they prey on.

In conclusion, this study confirmed the presence of microplastic contamination in the uMhlathuze and uThukela systems. Microplastic contamination was linked to various sources such as the improper disposal of hygiene products or products made of plastic, effluent discharge from industries, WWTWs, aquaculture and domestic waste. This work provides a useful baseline for future studies in the uMhlathuze and uThukeka systems and is an important first step for future assessment focusing on the characterization of sources, fate, transport, and seasonal dynamics of contaminants in the northern KwaZulu-Natal rivers. The behaviour of microplastics in the studied systems, coupled with the transport dynamics and trends in the accumulation zones, can be attributed to the links between different factors. For example, links between land use, hydrological (summer and winter rainfall) and meteorological factors (storms,

temperature, wind), location within a river network (upstream and downstream) and anthropogenic factors (human and industrial activities) in these systems all played a role. More studies investigating these links are recommended.

4.3.5. Occurrence of engineered nanomaterials (ENMs) in surface water samples from uMhlathuze and uThukela River-Estuaries

Generally, the modal sizes obtained from nanoparticle tracking analysis (NTA) were within the conventional 1–100 nm nano definition. Overall, there was relatively good consistency in size properties obtained with the NTA and the transmission electron microscope (TEM), although differences were expected because of the different measurement techniques. The NTA measures the hydrodynamic size whereas TEM measures the dry phase. Elemental profiling indicated the presence of titanium dioxide (TiO_2), silicon dioxide (SiO_2), zinc oxide (ZnO), iron oxide (Fe_2O_3) and copper oxide (CuO) nanoparticles. TiO2 and SiO_2 NPs dominate the South African market of nano-enabled products due to photocatalytic and coating application respectively (Moeta *et al.*, 2019). The dominance of SiO_2 and Fe_2O_3 elements in Mhl (August) suggests that phyllosilicate clay minerals were dominant mineral components of the collected particles (Rand *et al.*, 2020).

The widespread presence of TiO_2 may be attributed to its extensive application in coating, paints, and cosmetic products. They are released in the environment mainly from domestic and industrial effluent or direct disposal of Ti-containing products. This situation seems to be most likely at the uMhlathuze River-Estuary as a relatively high concentration of Ti in water samples was detected, and titanium mining at Richards Bay could be a potential source. ENMs that are applied in batteries and electronics such as CuO and SiO_2 are rarely detected in the environment because they seldom come into contact with water and are strongly bound in the product matrix (Rand *et al*., 2020). However, in this study they were found extensively, suggesting other potential sources. ENMs reach the environment through different point and non-point sources. This includes ENMs released during household activities such as bathing, laundry, food preparation and some fertilizers. In addition, ENMs may also be released into water from urban runoff such as storm water, which may contain ENM paint products, and sanitary sewer outflows which may contain ENMs. In addition, direct

ENMs released during recreational activities involving sunscreens and other personal care products can be washed off and enter aquatic environments directly (Good *et al*., 2016). The wide application of ENMs may be due to their properties and uses, summarised in Table 4.5.

Table 4. 5: Summarised properties and uses of ENMs detected in this study (Adapted from Good *et al*., 2016).

Material category	ENMs	Properties	Uses
Metals	Cu	Antimicrobial, conductivity.	Electronics, cosmetics, textiles, and personal care products.
	Fe	Reductive reactivity	Filters, water, and soil remediation.
Semi-conductors	TiO_2	Photocatalytic	Paints, coatings, cosmetics, and packaging.
	ZnO	Antimicrobial, photocatalytic	Cosmetics, personal care products and packaging.
Ceramic	SiO_2	Mechanical strength, functionalizable surfaces	Paints, plastics, cosmetics, polishing agents and food packaging.

CHAPTER 5:

ACCUMULATION OF METALS IN SURFACE WATER AND CRABS, BIOMARKER RESPONSE OF THE CRAB *CHIROMANTES EULIMENE*

5.1. INTRODUCTION

Metal contamination may be commonly derived from industrial, domestic, and other related anthropogenic activities. Metals also occur naturally in trace concentrations released from windblown dust, vegetation, and ore-bearing rocks (Majola *et al.*, 2020). Exposure to such metal contamination can affect the survival of aquatic animals through direct and indirect toxicity. Over recent decades, many issues have been associated with the accumulation of metals in aquatic compartments (Miller *et al.*, 2019). Some implications of metals contamination on organisms have been investigated, although they are commonly derived based on exposure concentration levels measured in water or sediment to the organisms (Newman *et al*., 2015, Miller *et al.*, 2019). For example, at the uMhlathuze Estuary, bioaccumulation of metals (aluminium (Al), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), lead (Pb) and zinc (Zn) from water and sediment to fish species (*Liza dumerelii*) was investigated by Mzimela *et al*. (2003) and Mzimela *et al*., (2014). Recent studies also investigated metal concentration in surface sediment, water samples and different species of crabs from the uMhlathuze Estuary and Richards Bay Harbour (Adeleke *et al*., 2020, Izegaegbe *et al*., 2020a, Izegaegbe *et al*., 2021). In addition, Majola *et al*., (2020) conducted a passive metal bioaccumulation and energy biomarker study in order to assess the impact of anthropogenic activities in the tissues of crabs, *Chiromantes eulimene*, from Richards Bay harbour. Despite this existing historical and current information on KZN estuarine systems, very little is known about the presence and dynamics of organic and inorganic contamination in the northern KwaZulu-Natal aquatic systems, predominantly the relationship between metals and biochemical effects.

Therefore, this study adopted mud crabs as sentinels for monitoring metal contamination in the uMhlathuze and uThukela Estuaries. Generally, invertebrates are preferred for this type of study. In particular, crabs are known as key species in the benthic environment and for their capability to accumulate both heavy and trace metals in their tissues and they are, therefore a suitable bioindicator for examination of the

contamination level in the aquatic environment (Cheng *et al*., 2017, Cheng *et al*., 2018). In addition, crabs were selected as good indicator organisms within these systems due to their abundance, mostly in terms of numbers and biomass, amongst other groups of aquatic sediment macrofauna. The mud crab *Chiromantes eulimene* is known for being a good indicator species (Thwala *et al*., 2011). The mud crab *C. eulimene* was originally a Western Indian Ocean species that is commonly found in mangrove forests, where they burrow in the intertidal salt marshes (Guerao *et al*., 2011, Majola *et al.*, 2020). It is known as a semi-terrestrial crab and tolerates a salinity range of 0–65 (Majola *et al*., 2020).

The biomarker response of an organism to chemical contaminants is necessary to efficiently measure the degree of pollutant exposure and biological effects, particularly, biomarkers integrating both exposure and biological effects, such as acetylcholinesterase (AChE) (Dalzochio *et al*., 2016). Acetylcholinesterase analysis is a widely used technique applied in biomonitoring to give insight into environmental and pathological perspectives (discussed in Chapter 2). Measurements of AChE in the brain, ventral ganglion, hepatopancreas, gills, and muscles have been the primary means of monitoring exposure in crustaceans (Fulton and Key, 2001). Therefore, this study selected gills as a useful source of enzymes to study the effects of exposure to contaminants. This chapter aimed to determine concentrations of aluminium (Al), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), silicon (Si), titanium (Ti) and zinc (Zn) in surface water and crabs from the uMhlathuze and uThukela systems, and to assess the contamination effects (exposure) through investigation of a biomarker response (acetylcholinesterase (AChE)).

5.2. RESULTS

5.2.1. Accumulation of metals in surface water samples

5.2.1.1. uMhlathuze River-Estuary

The concentrations of aluminium (Al), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), silicon (Si), titanium (Ti) and zinc (Zn) are presented in Table 5.1 and Figure 5.1. The average metal concentrations were recorded in higher levels at Mhl 2 than Mhl 1, except for Si. Lower average metal concentrations were recorded at Mhl 3 compared to Mhl 2 except for Mn, Pb, and Si. Levels of Mn, Pb and Si showed an increasing trend from Mhl 1 to Mhl 3. Levels of Al,

Cr, Fe, Pb and Ti showed a decreasing trend from Mhl 1 to Mhl 3 during summer (February). Manganese, Pb and Si showed an increasing trend from Mhl 1 to Mhl 3 during winter (August).

Table 5.1: Metal concentrations (µg/L) in surface water samples collected in different seasons at uMhlathuze River-Estuary

Site	Al	Cr	Cu	Fe	Mn	Ni	Pb	Si	Ti	Zn
Mhl 1 Feb	2470,36	11,70	12,90	1581,20	34,76	12,88	3,74	576,40	124,35	15,35
Mhl 1 Aug	1833,60	10,00	12,64	795,48	30,11	14,80	2,63	960,33	96,05	14,12
Mhl 1 Oct	2771,63	9,32	13,68	870,84	23,19	15,60	15,88	980,11	54,97	23,81
Average	**2358,53**	**10,34**	**13,07**	**1082,50**	**29,35**	**14,43**	**7,42**	**838,95**	**91,79**	**17,76**
Mhl 2 Feb	2045,30	10,56	14,10	1290,33	55,38	13,01	3,20	492,66	75,17	22,90
Mhl 2 Aug	3212,84	17,35	23,90	1909,54	46,28	18,67	5,37	966,85	215,50	43,51
Mhl 2 Oct	2756,69	11,67	13,31	722,02	78,11	16,70	27,43	1018,32	89,48	24,20
Average	**2671,61**	**13,19**	**17,10**	**1307,30**	**59,92**	**16,13**	**12,00**	**825,94**	**126,72**	**30,20**
Mhl 3 Feb	642,27	3,53	4,58	262,96	35,77	6,11	0,65	841,16	56,27	2,89
Mhl 3 Aug	2805,38	7,26	7,57	1640,80	173,13	9,68	6,90	1053,70	106,20	8,17
Mhl 3 Oct	4114,23	8,33	15,55	912,69	11,95	14,57	30,14	1038,92	98,87	19,49
Average	**2520,63**	**6,38**	**9,23**	**938,82**	**73,61**	**10,12**	**12,56**	**977,93**	**87,11**	**10,19**

Mhl (uMhlathuze River-Estuary); Feb (February); Aug (August); Oct (October)

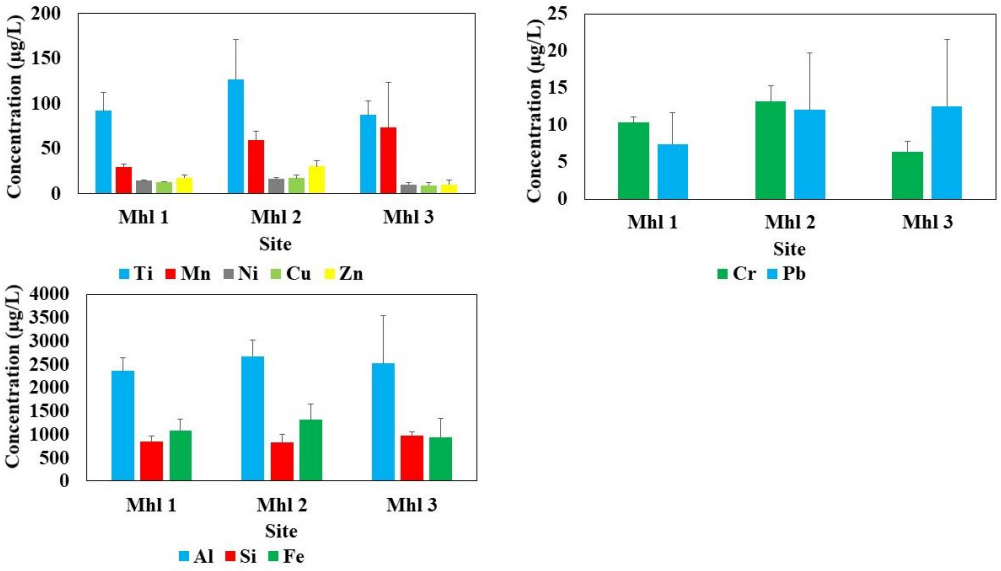


Figure 5.1: Average metal concentrations (mean ± SE) in surface water samples collected in different sites at uMhlathuze River-Estuary

To determine the relationship between the accumulation of metals in surface water samples and physicochemical parameters, a principal component analysis (PCA) was performed. The two axes of PCA from uMhlathuze River-Estuary explained 65,9% of the variation. All metals correlated with each other except for Mn, Fe, Ti, Cr, Cu and Zn, which correlated and formed their own group which closely correlated with turbidity. Lead and Si closely correlated with pH. Temperature and dissolved oxygen did not correlate with metal concentrations. Salinity and Mn were closely correlated (Figure 5.2).

