The Effect of Seasonal Variation on Nutrient Removal from Municipal Wastewater Using a Constructed Wetland Microcosm

By

Ndumiso Clement Talente Zulu

A Dissertation Submitted for the Requirement for the Degree of Masters of Science (Microbiology)

In the

Department of Biochemistry and Microbiology

Faculty of Science and Agriculture

University of Zululand



2018

KwaDlangezwa, South Africa

Supervisors: Dr Mathews Simon Mthembu

Prof Albertus Kotze Basson

The Effect of Seasonal Variation on Nutrient Removal from Municipal Wastewater using a Constructed Wetland Microcosm

This work is submitted in complete fulfilment for the degree of Masters (Microbiology) in the Department of Biochemistry and Microbiology, Faculty of Science and Agriculture at the University of Zululand, KwaDlangezwa, South Africa

By

Ndumiso Clement Talente Zulu

2018

Supervisor : Dr Mathews Simon Mthembu

Co-supervisors: Prof Albertus Kotze Basson

I declare that the dissertation herewith submitted for the Masters: Microbiology at the University of Zululand is my original work and has not been previously submitted for a Degree at any other University. I further declare that all the sources cited or quoted are acknowledged and indicated by means of comprehensive list of references

Ndumiso Clement Talente Zulu

I hereby approve the final submission of the following dissertation.

Dr. M.S. Mthembu

Prof. A.K Basson

This ______ day_____ of 2018, at the University of Zululand.

TABLE OF CONTENTS

DECLARATIONi
TABLE OF CONTENTS ii
ABSTRACTv
DEDICATION
ACKNOWLEDGEMENTSix
LIST OF FIGURESx
LIST OF TABLESxiv
ABBREVIATIONS
PREFACExvi
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW
1.1 INTRODUCTION
1.2 AIM AND OBJECTIVES OF THE STUDY
1.3 LITERATURE REVIEW
1.3.1 Introduction4
1.3.2 Overview and Water Scarcity in South Africa
1.3.3 Wastewater Effluent as a Source of Nutrient Pollution
1.3.4 Constructed Wetlands
1.3.5 Classification of Constructed Wetlands
1.3.6 Role of Macrophytes in Constructed Wetlands
1.3.7 The Role of Microbes in Constructed Wetlands
1.3.8 Macrophytes-Microbe's Interaction in Constructed Wetland
1.3.9 Mechanisms of Nitrogen Transformation in Constructed Wetlands
1.3.10 Mechanisms of Phosphorus Transformation in Constructed Wetland
1.3.11 Factors Affecting Nutrient Transformation in Constructed Wetlands
1.4 CONCLUSION
1.5 REFERENCES
CHAPTER 2: DETERMINATION OF NUTRIENT REMOVAL AND THE SEASONAL
EFFECT OF PHYSIOCHEMICAL PARAMETERS ON NUTRIENT REMOVAL IN A
MICROCOSMIC WETLAND 44

2.1 INTRODUCTION	44
2.3 METHODOLOGY	46
2.3.1 Constructed Wetland Microcosm Setup	
2.3.2 Water Sampling and Physiochemical Parameter Analysis	47
2.3.3 Organic Matter Analyses	47
2.3.4 Statistical Analysis	49
2.4 RESULTS AND DISCUSSION	50
2.4.1 Physiochemical Parameters Analysis	50
2.4.1.1 Potential of hydrogen (pH)	50
2.4.1.2 Temperature	52
2.4.1.3 Dissolved oxygen (DO)	54
2.4.1.4 Chemical oxygen demand (COD)	
2.4.2 Nutrient Removal	58
2.4.2.1 Ammonia	58
2.4.2.2 Nitrite	60
2.4.2.3 Nitrate	62
2.4.2.4 Phosphorus	64
2.4.3 The Effect of Physiochemical Parameters on Nutrient Removal in the Microcosms	66
2.4.3.1 Effect of pH on nutrient removal	66
2.4.3.2 Effect of temperature on nutrient removal	
2.4.3.3 Effect of dissolved oxygen on nutrient removal	73
2.4.3.4 Effect of COD on nutrient removal	76
2.5 CONCLUSION	78
2.6 RECOMMENDATION	79
2.7 REFERENCES	80
CHAPTER 3: MICROORGANISMS, POPULATION SHIFT AND THEIR ROLE IN	
NUTRIENT TRANSFORMATION IN THE MICROCOSM	85
3.1 INTRODUCTION	85
3.2. AIM AND OBJECTIVES	
3.3.1 Sample Collection	88
3.2.2 DNA Extraction	88
3.2.3 DNA Analysis	89
3.2.4 DNA Amplification	