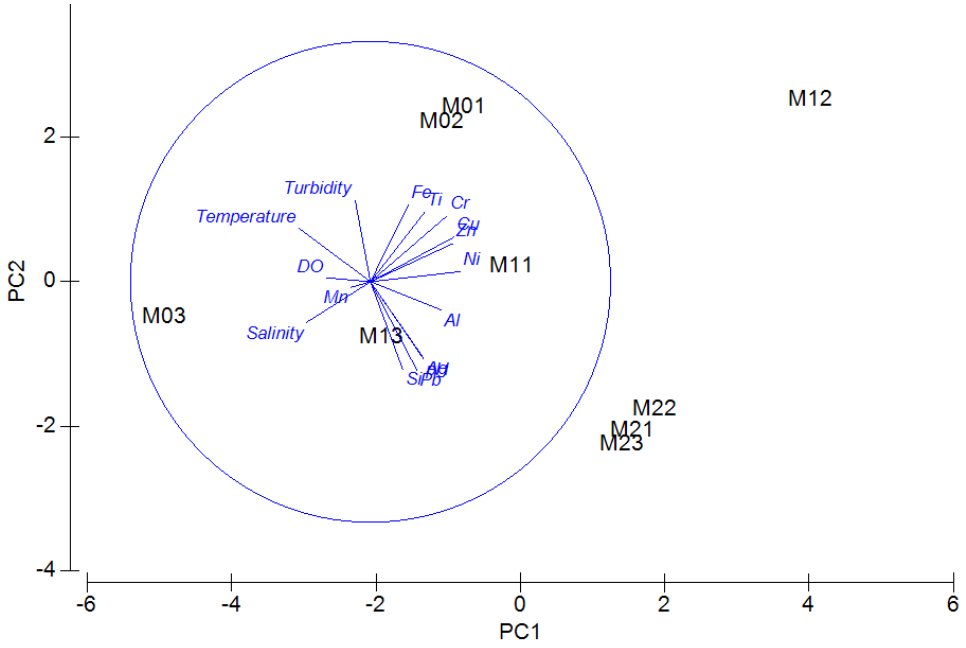


Figure 5.2: The PCA biplots illustrating the relationship between metal concentrations in surface water and physicochemical parameters from uMhlathuze river-estuary. M (uMhlathuze River-Estuary), 0 (February), 1 (August), 2 (October), 1,2,3 (sites: 1,2,3)

5.2.1.2. UThukela River-Estuary

Concentration of aluminium (Al), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), silicon (Si), titanium (Ti) and zinc (Zn) are presented in Table 5.2 and Figure 5.3. Lower concentrations were generally measured at Mnd 2 compared to Mnd 1, except for Cu, Si and Ti. Markedly higher concentrations of Al, Cu, Fe, Mn, Si, Pb, Ti and Zn were recorded at the estuarine site (Mnd 3) than at Mnd 2. The mean concentration of Cr showed a decreasing trend towards the estuarine site, whilst all other metals increased towards the estuarine site. The concentrations of Fe, Ni, Pb, Zn and Al displayed a decreasing trend toward the estuarine site during summer (February) whereas Si and Ti concentrations increased towards the estuarine site. During winter, levels of Al, Cr, Fe and Zn showed a decreasing trend, while levels of Si displayed an increasing trend towards the estuarine sites (Figure 5.3).

Table 5.2: Metal concentrations (µg/L) in surface water samples collected in different seasons at uThukela River-Estuary

Site	Al	Cr	Cu	Fe	Mn	Ni	Pb	Si	Ti	Zn
Mnd 1 Feb	1849,00	11,22	18,45	1673,44	213,81	14,73	5,66	69,56	69,56	220,40
Mnd 1 Aug	2346,44	14,32	20,71	2141,55	328,84	15,47	8,72	1003,00	102,71	242,82
Mnd 1 Oct	3746,80	21,23	17,64	2087,85	194,68	18,16	14,52	851,15	78,27	475,22
Average	**2647,41**	**15,59**	**18,93**	**1967,61**	**245,78**	**16,12**	**9,63**	**641,24**	**83,51**	**312,81**
Mnd 2 Feb	1652,00	15,64	23,57	973,07	83,86	14,29	3,09	853,76	101,08	92,14
Mnd 2 Aug	1589,52	13,34	22,88	868,26	89,96	14,48	9,11	1005,37	49,53	74,98
Mnd 2 Oct	3046,06	9,25	13,91	566,03	19,47	15,55	11,68	1054,39	102,27	46,74
Average	**2095,86**	**12,75**	**20,12**	**802,45**	**64,43**	**14,77**	**7,96**	**971,17**	**84,29**	**71,29**

Mnd (uThukela River-Estuary); Feb (February); Aug (August); Oct (October)

Table 5.3 continued: Metal concentrations (µg/L) in surface water samples collected in different seasons at uThukela River-Estuary (*continuation*)

Site	Al	Cr	Cu	Fe	Mn	Ni	Pb	Si	Ti	Zn
Mnd 3 Feb	1584,05	8,11	10,48	713,07	183,50	12,89	2,51	946,63	111,64	20,54
Mnd 3 Aug	1790,72	8,72	12,66	701,33	152,32	14,29	6,63	1016,45	196,86	21,66
Mnd 3 Oct	4859,12	19,75	24,99	3270,87	206,54	17,80	22,11	1301,11	151,82	331,84
Average	**2744,63**	**12,20**	**31.47**	**1561,76**	**180,79**	**14,99**	**10,42**	**1088,06**	**153,44**	**124,68**

Mnd (uThukela River-Estuary); Feb (February); Aug (August); Oct (October)

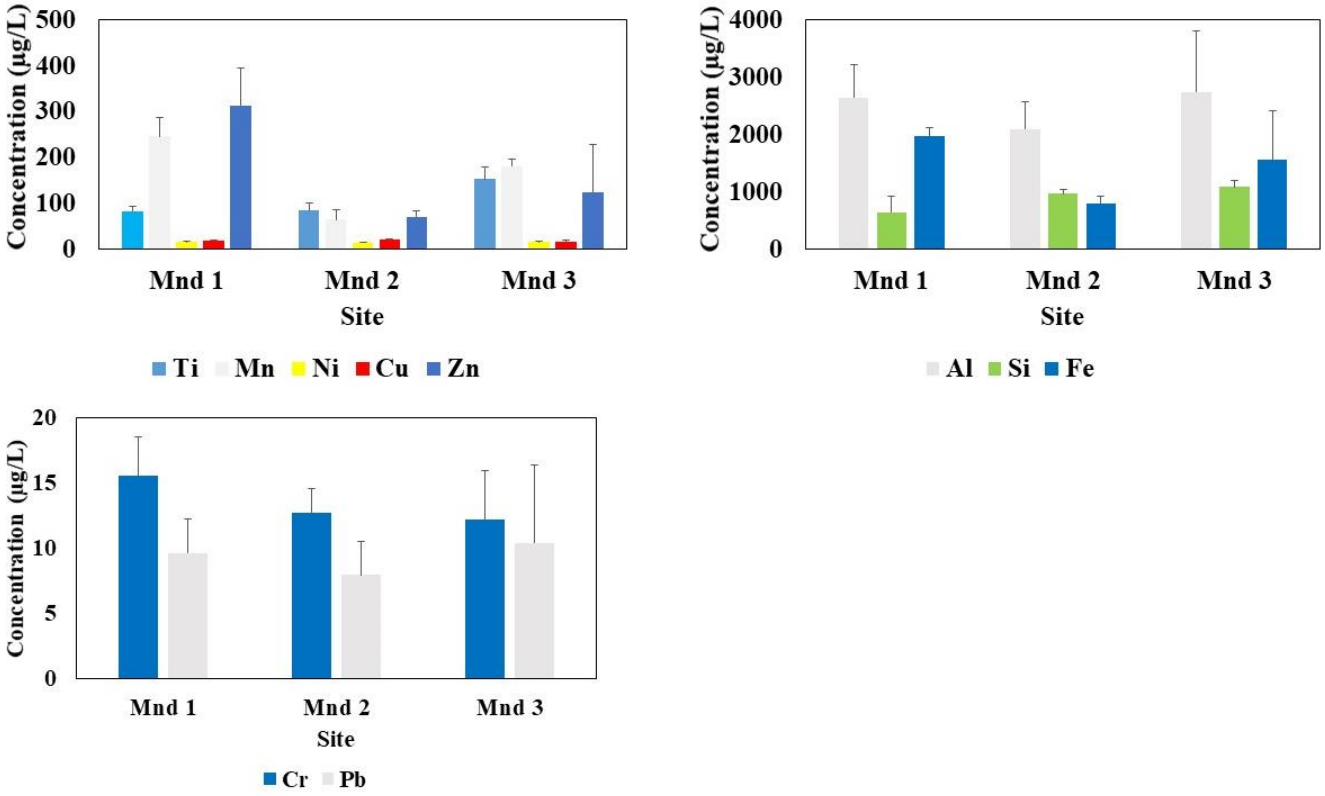


Figure 5.3: Average concentrations (mean ± SE) in surface water samples collected in different sites at uThukela River-Estuary.

To determine the relationship between the accumulation of metals in water samples and physicochemical parameters, a PCA was performed. The two axes of PCA at the uThukela River-Estuary explained 66,6% of the variation. All metals correlated with each other except for Si and Ti. Temperature and DO did not correlate with metal concentrations. Silicon and Ti closely correlated with salinity. Turbidity was closely correlated with Zn, Mn, Fe, Cr, Al, Cu and Ni. Lead closely correlated with pH. Only site Mnd 1 October (T21) and Mnd 3 October (T23) correlated with metal concentrations (Figure 5.4).

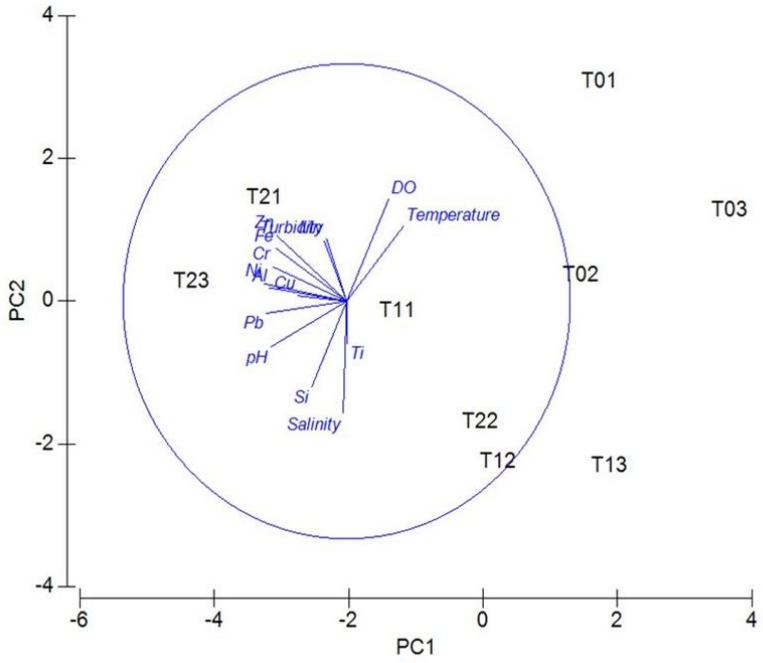


Figure 5.4: The PCA biplots illustrating the relationship between metal concentrations in surface water and physicochemical parameters from uThukela River-Estuary. T (uThukela River-Estuary), 0 (February), 1 (August), 2 (October), 1,2,3 (sites 1,2,3)

5.2.2. Passive accumulation of metals in the mud crab *Chiromantes eulimene*

5.2.2.1. UMhlathuze Estuary

Mean metal concentrations of chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), titanium (Ti) and zinc (Zn) in female and male crabs are presented in Table 5.3 and Figure 5.5. A significant difference was observed in metal accumulation between crabs from different seasons in all metals except for Fe.

Multiple comparisons using post hoc testing (Tukey HSD) showed no gender variation in the accumulation of all metals. However, metals such as Fe, Mn, Ni, Pb, Zn and Ti accumulated at slightly higher concentration in males than in females (Table 5.3). During high flow, the mean accumulation pattern of metals in *C. eulimene* was observed as Fe > Zn > Mn > Cu > Ti > Cr > Pb > Ni in both male and female crabs. During low flow, metal accumulation was observed in the order Fe > Mn > Ni > Zn > Pb > Cu > Ti > Cr in males, whilst in females, metals accumulated in the order Fe > Mn > Ni > Pb > Zn > Cu > Ti > Cr (Figure 5.5).

Table 5.4: Metal concentrations (mg/g) in crabs of *Chiromantes eulimene* collected from uMhlathuze Estuary. The ANOVA result (*F* values) of the differences in metal concentration between seasons, *df* value was 3, * indicates the significant differences ($p < 0.05$)

Metal	System	F value	Gender	High flow	Low flow
Cr	Mhl	919.06*	F	5.71± 0.31	68.30± 2.14
			M	5.53±0.65	72.55±1.83
Cu	Mhl	12.21*	F	69.77±15.40	110.70±13.10
			M	67.02±5.22	126.67±11.98
Fe	Mhl	0.12	F	597.44±44.17	535.38±94.14
			M	606.64±101.46	595.43±109.61
Mn	Mhl	8.86*	F	70.18±12.25	311.60±76.45
			M	72.89±8.40	305.14±78.15
Ni	Mhl	44.00*	F	2.77±0.40	224.8±20.59
			M	2.94±0.66	249.14±38.96
Pb	Mhl	81.04*	F	2.98±0.10	164.83±18.36
			M	3.56±0.12	179.12±18.81
Zn	Mhl	14.43*	F	89.88±3.81	149.26±22.46
			M	94.22±4.57	180.34±13.79
Ti	Mhl	43.66*	F	43.29±5.00	86.58±6.90
			M	43.67±1.41	99.66±2.92

Mhl (uMhlathuze Estuary), F (Female) M (Male), n (7-10 individuals, pooled).

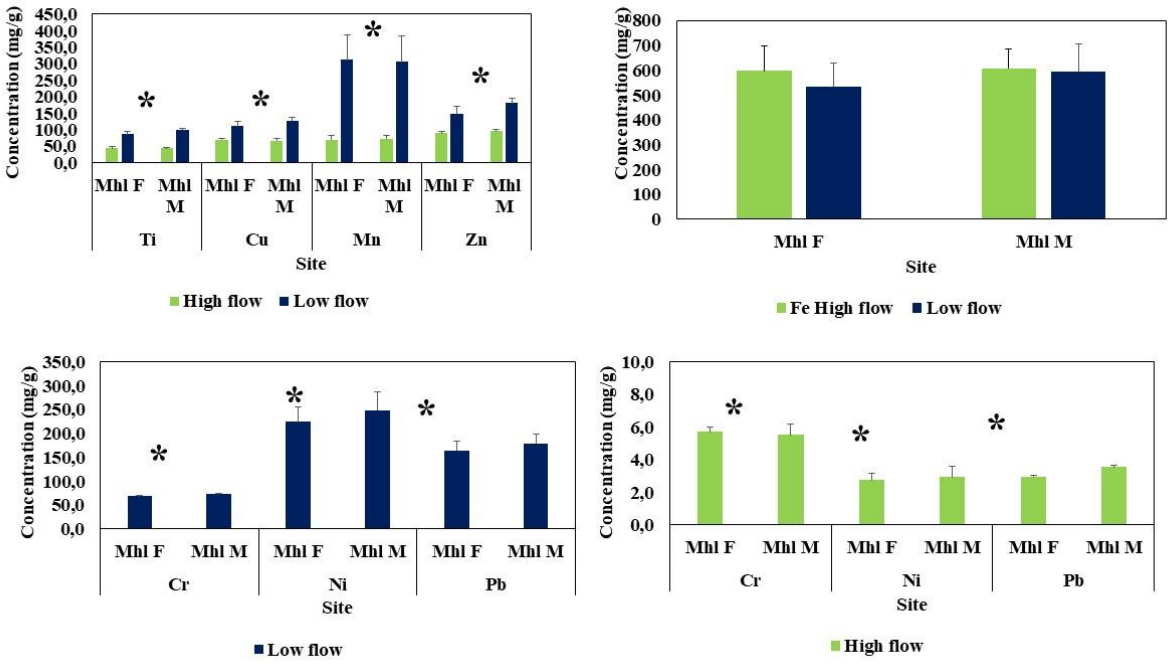


Figure 5.5: The metal concentration (mean ± SE) of the *Chiromantes eulimene* from uMhlathuze Estuary, * indicates a significant difference between crabs collected in high and low flow conditions.

The relationship between metal concentration in surface water samples (Mhl 3 only) and mean metal concentration in crabs during summer and winter are presented in Figure 5.6. The two axes of PCA at the uMhlathuze Estuary explained 96.6% of variation. Copper and Zn were closely correlated. Lead, Cr and Ni were closely correlated. Manganese, Ti and Fe were sparsely correlated.

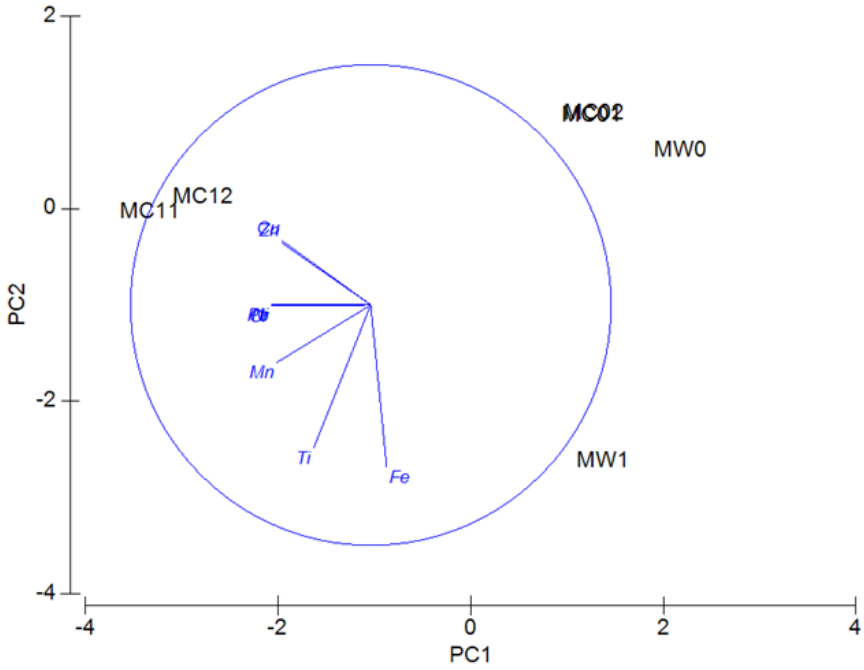


Figure 5.6: The PCA biplots illustrating the relationship between metal concentrations in surface water and crabs from uMhlathuze Estuary. M (uMhlathuze Estuary), W (Water, 0-February, 1 August), C (crabs, 0-high flow, 1-low flow or male, 2-female).

5.2.2.2. UThukela Estuary

Mean metal concentrations of chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), titanium (Ti) and zinc (Zn) in female and male crabs are presented in Table 5.4 and Figure 5.7. A significant difference was noted in all metals between high and low flow conditions. Multiple comparisons showed that there was some gender variation in the accumulation of metals in crabs. Females accumulated higher concentrations of Cr, Ni and Fe

than males (Table 5.4). During high flows, mean metal accumulation was ordered as Fe > Ti > Zn > Cu > Mn > Cr > Pb > Ni in females, while in males, metals accumulated in the order Fe > Ti > Zn > Cu > Mn > Cr > Ni > Pb. During low flows, the order of metal accumulation in females was Fe > Mn > Ni > Pb > Zn > Cu > Ti > Cr, whilst in males, accumulation was in the order Fe > Mn > Cu > Pb > Ni > Zn > Ti > Cr (Figure 5.7).

Table 5.5: Metal concentrations (mg/g) in crabs of *C. eulimene* collected in uThukela Estuary. The ANOVA result (*F* values) of the differences in metal concentration between seasons, *df* value was 3, * indicates the significant differences ($p < 0.05$).

Metal	System	F value	Gender	High flow	Low flow
Cr	Mnd	565.90*	F	9.88± 1.88	77.29±2.44
			M	3.67±0.91	71.44±0.80
Cu	Mnd	110.95*	F	44.69±6.70	179.09±8.23
			M	33.61±6.27	183.43±10.49
Fe	Mnd	13.84*	F	951.43±80.47	1531.77±221.60
			M	439.61±70.83	1166.63±131.67
Mn	Mnd	42.72*	F	40.68±6.58	229.60±33.28
			M	26.59±4.80	206.97±48.12
Ni	Mnd	298.83*	F	3.69±0.44	221.03±8.80
			M	2.96±0.34	174.4±13.26
Pb	Mnd	320.57*	F	8.26±2.55	186.86±10.03
			M	1.93±0.41	177.71±8.800
Zn	Mnd	63.60*	F	73.04±7.00	186.74±15.43
			M	47.06±7.28	177.03±3.83
Ti	Mnd	4.30*	F	84.11±9.30	109.60±18.86
			M	66.67±9.58	117.60±4.96

Mnd (uThukela Estuary), F (Female) M (Male), n (7-10 individuals pooled)

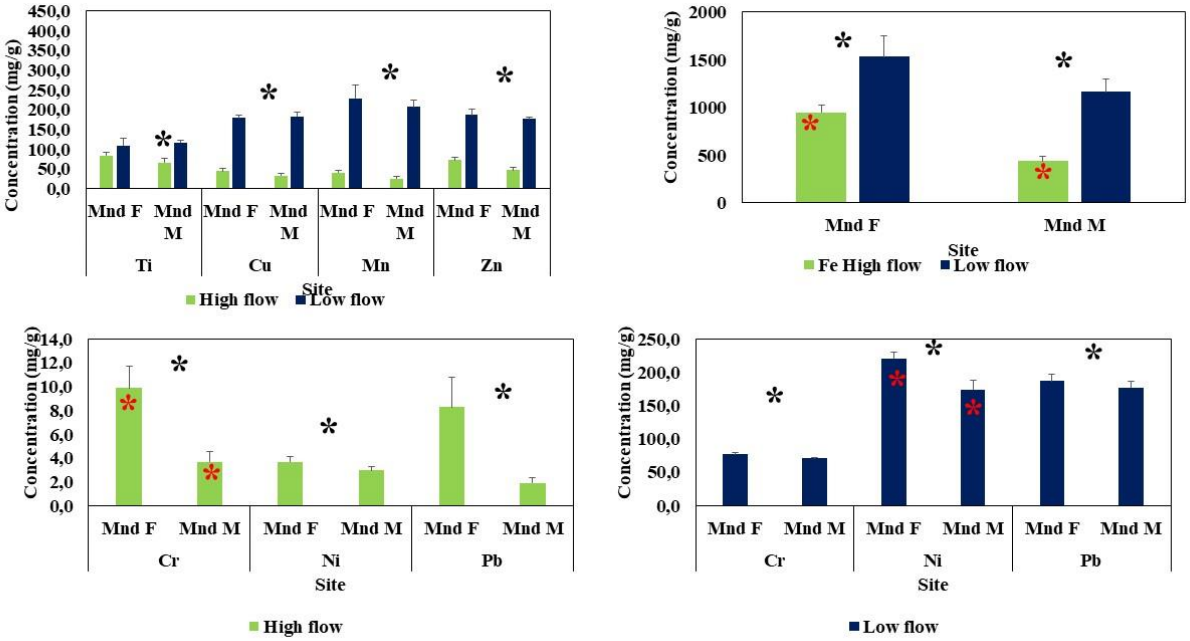


Figure 5.7: The metal concentration (mean ± SE) of the *Chiromantes eulimene* from uThukela Estuary, * indicates the significant difference between crabs collected in high and low flow, **indicates the significant difference between genders per metal.