3.2.5 DNA Purification from Agarose Gel and Sequencing	91
3.4 RESULTS AND DISCUSSION	92
3.4.1 DNA in Water Samples	92
3.4.2 Microorganisms Identified in Water Samples	97
3.5 CONCLUSION	102
3.6 RECOMMENDATIONS	.103
3.6 REFERENCES	104
CHAPTER 4: DISCUSSION, CONCLUSION AND RECOMMENDATIONS	107
4.1 INTRODUCTION	107
4.2 NUTRIENT REMOVAL	107
4.3 THE EFFECT OF SEASONAL CHANGES AND PHYSIOCHEMICAL PARAMETERS ON	
NUTRIENT REMOVAL	108
4.4 THE ROLE OF MICROORGSNISM IN NUTRIENT REMOVAL	109
4.5 CONCLUSION	110
4.6 RECOMMENDATIONS	110
4.7 REFERENCES	111
APPENDICES	113

South Africa is facing a severe water scarcity due to the exponential deterioration of natural water bodies such as dams and lakes. The deterioration of natural waterways is fuelled by anthropogenic activities that produce bulk amount of wastewater. The haphazard disposal of nutrient-rich wastewater from household, industries and institutions may lead to the occurrence of eutrophication which impair the integrity and quality of water in natural water bodies. This has resulted in the urgent need for the development and implementation of new innovative green technology for wastewater treatment.

Constructed wetlands have proven to be an ideal alternative technology for wastewater treatment. This is because they are environmentally friendly and economically sustainable treatment systems. In addition, these systems have a potential of reducing contaminants to acceptable levels that pose no threat to human and environmental health. Despite these advantages, their application is still challenging in some parts of the world. This is due to the limited information about the seasonal performance of these systems and poor understanding of the influence of environmental parameters in pollutant assimilation. This study delineates the effect of seasonal variation in microbial community structures in wetland microcosm. In addition, this study also investigated the seasonal effect of physiochemical parameters on nutrient assimilation in these systems.

The constructed wetland microcosms were setup at the Empangeni (University of Zululand), and was divided into planted (planted with *Amaranthus hybridus* and *Bidens pilosa*) and unplanted (reference) section. These systems were operated in warm and cold seasons for one month. The physiochemical parameters (dissolved oxygen, pH and temperature) were monitored. The removal efficiency of chemical oxygen demand, ammonia, nitrite, nitrate and

phosphorus were measured pre- and post-treatment using spectrophotometric methods. The spectrophotometer was used with commercial kits (Merck) following manufacturers protocol.