The relationship between metal concentration in surface water samples (Mnd 3 only) and the mean metal concentration in crabs during summer and winter is presented in Figure 5.8. The two axes of PCA showed 99,1% of variation. All metals correlated with each other except for Fe. Zinc and Ni were close to each other. Chromium correlated with Ti. Lead and Cu closely correlated with each other (Figure 5.8).

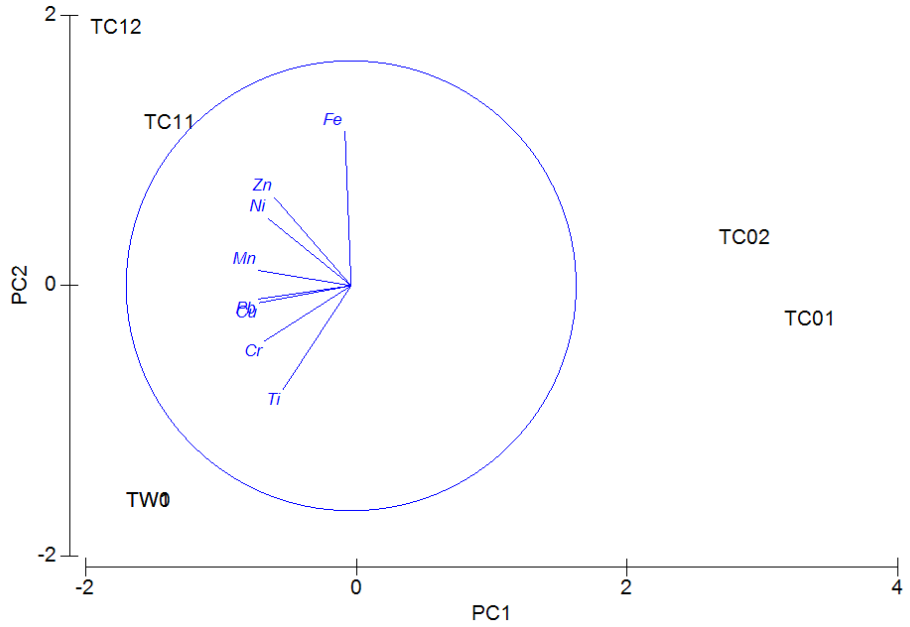


Figure 5.8: The PCA biplots illustrating the relationship between metal concentrations in surface water and crabs from uThukela Estuary, T (uThukela Estuary), W (Water, 0-February, 1 August), C (crabs, 0-high flow, 1-low flow/male, 2-female).

5.2.3. Acetylcholinesterase (AChE) activity in the crab *Chiromantes eulimene* from uMhlathuze and uThukela Estuary

Acetylcholinesterase (AChE) activity in the gills of male crabs, *Chiromantes eulimene*, collected during high and low flows from the uMhlathuze and uThukela Estuaries are presented in Figure 5.9. One-way ANOVA revealed a significant difference in the AChE activity of crabs from the uMhlathuze Estuary between summer and winter seasons (df = 1, F = 18.3, p < 0.003). Significantly lower AChE activity was measured in the gills of crabs from the uMhlathuze Estuary collected in summer (26.73± 1.84), compared to the winter samples (158.09 ± 30.63; Figure 5.9). The mean AChE activity in the gills of crabs from the uThukela Estuary was 128. 71 ± 7.17 and 110.23 ± 14.32 in summer and winter, respectively. There was no seasonal difference in AChE activity in the gills of crabs from the uThukela Estuary (df =1, F = 1.3, p > 0.05) (Figure 5.9).

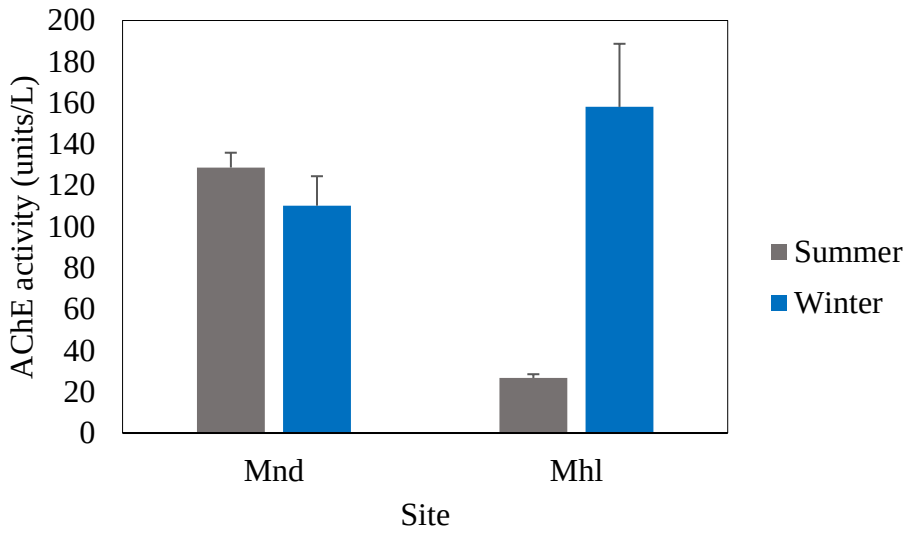


Figure 5.9: Acetylcholinesterase (AChE) activity (mean ± SE) at uMhlathuze and uThukela Estuaries.

5.3. DISCUSSION

5.3.1. Accumulation of metals in surface water samples

5.3.1.1. uMhlathuze River-Estuary

An increasing metal concentration from riverine to estuarine sites of Mn, Pb and Si indicates a continuous input along the river gradient. The highest Mn, Pb and Si concentrations in water recorded at Mhl 3 (estuarine site) implies that the river mouth is probably the sink for these metals in the estuary. Lower levels of other dissolved metals at Mhl 3 may be attributed to the natural attenuation factor along the river gradient. Variations in metals concentrations in different seasons may be attributed to different hydrological factors.

These results were compared with the existing water quality guidelines for freshwater and marine water (Table 5.5). The concentrations of Al, Cu, Pb and Zn exceeded DWAF guideline values for freshwater use during all seasons (Table 5.5). Levels of Cr exceeded the DWAF threshold value for freshwater use (12 µg/L) at Mhl 2 during August only. The levels of Cr, Cu, Ni, Pb obtained in Mhl 3 were above the DWAF threshold limits for the marine environment (Table 5.5) during all seasons, except for the value for Pb (Mhl 3 February). This comparison is significant as the sites studied

are located in riverine and estuarine areas. Levels of Al, Cu, Cr, Fe, Mn, Ni, Pb and Zn exceeded DWAF guidelines, suggesting that anthropogenic sources mostly influenced the concentrations. Guidelines for Si and Ti are currently not available. However, high concentrations were recorded, which means that they are metals of high concern in the uMhlathuze River-Estuary. Summarised freshwater and marine environment guidelines for metals in this study are presented in Table 5.5.

Table 5.6: South African water quality guidelines. Adapted from (DWAF, 1996, DWAF, 2018b).

Metal	**Source**	**Freshwater environment**	**Marine environment**
Al	-Originating from the combustion of coal. -Effluent from acid mine drainage, industries such as aluminium smelters, paper, metal construction, leather, and textile industries.	pH<6.5 – 5 pH > 6.5 -10 µg/L	Not available
Cd	-Natural weathering and erosion by rock. -waste from the manufacturing of plastics, glass, alloys. -Wastewater streams from mining operations, agriculture, urban areas and various industries.	SW- 0,15 MW- 0.25 HW -0.35 VHW 0.40 µg/L	0,12 µg/L
Cr	-Weathering and erosion of rocks, incineration and burning of fossil fuels. -Effluent from pigment production and tanning. -WWTPs effluents, landfill site leachate. -Effluent from mines.	12 µg/L	2 µg/L
Cu	-Waste from metal plating operations, jewellery, ornamental industries, electrical wiring industries, antifouling paints. -Leachate seepage, dumping of demolition material. -Stormwater from the landfill sites, informal settlement.	SW – 0.3 MW- 0.8 HW – 1.2 VHW 1.4 µg/L	3 µg/L
Fe	-Weathering of sulphide ores and igneous, sedimentary, and metamorphic rocks -Leaching from sandstones -Burning of coals and coke.	Not available	Not available

	-Effluent from acid mine drainage, sewage, mineral processing. -Landfill leachates. -Effluent from industries such as chlor-alkali, fungicide, petro-chemical and household chemical industry.		
Mn	-It is naturally found in salts and minerals. -Effluent discharge from industrial and agricultural sectors.	180 µg/L	Not available
Ni	-Geomorphological processes. -Atmospheric deposition -Effluents from landfill sites. -Waste from its application in alloy, stainless steel production, production of jewellery, nickel containing batteries and electric industry.	Not available	5 µg/L
Pb	-Weathering of rock, forest fires. -Emission from moto vehicles. -Effluent from the sewage treatment plant, dumping sites, industries and agricultural activities (i.e. impurities in fertilizers, metal-based pesticides, compose and manure). Metal plating and petroleum additives.	SW- 0.2 MW – 0.5 HW -1.0 VHW – 1.2 µg/L	2 µg/L
Si	-Weathering of silicate material. -Emanating from volcanic sources.	Not available	Not available
Ti	-Effluent from domestic and industries. -Direct disposal of Ti containing products.	Not available	Not available
Zn	-Weathering, erosion, deposition, and volcanic eruptions. -Effluent from pulp and paper manufacturing industries. -WWTPs and stormwater from industrial and domestic effluents. -Agricultural effluents. -Waste from paint, steel galvanisation, production of stainless steel, dye and Zn metal works.	2 µg/L	20 µg/L

SW-soft water; MW –medium water, HW – hard water, VHW- very hard water

5.3.1.2. uThukela River-Estuary

High concentrations of Al, Cr, Fe, Mn, Ni, Pb and Zn at Mnd 1 (upstream of the sewage plant) may be consequences of direct dumping of materials composed of metals, and surface runoff from dumping and or landfill sites. High concentrations of Cu, Si and Ti at Mnd 2 (downstream of the sewage plant) suggest that effluent from municipal waste and inefficiency of the WWTWs may be the major sources of metal contamination. The highest levels of Al, Cu, Fe, Ni, Mn, Si, Pb, Ti and Zn, recorded at Mnd 3, suggests that the mouth of the estuary may be the sink for these metals. Elevated concentrations of metals in this system may be a direct consequence of surrounding industrial and agricultural activities in the catchment.

These results were compared with the existing water quality guidelines for freshwater and marine water (Table 5.5). The concentrations of Al, Cu, Mn, Pb and Zn obtained at Mnd 1 and Mnd 2 exceeded the DWAF guidelines for freshwater, across both seasons. The levels of Cr at Mnd 1 and 2 exceeded DWAF threshold limits for freshwater use during August and October; February and August respectively. The concentrations of Cr, Cu, Ni, Pb and Zn recorded in Mnd 3 (all seasons) exceeded the DWAF threshold limits for marine environments.

Correlation of all metals except for Si and Ti was observed. Contamination by titanium could be due to a combination of untreated waste, leachate seepage, effluent from chemical, textile, paper and pulp industries in the Mandeni area and other natural processes. The concentrations of Mn and Zn in the sludge tended to decrease downstream of the river. Higher concentrations recorded at Mnd 1 points to the influence of industries. Si and Ti had higher concentrations at Mnd 3, corresponding to higher salinities that sometimes occur at this site. Salinity has been reported to increase metal concentrations in the dissolved phase (Mzimela and Izegaegbe, 2021).

5.3.2. Passive accumulation of metals in crabs *Chiromantes eulimene*

5.3.2.1. UMhlathuze Estuary

The higher accumulation of all metals in males compared to females may be due to the fact that males are more active than females. However, the lack of significant discrepancies between sexes, particularly with regard to accumulation of essential metals, may be attributed to their equal demands due to their significant role in metabolic processes. Higher concentrations of Ni were recorded in the uMhlathuze Estuary crabs, with males accumulating higher amounts than females. Izegaegbe *et al*., (2020) also measured a high concentration of Ni in the mud crab *Paratylodiplax blephariskios*. High levels of Ni and Cr have been reported for their potential toxicity in estuarine organisms due to levels which exceeded TEL and ERL sediment guidelines (adopted from Australia, New Zealand, and USA) in Richards Bay Harbour sediment (Izegaegbe *et al*., 2020). Ni and Cr levels in surface water samples in the present study were above the DWAF threshold limits for marine water. Thus, Ni and Cr are metals of concern.

5.3.2.2. UThukela Estuary

At uThukela Estuary, gender variation was observed in the accumulation of Fe, Cr and Ni in crabs, where females accumulated higher amounts. A higher accumulation of essential metals such as Fe may be due to its physiological importance in decapod crustaceans. Lead presented in higher concentrations in females than in male crabs during both seasons. Lead is known to play no role in biological functions in crabs, and therefore it could not be regulated through metabolic processes, only being accumulated. Lead tends to be detoxified by metallothioneins or phosphate granules and stored permanently in tissues (Saher and Siddiqui, 2017). High concentrations of copper were recorded in this study. Copper is one of the micronutrients that is essential for crustaceans in terms of blood pigment (haemocyanin) and exoskeleton hardening, thus slightly elevated levels of Cu could signify the normal regulation of metabolic activities, however, Cu tends to be toxic at higher concentrations.

5.3.3.3. Comparative analysis

Mean metal concentrations for Al and Mn in this study were higher than those recorded by Mzimela *et al*., (2003) and Mzimela *et al*., (2014). Concentrations of Cr, Pb, Zn and Cu recorded in the present study were lower than those recorded in other studies (Table 5.6). Levels of Fe in this study correlated with those recorded by Mzimela *et al*., (2014) at the uMhlathuze Estuary. The difference between systems with regards to metal accumulation is governed by various factors such as seasonality, hydrology, locality, physicochemical parameters, and anthropogenic inputs within that studied system. In the uThukela River-Estuary, few studies have focused on the accumulation of metals. However, Vetrimugan *et al*., (2019) reported high concentrations of Cr, Cu, Ni, Cd and Zn in the sediment of the uThukela Beach (Table 5.6.).

These results showed the accumulation of Cr, Cu, Fe, Mn, Ni, Pb, Ti and Zn in males and females of *C. eulimene* from the uMhlathuze and uThukela Estuaries. There were higher concentrations of Mn in females than in male crabs from the uThukela Estuary. These findings agree with those of Chen *et al.,* (2015), who found that female crabs had higher concentrations of Mn; this is attributed to the nature of rock crabs, which carry externally fertilized eggs for at least one month during their reproduction period, and thus consume a more Mn-enriched diet to meet energy demands during this stage. The study found inter-sexual differences in the Fe, Ni and Cr concentrations in the crabs, with females accumulating higher concentrations than males in the uThukela Estuary. This is contrary to the results of Saher and Siddiqui *et al.,* (2017) who demonstrated that male fiddler crabs accumulated higher concentrations of Fe, Ni and Pb than females, but comparable to other studies that have been conducted in the uMlalazi Estuary, Durban Harbour, Richards Bay Harbour, Bhizolo Canal, Mzingazi Canal and the uMhlathuze Estuary (Table 5.6). The variation in metal accumulation in this study and others may be attributed to different localities coupled with surrounding activities, resulting in metal differences to bioavailability.

Table 5.7: Comparison of metal concentration in surface water (µg/L), sediment (mg/g) and crabs (mg/g) samples

Matrix	System	Al	Cr	Cu	Fe	Mn	Ni	Pb	Zn	References
Water	uMhlathuze Estuary	990	48	39	907	48	-	130	66	(Mzimela *et al.*, 2014)
	Richard Bay	-	-	117	-	-	-	39	51	(Adeleke *et al*., 2020)
	Durban Bay	-	-	141	-	-	-	59	-	
	uMlalazi Harbour	-		113	-	-	-	61	163	
Sediment	uMhlathuze Estuary	18677	24	51	20607	13	-	45	46	(Mzimela *et al*., 2014)
	Richards Bay	-	-	16	-	-	-	11	26	(Adeleke *et al*., 2020)
	Richard Bay CoalTterminal	14873	104	16	19298	527	16	15	38	(Izegaegbe et al., 2021)
	uMlalazi Estuary	-	-	36	-	-	-	33	56	(Adeleke *et al*., 2020)
	Durban Harbour	-	-	16	-	-	-	12	39	
	uMhlathuze Estuary	3029	16	3	2949	39	12	2	2	(Majola *et a*l., 2020)
	Bhizolo Canal	8773	27	6	8281	113	22	4	16	
	Tugela Beach	-	378	71	6291	97	36	11	71	(Vetrimurugan *et al*., 2020)

Crabs										
*Chiromantes eulimene, g*ills	Bhizolo Canal	2096	399	107	3074	74	242	23	220	(Majola *et al*., 2020)
	uMhlathuze Estuary	245	392	83	1675	54	235	14	122	
Chiromantes eulimene, pincers	Bhizolo Canal	2251	180	35	3032	50	107	4	117	
	uMhlathuze Estuary	56	56	14	235	16	32	2	33	
Chiromantes eulimene, digestive glands	Bhizolo Canal	1480	339	154	2643	76	203	9	141	
	uMhlathuze Estuary	391	326	91	2263	67	272	13	82	
Chiromantes eulimene, carapace	Bhizolo Canal	1499	317	69	2481	78	192	13	100	
	uMhlathuze Estuary	136	311	47	1555	59	247	8	52	
Paratylodipax blephariskios	Bhizolo Canal	10684	54	99	8919	2287	35	29	126	(Izegaegbe *et al*., 2021)
	Richard Bay Coal Terminal;	7090	46.5	77.4	7364	800	25	17	101	
	Mzingazi Canal	7605	56	73	7212	1256	27	31	106	

Dotilla fenestrata	Richards Bay	-	-	28	-	-	-	1	10	(Adeleke *et al*., 2020)
	uMlalazi Estuary	-	-	19	-	3	-	7	-	
	Durban Harbour	-	-	83	-	-	-	2	10	

5.4.3. Acetylcholinesterase (AChE) activity as biomarker of exposure and effects to contaminants in the crabs (*Chiromantes eulimene*) from uMhlathuze and uThukela Estuary

Contaminants such as microplastics, pharmaceuticals, pesticides, ENMs and metals can be present in various aquatic compartments in varying concentrations depending on their usage and exposure route. The ecotoxicological modes of actions of these contaminants are not well understood. Various ecotoxicological tools are employed for rapid detection to predict the toxicity of these contaminants in aquatic ecosystems. The biomarker acetylcholinesterase (AChE) was adopted for this study to predict early signals of toxicity.

Findings revealed that AChE activity was reduced in the gills of crabs from the uMhlathuze and uThukela Estuaries. In general, AChE inhibition is known to be caused by exposure of organisms to various organic (e.g., atrazine, carbamazepine) and inorganic (e.g., cadmium, copper) contaminants (Juhel *et al*., 2017, Haque *et al*., 2019, Perić and Burić, 2019, Parra *et al.*, 2021). This study found various contaminants in the uMhlathuze and uThukela systems, including microplastics, pharmaceuticals, pesticides and metals, and reduction of AChE activity in *Chiromantes eulimene* could be due to its exposure to these contaminants. Reduction of AChE activity is mainly due to the fact that contaminants bind to a functional group of enzyme, subsequently leading to enzyme activity inhibition and further overstimulation of the acetylcholine (ACh) receptors (Gusso-Choueri *et al*., 2015). This results in changes to the neurotransmission, coupled with movement impairment, respiratory failure, reproduction failure, loss of prey capture ability and hypertension (Gusso-Choueri *et al*., 2015, Deidda *et al*., 2021).

Literature reports that the high affinity of Cu to sulphur donor groups can lead to AChE reduction (Gomes *et al*., 2011). Given the high concentrations and occurrence of Cu in nano and ionic form in this study, one can deduce that Cu is a potent inhibitor of AChE activity. It was difficult to compare these results with other studies because there are limited studies of this nature in these regions, but there have been some studies confirming AChE inhibition activity elsewhere in the world. For example, Juhel *et al*., (2017) investigated the toxicity of emerging contaminants (carbamazepine, bisphenol

A and atrazine) in the green mussel (*Perna viridis*) and found that AChE activity was strongly inhibited by carbamazepine and atrazine. Yu *et al*., (2018) exposed the crab *Eriocheir sinensis* to different concentrations of microplastic particles (polystyrene) for 7 days to assess their effect on oxidative stress in the liver, and the findings showed a reduced activity of AChE in crab tissue. Gusso-Choueri *et al*., (2015) reported the reduction of AChE in the liver and muscle of *Cathorops spixii* due to exposure to Pb, Mn, Zn, and Cr. Parra *et al*., (2021) reported that Cd, microplastics and their mixture causes neurotoxicity in the gills and digestive glands of the Asian clam, *Carbicula fluminea*. In addition, neurotoxic effects reflected as AChE reduction were observed in gills of mussels, *Mytilus galloprovincialis*, which were exposed to CuONPs (10 µg/L) for 15 days (Gomes *et al*., 2011). These findings demonstrate that exposure of organisms to various mixtures of contaminants may lead to the reduction of AChE activity.

CHAPTER 6

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary and conclusions

The aim of this study was to examine the extent of occurrence by selected contaminants of emerging concern (CECs) in the uMhlathuze and uThukela aquatic systems using the source-to-sea approach. The study had three objectives, and a summary of major findings per objective are provided in the sections that follow.

Objective 1: Qualitative screening of the occurrence of pharmaceuticals, lifestyle drugs and pesticides in surface water samples collected from uMhlathuze and uThukela River-Estuary systems

The presence of target synthetic organic compounds in the surface water of the uMhlathuze and uThukela systems was confirmed using GC-MS. Thirty-eight compounds belonging to different classes of pharmaceutical and lifestyle drugs were detected at the uMhlathuze River-Estuary, and 20 compounds were found in the uThukela River-Estuary, belonging to 13 therapeutic compound classes. Twenty-seven compounds belonging to three classes of pesticides were found. The sites with the highest detection of pesticides and pharmaceuticals were Mhl 2 and Mnd 2, respectively, in uMhlathuze and Mandeni Rivers. This was probably caused by effluent discharges from wastewater treatment works and /or sewage plant. Across all sites, pharmaceuticals and lifestyle drugs, including their metabolites, were detected most frequently. This included cocaine, benzoylecgonine, threoaminobupropion, hydroxybupropion and erythroaminobupropion, threoaminobupropion and hydroxybupropion. In the case of pesticides, terbuthylazine, terbuthylazine desethyl, sebuthylazine desethyl, atrazine and 4-deethyl atrazine were commonly detected.

Objective 2: To characterize microplastics and engineered nanomaterials (ENMs) in surface water samples collected from the uMhlathuze and uThukela River-Estuaries during low and high flow conditions

There was a general decrease of microplastic concentration from riverine to estuarine sites when seasons were combined. Morphologically, fibres dominated the microplastics over films in all samples. Overall the microplastics comprised various polymers including poly(propylene) (PP), polycarbonate (PC), polystyrene (PS), nylons (NY), low-density poly(ethylene) (LDPE), high-density poly(ethylene) (HDPE), poly (ethylene terephthalate) (PETE), polyvinyl chloride (PVC), acrylonitrile butadiene styrene (ABS), latex (LX), nitrile (NT), poly(ethylene vinyl acetate) (EVA), poly(ethylene vinyl acetate) + poly (cyclohexanone) (EVA + poly(cyclohexanone)), poly (butyl acrylate) (PBA), polyurethane (PU) and polyethylene (PE). The presence of various polymer forms pointed to varying anthropogenic sources, possibly industrial and agricultural activities, as most of the observed polymers are extensively applied in these industries. There was a seasonal variation of microplastic density in both river systems.

The commonly detected engineered nanomaterials were titanium dioxide (TiO_2), silicon dioxide (SiO_2), zinc oxide (ZnO), iron oxide (Fe_2O_3) and copper oxide (CuO) nanoparticles; this was confirmed through elemental mapping. TiO_2 and SiO_2 NPs are known to dominate the South African market of nano-enabled products due to their use in photocatalytic and coating applications, respectively. As such, these forms of pollutants were likely released from products and industrial operations as waste. This study is among the first to report the occurrence of nanopollution in environmental water samples in South Africa.

Objective 3: Examination of bioaccumulation and biomarker response (acetylcholinesterase enzyme) in order to assess contaminant exposure to the mud crabs *Chiromantes eulimene* in the uMhlathuze and uThukela Estuaries

The amount and type of metal concentrations varied between the systems in different seasons, depending on anthropogenic activities along the sites. The levels of Al, Zn, Cr, Ni, Cu and Pb exceeded freshwater and marine DWAF guidelines. Elevated levels

of metals in this study were attributed to various industrial and agricultural activities dominating within these systems.

Metal accumulation in crabs varied between seasons. Chromium, Ni and Fe accumulation was gender dependent, with accumulation in females higher than in males in the uThukela Estuary. The gills of crabs collected in summer from the uMhlathuze Estuary showed extremely reduced acetylcholinesterase (AChE) activity compared to those collected in winter. However, at the uThukela Estuary, lower activity was noted in winter compared to summer. This is probably due to varying levels of pollution of the habitats between these two systems. Moreover, agricultural and industrial activities within these two systems are probable sources of contaminants which further inhibit AChE levels. The gills from male crabs appear to be a useful source of enzymes to study the effects of exposure to contaminants. Contamination of contaminants of emerging concern in these systems was confirmed through biomarker analysis. This study serves as a baseline for the occurrence, bioaccumulation, and potential effects of contaminants of emerging concern in these regions.

6.1.2. Recommendations

This study found organic contamination in surface water through qualitative analysis. Therefore, future studies should quantify concentrations even in sediment and organisms. Moreover, attention must be given to organic compounds known as endocrine disrupting compounds (EDCs). Information on these would reduce data gaps and aid in the prioritization and risk assessment of organic compounds. More research should also be directed towards the possible contamination of sediments, and the bioaccumulation rate of microplastics and engineered nanomaterials in freshwater and estuarine organisms in northern KwaZulu Natal systems. More species, with different uptake routes and from different trophic levels, must be used to monitor the bioavailability of the contaminants of emerging concern in both freshwater and estuarine ecosystems.

The presence of various contaminants of emerging concern in surface waters and accumulation coupled with effects in organisms has been confirmed. Therefore, more

frequent monitoring studies by relevant government institutions, researchers, and industries operating in the northern KwaZulu Natal are recommended.

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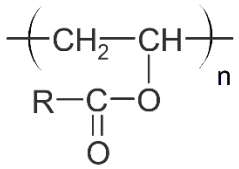
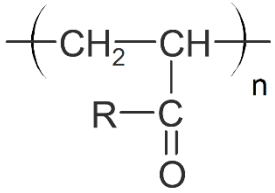
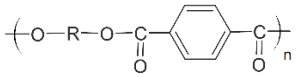
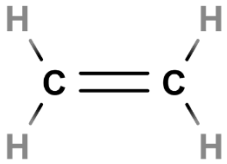
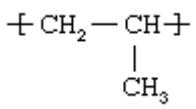
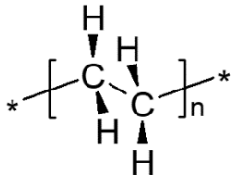
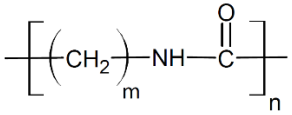
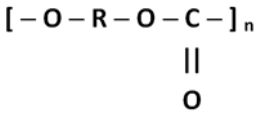
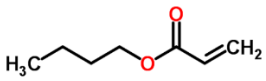
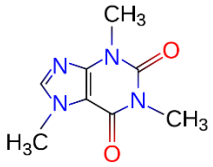
APPENDICES

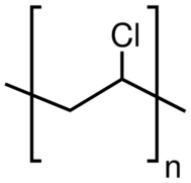
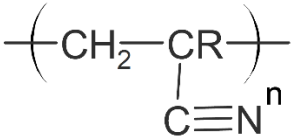
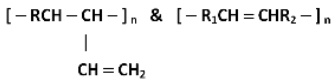
Appendix A1: List of important absorption bands representing vibration modes and assignment for the ATR FT-IR spectra for the present identified polymers.

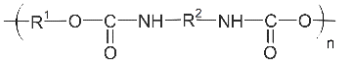
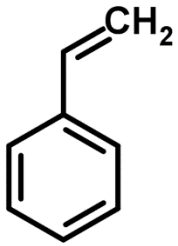
Polymer	Common application/uses	Chemical structure	Absorption band	Assignment
Poly(ethylene vinyl acetate) (EVA)	Product packaging, a wide range of industrial sectors (footwear, medical and agriculture)		2917, 2915, 2848, 1470, 1370	C–H stretch
			1740	C = O
			1469	CH_2 bend, CH_3 bend
				C(=O) O stretch
			1239, 1241	C–O stretch
			1020	
			718	CH_2 rock
Poly(ethylene vinyl acetate) (EVA) + poly (cyclohexanone)	Product packaging, a wide range of industrial sectors (footwear, medical and agriculture)		2917, 2849	CH_2

			1024	O-C
			1242,1058	[C-C (O)-O]
			1736	C=O
			1375, 1467, 1451	C-H
			2849, 2017	CH_2
			720	C-H (CH_2)
			960	C-C
			1713	C=O
Poly (ethylene terephthalate) (PETE)	Bottles, strapping, fabric manufacturing.		1712, 1713	C=O
			2965,2923,2853	CH_2
			1615, 1505, 1577, 1580,1454	C=C aromatic ring
			1470, 1471, 1408, 1410,1371, 1372, 1339, 1340	C-H
			1016, 970, 870, 871,719	C-H aromatic ring
			1098,1094,1118,1241	O-C
			847	(C-H(-CH_2)-)
			2908, 2962,2917	CH_2
Low-density poly(ethylene) (LDPE)	Plastic bags, six-pack rings, bottles, netting, drinking straws, stretch wraps, agriculture.		1470, 1377	CH_3 bend
			1375, 1467, 1462	CH_2 bend
			2915, 2917, 2845	CH_2 stretch

			717	CH_2 rock
Poly(propylene)(PP)	Rope, bottle caps, gear, strapping		1455, 1358	C-H(-CH_2)-)
			1375, 1436	C-H(-CH_3)-)
			1166	CH bend, CH_3 rock, C-C stretch
			898, 840, 997	CH_2 rock, C-CH_3 stretch
			808, 1153	CH_2 rock, C-C, C-CH stretch
			2915, 2950, 2838	C-H
High-density poly(ethylene), (HDPE)	Milk and juice jugs		1462, 1471, 1472, 2913, 2847	CH_2 bend
			2915,2845	C-H stretch
			730,717	CH_2 rock
Nylon (NY)	Fishing nets, rope		1637	N-H bend, C=O
			1538, 1537, 1260	N-H bend, C-N stretch
			1260	C-N bend, N-H
			1474	CH_2, N-H
			1464, 1372, 2931, 2860, 1199	CH_2 bend
			3298, 3300, 3078	N-H stretch

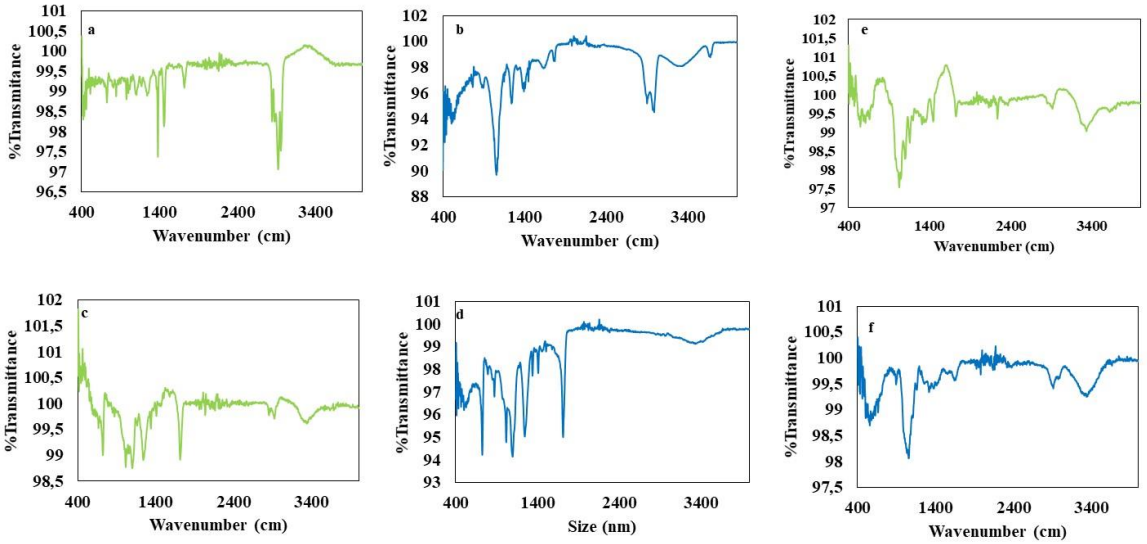
			720, 729	N-H bend
			2932, 1462, 1438, 1371, 1198, 1169	C-H stretch
			1634	C=O stretch
			1417	CH_2 C-O
Polycarbonate (PC)	Agricultural, electronics and automotive industries,		1409, 1503, 1454, 1593	C-C Aromatic ring stretch
			1364, 2872, 2966	CH_3 stretch
			1158, 1218, 1013	C-O stretch
			1464, 1386, 2853	CH_2
			1768	C=O bend
			828, 886, 3057, 3040	Aromatic CH
Poly (butyl acrylate) (PBA)	Encapsulation of herbicides, medical devices, paints.		1379, 2959, 2869,	CH_3
			1241, 1158	C-O stretch
			1065, 848	C-H butyl group
			716, 716, 731, 1447, 1395,	CH_2
			1728	C=O
			1461, 1024	C-C
Latex (LX)	Gloves, rubber band products		1376	CH_3 bend

			2960, 2920, 2855	C-H stretch
			1167	C=C stretch
			1447	CH_2 bend
			1099	C-C bend
Polyvinyl chloride (PVC)	Plastic film, bottles, pipe, containers		2970	
			1427	CH_2 bend
			1331, 1255	C-H bend
			966	CH_2 rock
			616, 630	C-Cl stretch
Nitrile (NT)	Protective gloves, fuel and oil handling hoses, seals and grommets.		1360, 1197, 1440	CH_2 bend
			2917, 2849	=C-H stretch
			1605	C=C stretch
			2237	C-N stretch
			967	=C-H bend
			2922	C-H stretch
Acrylonitrile butadiene styrene (ABS)	Automotive industries, Plastic sheet, vacuum forming		1452	CH_2 bend
			1494	Aromatic ring stretch

			759	Aromatic C-H, =CH bend
Polyurethane (PU)	Medical and agricultural uses, building, construction,		1451	CH_2 bend
			1531	C-N stretch
			1223	C(=O)O stretch
			2865	C-H stretch
Polystyrene (PS)	product packaging, laboratory equipment, construction, aquaculture, fisheries.		1492, 1601	Aromatic ring stretch
			1451	CH_2 bend
			1027	C-H aromatic ring bend
			3024	C-H aromatic ring stretch

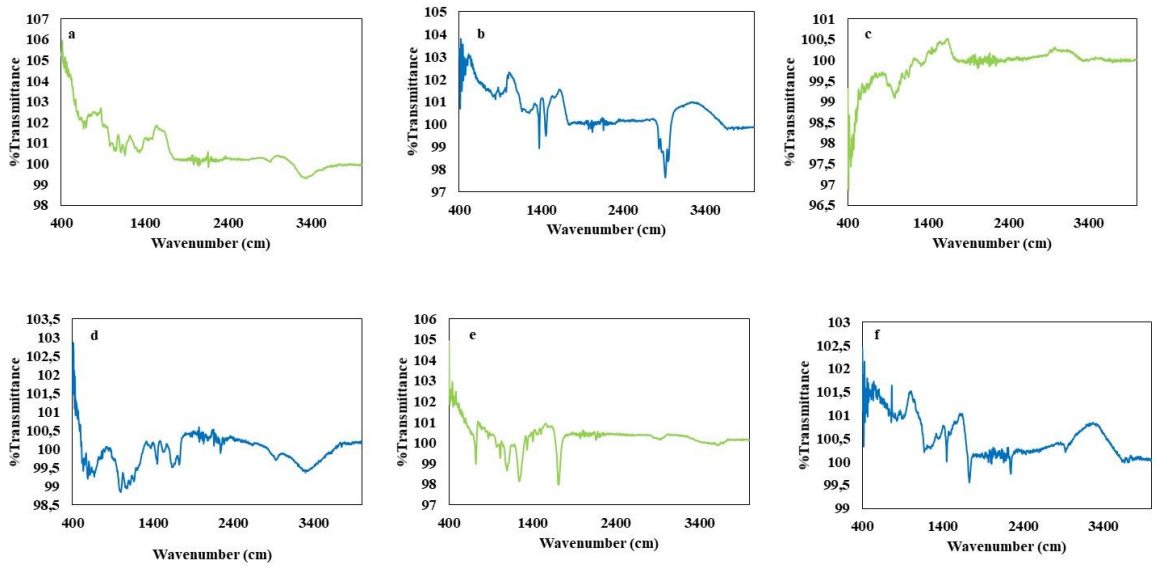
Absorptions bands are adopted from Jung *et al*, 2018 and Asensio *et al*., 2009, structures were adopted from: http://polymerdatabase.com/class%20index%20GL.html (Accessed 23/09/ 2021).

Appendix A2



Appendix A2: FTIR spectra in water samples collected from uMhlathuze (Mhl) River-Estuary: Mhl 1 February (a), Mhl 1 October (b), Mhl 2 February (c), Mhl 2 October (d), Mhl 3 February (e) and Mhl 3 October (f).

Appendix A3



Appendix A3: FTIR spectra in water samples collected from uThukela (Mnd) River-Estuary: Mnd 1 February (a), Mnd 1 October (b), Mnd 2 February (c), Mnd 2 October (d), Mnd 3 February (e) and Mnd 3 October (g).

Appendix A4

Appendix A4: Pharmaceutical compounds detected in water samples of uMhlathuze and uThukela River-Estuary systems and respective median toxicity levels to aquatic species.

PHARMACEUTICAL GROUPS	COMPOUND	COMMON NAME	USE/ACTIVITY	AQUATIC SPECIES AND LC/EC_{50}
Analgesic	Acetaminophen	Paracetamol	Nonsteroidal anti-inflammatory activity	*Daphnia magna;* 48 hr EC_{50}: 34.99 mg/L (Minguez *et al*., 2016)
	Hydrocodone	Lortab, Vicodin, Hycodan	Pain reliever through inhibition of nociceptive pain reflexes.	No data
	Tramadol	Ultram	Pain reliever for ongoing pain from moderate to moderately severe pain	*Carrassius auratus:* 96 hr LC_{50}: 6.2 mg/L (Mankes and Charles, 2017)
Angiotensin blocker	Irbesartan	Avapro	Angiotensin receptor blocker that prevents anticipated kidney problems, cardiovascular diseases, hypertension.	*Daphnia magna*: 48 hr EC_{50}: >100 mg/L. (Minguez *et al*., 2016)
	Losartan	Cozaar	Angiotensin II receptor antagonist utilized in therapies of hypertension and diabetic nephropathy.	*Lemna minor*: 168 hr EC_{50}: 64.6 mg/L (Godoy *et al*., 2015)

	Telmisartan	Micardis	Angiotensin II receptor blocker against symptoms of hypertension and other cardiovascular diseases.	No data
	Valsartan	Diovan	Angiotensin II receptor antagonist against high blood pressure and heart failure.	*Daphnia magna:* 48 hr EC_{50}: >100 mg/L (Minguez *et al*., 2016)
Antibiotic	Sulfamethazine	Sulfamethazine	An antimicrobial agent that stops bacterial growth.	*Daphnia magna*: 48 hr EC_{50} :174.4 mg/L (Kim *et al.*, 2007)
Antiarrhythmic	Disopyramide	Trade names (Norpace and Rythmodan)	Treats life-threatening ventricular arrhythmias.	No data
Antiemetic drug	Mitoclopramide	Reglan	Dopamine antagonistic drug that prevents nausea and vomiting.	No data
Antiepileptics	Carbamazepine	Tegretol	Prevents epilepsy, trigeminal neuralgia, and bipolar mania	*Ceriodaphnia dubia*: 48 hr EC_{50} :77.7 µg/L (Ferrari *et al*., 2003)
	Gabapentin	Neurontin	Anti-seizure medication	*Daphnia magna:* >100 mg/L (Minguez *et al.*, 2016)
	Phenytoin	Dilantin	Generally used for management of partial and complex epilepsy diseases.	No data

Antifungal	Fluconazole	Diflucan	Bis triazole agent that prevents fungal infections.	*Gobiocypris rarus:* 72 hr LC_{50} :4.91 mg/L (Zhu *et al*., 2014a)
Antihistamines	Cetirizine	Zyrtec	Second-generation histamine H1 antagonist widely used for relieve of various allergies.	*Daphnia magna*: 48 hr EC_{50}: >100 mg/L (Minguez *et al.,* 2016)
	Chlorpheniramine	Maleate	First-generation histamine H1 antagonist widely used for relieve of various allergies.	Algae species: 72 hr EC_{50} :5.05 mg/L (Guo *et al*., 2016)
	Diphenhydramine	Benadryl, Genahist, Sominex.	First-generation H1 receptor used for the treatment of allergies.	*Danio rerio*: 5 days LC 50: 16.6 mg/L (Mankes and Charles, 2017)
	Fexofenadine	Allegra	Second-generation antihistamine drugs that are effective in treating allergy symptoms.	*Daphnia magna*: 48 hr EC_{50}: 58 mg/L (Golovko *et al.*, 2020)
Antihelmintics	Flubendazole	Flutelmium	Clinically effective in treating infections of gastrointestinal parasites	*Daphnia magna*: 48 hr EC_{50}: 0.045 mg/L (Wagil *et al*., 2015)
Antipsychotic	Sulpiride	Dogmatil	Widely used as a psychotherapeutic agent against a wide range of psychiatric illnesses.	No data

Antiretroviral	Nevirapine	Viramune XR	Non-nucleoside reverse transcriptase inhibitor, which stops replication of the virus within the cells.	No data
Antiviral agent	2-aminoadamantane	Symmetrel	Used for treatment of illnesses like influenza A, and the symptoms of Parkinson's disease.	No data
Beta-receptor blockers	Acebutalol	Sectral	beta-adrenoceptor antagonists are effective in the management of cardiovascular disorders.	*Daphnia magna*: 48 hr EC_{50}: >100 mg/L (Minguez *et al.*, 2016)
	Atenolol	Tenormin	Second-generation beta-1-selective adrenergic antagonist used for the treatment of cardiovascular diseases.	*Daphnia magna*: 48 hr EC_{50}: >100 mg/L (Minguez *et al*., 2016)
	Bisoprolol	Zebeta	Cardioselective β1-adrenergic antagonistic agent effective for treatment of various cardiovascular related disease.	*Daphnia similis*: 48 hr $LC_{50:}$ 93.1 mg/L (Godoy *et al.,* 2019)
	Sotalol	Betapace	Widely used for the treatment of atrial and ventricular arrhythmias.	*Daphnia magna*: 48 hr EC_{50}: >100 mg/L (Minguez *et al*., 2016)

Cardiac glycoside	Convallatoxin	Corglycone	A veterinary drug used as a diuretic and cardiac stimulant.	No data
Anti-diabetic drug	Metformin	Glucophage	Potent antihyperglycemic agent that is used mostly in the treatment of type II diabetes.	*Daphnia similis*: 48 hr LC_{50}: 14.3 mg/L (Godoy *et al*., 2019)
Diuretic drug	Chlorothiazide	Diuril	Principally used to treat edema and associated diseases.	No data
Psychoanaleptics	Amphetamine	Amphetamine	Central nervous system stimulants primarily used for the treatment of attention-deficit/ hyperactivity disorder, narcolepsy, and obesity.	*Oncorhynchus mykiss*: 3 hr EC_{50}: 53.05 mM (Lilius *et al*., 1994)
	Benzoylecgonine	Benzoylecgonine	metabolite of cocaine.	No data
	Cocaine	Coca, Coke	Anaesthetic drug that enhances activities of dopamine	No data
	Ephedrine/Pseudo	Ephedrine	Stimulants used to relieve allergic rhinitis.	No data
	Erythroaminobupropion	Erythroaminobupropion	Major metabolites of antidepressant bupropion that treat major depressive disorders through inhibition of dopamine and norepinephrine	No data
	Hydroxybupropion	Hydroxybupropion		No data
	Threoaminobupropion	Threoaminobupropion		No data

Ketamine	Ketalar	Medical prescriptions are used as an anaesthetic and used to treat depression	*Daphnia magna*: 48 hr LC_{50}: 30 93 mg/L (Li *et al*., 2017)
Methamphetamine	Methamphetamine	Recreational stimulant drug of the central nervous system and primarily used for the treatment of attention-deficit/ hyperactivity disorder, narcolepsy, and obesity	No data
Mirtazapine	Remeron	Antiemetic, anxiolytic stimulant agent mainly used as an antidepressant drug.	*Daphnia magna*: 48 hr LC_{50}: 100 mg/L (Golovko *et al*., 2020)
Moclobemide	Aurorix	The antidepressant drug is primarily used to treat mental disorders through the inhibition of monoamine oxidase-A (MAO) enzyme.	No data
Nicotine	Nicotine	Recreational drugs commonly used as a stimulant and anxiolytic	*Oncorhynchus mykiss*: 96 hr LC_{50} :4 mg/L *(*Mankes and Charles, 2017)
THC-OH		Therapeutic and hallucinogenic drug that is known as the main metabolite of	No data

			tetrahydrocannabinol (marijuana).	
	Venlafaxine	Effexor	The antidepressant drug that is primarily used for the treatment of depression, social anxiety disorder, and cataplexy.	*Daphnia magna*: 48 hr EC_{50} :141.28 mg/L (Minguez *et al*., 2016)
Psycholeptics	Termazepam	Restoril	Benzodiazepine drugs are mainly used to treat anxiety disorders and insomnia.	No data
	Oxazepam	Serax	Benzodiapine drug principally used to manage anxiety and alcohol withdrawal symptoms.	No data
Phenethylamine	Methaqualone	Quaaludes	Sedative- hypnotic agent generalized for treatment of insomnia.	No data
	Methylphenidate	Ritalin	The central nervous stimulant drug used to treat attention deficit hyperactivity disorder and narcolepsy.	No data
Steroid hormone	Testosterone	Aveed	Potent androgen that is therapeutically used in androgen male circulation.	No data

LC_{50} (Lethal concentration that is fatal to 50% of the test organisms); EC_{50} (concentration observed to cause an adverse biological effect on 50% of test organism).

Appendix A5

Appendix A5: Toxicities of pesticide compounds detected in water samples of uMhlathuze and uThukela River-Estuary systems to aquatic species and respective median toxicity levels to aquatic species. LC_{50} (concentration that is fatal to 50% of the test organism; EC_{50} (concentration observed to cause an adverse biological effect on 50% of test organism).

USE/activity	CHEMICAL NAME	COMMON / TRADE NAME	ACTIVITY	AQUATIC SPECIES AND LC_{50}; EC_{50}
Herbicide	4-deethyl atrazine	Atrazine degrade	Photosynthesis inhibition	No data
	Atrazine	Atrazine	Photosynthesis inhibition	*Oncorhynchus mykiss:*48 hr LC_{50} :4.5-43 ppm (Mischke and Avery, 2013)
	Ametryn	Evik	Pre- and post-emergence control of annual grasses and broad-leaved weeds in crops	*Oncorhynchus mykiss*:96 hr LC_{50} :3.2-13.5 ppm (Mischke and Avery, 2013)
	Bromacil	Borea	Control of annual and perennial grasses and broadleaf weeds I agricultural food crops	*Daphnia magna*: 48 hr LC_{50} :121 ppm (Mischke and Avery, 2013)
	Clomazone	Clomazone	Control a variety of broadleaf weeds and grasses on agricultural crops and forests.	*Daphnia magna*: 48 hr LC_{50} :5.2 ppm (Mischke and Avery, 2013)
	Dimethachlor	Dimethachlor	It prevents seedling of shoot from growing	No data
	Hexazinone	Velpar	Photosynthesis inhibition.	*Daphnia magna*: 48 hr LC_{50}: 85-152 ppm (Mischke and Avery, 2013)
	Metolachlor	Metolachlor	Seedling shoot growth inhibitor	*Daphnia magna:* 48 hr LC_{50} :1.1-26 ppm (Mischke and Avery, 2013)
	Metribuzin	Lexone, Sencor	Photosynthesis inhibition	*Daphnia magna*: LC_{50} :35.360 ppm (48 hr) (Mischke and Avery, 2013)

	Prometryn	Caparol	Inhibits photosynthesis.	*Daphnia magna*: 48 hr LC_{50} :9.7 -18.59 ppm (Mischke and Avery, 2013)
	Propazine	Milocep, Milogard	Pre-emergence herbicide used for Photosynthesis inhibition	No data
	Sebuthylazine desethyl	Sebuthylazine desethyl	Photosynthesis inhibition	No data
	Tebuthiuron	Tebuthiuron	Inhibits photosynthesis.	No data
	Terbuthylazine		Photosynthesis inhibition	*Lemna minor*: 7 days $EC_{50:}$ 35 µg/L (Cedergreen and Streibig, 2005)
	Terbuthylazine desethyl	Terbuthylazine degrade	Photosynthesis inhibition.	No data
	Terbutryn	Terbutryn (Trade names: Prebane,Igran)	Photosynthesis inhibition.	*Chlamydomonas geitleri* 72 hr EC_{50}:7.2 µg/L
Fungicide	Thiabendazole	Mintezol	It inhibits the helminth-specific enzyme fumarate reductase on the parasite.	No data
	Azoxystrobin	Amistar	Active agent against a wide range of fungi on crops.	No data

	Carbendazim	Delsene	Minimise the impact of different fungal diseases on the different crops.	*Oncorhynchus mykiss*: 96 hr LC_{50}: 0.85-1.75 mg/L (Singh *et al*., 2016)
	Metalaxyl	Ridomil	Used to control a wide range of fungal diseases.	*Daphnia magna:* 24 hr LC_{50} :176.40 mg/L (Yao *et al*., 2009)
Insecticide	Carbofuran carbamates	Furadan	Protects a variety of agricultural crops against insects and nematodes.	*Perca flavescens*: 96 hr LC_{50} :147 (115-118 µg/L) (Murthy *et al*., 2013)
	Imidacloprid,	Marathon	Insecticidal activities against target pests on various field crops	*Daphnia magna* 48 hr LC_{50}: 10.44-64.873 ppm (Mischke and Avery, 2013)

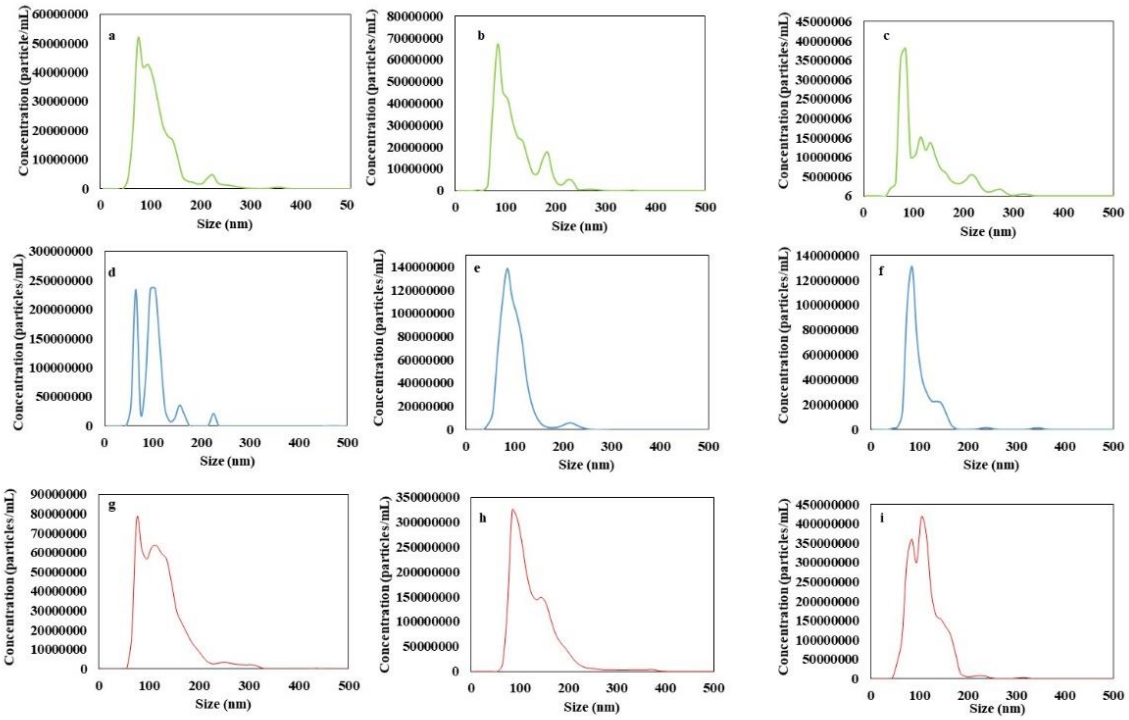
Appendix A6

Appendix A6: Particle size (mm) of extracted ENMs from water samples from uMhlathuze and uThukela River-Estuary, as determined by NTA.

SAMPLE DESCRIPTION	PARTICLES (nm)	CONCENTRATION (particles/mL)
MHL 1a	92.1 +/- 4.9	1.14e+09 +/- 6.32e+07
b	83.2 +/- 5.3	3.26e+08 +/- 1.63e+07
c	88.6 +/- 11.0	6.35e+08 +/- 1.51e+07
MHL 2 a	83.3 +/- 2.5	7.65e+08 +/- 1.48e+07
b	87.6 +/- 3.2	3.66e+08 +/- 2.52e+07
c	94.4 +/- 4.0	2.22e+09 +/- 1.02e+08
MHL 3a	78.0 +/- 2.8	4.76e+08 +/- 1.20e+07
b	80.7 +/- 0.6	1.99e+08 +/- 1.58e+07
c	106.3 +/- 3.5	2.83e+09 +/- 3.31e+08
MND 1a	85.3 +/- 5.6	3.28e+08 +/- 1.61e+07
b	72.1 +/- 3.3	1.95e+08 +/- 1.90e+07
c	88.6 +/- 11.7	5.12e+08 +/- 2.15e+07
MND 2a	119.2 +/- 2.0	7.60e+08 +/- 5.48e+07
b	89.6 +/- 3.1	8.27e+08 +/- 2.76e+07
c	100.6 +/- 15.8	1.57e+09 +/- 6.36e+07
MND 3 a	85.7 +/- 5.0	3.28e+08 +/- 6.22e+06
b	113.7 +/- 18.1	7.83e+08 +/- 5.90e+07
c	86.8 +/- 8.2	1.04e+09 +/- 2.44e+07

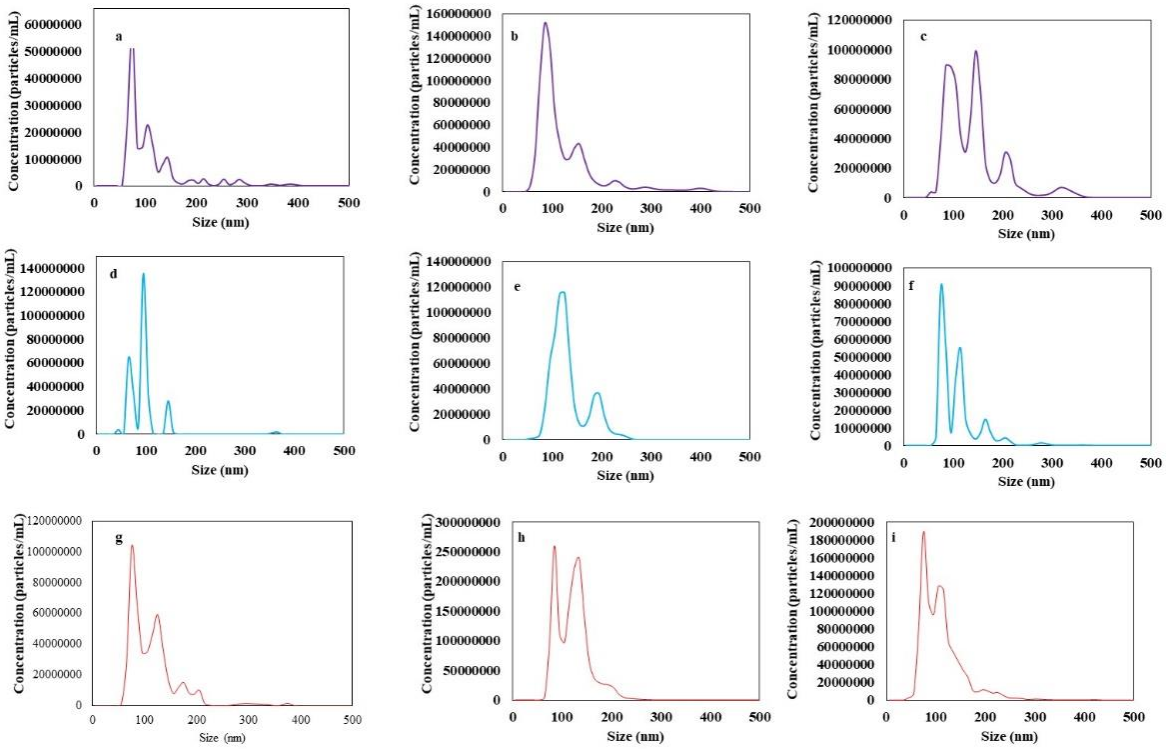
Mhl (uMhlathuze River-Estuary); Mnd (uThukela River-Estuary); a (February 2020); b (August 2019); c (October 2020).

Appendix A7



Appendix A7: NTA size distribution of particles in water samples collected from uMhlathuze River-Estuary (Mhl) : Mhl 1 August (a), Mhl 1 February (b), Mhl 1 October (c), Mhl 2 August (d), Mhl 2 February (e), Mhl 2 October (f), Mhl 3 August (g), Mhl 3 February (h) and Mhl 3 October (i).

Appendix A8



Appendix A 8: NTA size distribution of particles in water samples collected from uThukela River-Estuary (Mnd): Mnd 1 August (a), Mnd 1 February (b), Mnd 1 October (c), Mnd 2 August (d), Mnd 2 February (e), Mnd 2 October (f), Mnd 3 August (g), Mnd 3 February (h) and Mnd 3 October (i).