Nutrient removal was seasonal and varying degree of nutrient removals were observed in planted and reference section of the wetland microcosms. The highest reduction efficiencies were obtained in warm than cold seasons. In warm season, the highest removals were 97%, 95%, 90%, 70% and 74% for ammonia, nitrite, nitrate, phosphorus and COD in planted section, while in reference section the removals were 69%, 69%, 82%, 57% and 59% for ammonia, nitrite, nitrate, phosphorus and COD respectively. In cold season, the removals were 60%, 73%, 65%, 68% and 64% for ammonia, nitrite, nitrate, phosphorus and COD in planted section, while in reference section the removals were 42%, 64%, 50%, 46% and 50% ammonia, nitrite, nitrate, phosphorus and COD respectively. The increase in physiochemical parameters was directly proportional to nutrient reduction in the microcosms. The correlation of physiochemical parameters with the nutrients removal ranged from very poor (temperature ($0.11 \le r \le 0.95$), moderate negative (COD (- $0.44 \le r \le 0.94$) (pH (- $0.45 \le r \le 0.89$) and to a very strong positive correlation (DO (- $0.72 \le r \le 0.89$). Based on the discharge limits of nutrients, the effluent for nitrite, nitrate and phosphorus were within the discharge limit while ammonia did not meet the discharge standards in both seasons as per South Africa's Department of Water and Sanitation.

Microbial community structure and diversity occurred in the microcosms. However, their occurrence was seasonal with warm season showing high abundance than the cold season. Furthermore, the planted sections showed high microbial abundance and diversity than the reference sections. This indicated that macrophytes supported the growth, diversity and activity of microorganisms within these systems. This was supported by the high removal of nutrients in the planted sections than in the reference sections. *Nitrosomonas* and *Nitrobacter* were the most dominant nitrifiers in the microcosms in both seasons while *Thauera*, *Pseudomonas* and

Acidovorax were the dominant denitrifiers in the both warm and cold seasons. Phosphate accumulating microorganisms were dominated by Accumulibacter in warm season and Bacillus in cold season.

This work is dedicated to my grandparents Ntombizodwa Zulu and the late Lindiwe Mbongwa. This work is also dedicated to my mother's Nonhlanhla Sbongile Makhanya, Nobukhosi Charity Zulu, Londiwe Makhanya and my Father Mbongeni Cosmos Zulu not forgetting my great childhood friend Sphamandla Ngcobo for teaching me the meaning of hard work, dedication and perseverance. Your words of inspiration and encouragement in pursuit of excellence, still dawdle on. This work is further dedicated to my three families, the Zulu family, Makhanya Family and my academic family. Most importantly, this work is dedicated to all those who have contributed positively to the building of my life.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God almighty for giving me strength and courage throughout this study, for without him, I would not have managed and pulled through. I would like to express my deepest appreciation and gratitude to my Supervisors Dr. Mathews Simons Mthembu and Prof Albertus Kotze Basson for their continuous support throughout this study. If it was not for your support, I would not have achieved this dream. Thank you for your patience with me, showing me the right path when I was lost and giving advices not only based on school work but life in general. I would also like to thank my parents Sibongingile Makhanya, Nobukhosi Zulu, Londiwe Makhanya and Mbongeni Zulu for supporting me from the genesis to the execution of this work. This is for you not forgetting my grandparents Ntombizodwa and the late Lindiwe Makhanya and Bleki Zulu. To my siblings Thabiso Makhanya, Cebolenkosi Zulu, Lusanda Zulu and Nhlalwenhle Zulu, as and elderly brother I have played my role and led by example. I hope and pray this will encourage you to do better than me. Education is no easy journey but it is a journey worth taking. Special thanks to my friend Sphamandla Ngcobo for you are the one who began this journey back in 2012. You supported and encouraged me to chase after my dreams and for that I'm eternally grateful Mapholoba my brother. I would also like to thank all my lab mates particular Fanele Ndulini and Gciniwe Sithole for your advice from the beginning to the execution of this work. Without your constant advice and support I would not have reached the finishing line. I would like to thank everyone who contributed positively throughout this study particularly Nqobile Zondo, Noxolo Hlongwa and Nathi Nxumalo. I would also like to thank all the staff members in the Department of Biochemistry and Microbiology. Also, I would like to express my gratitude to the Department of Agriculture for allowing me to use some of their equipment during this study.

LIST OF FIGURES

Figure 1.1: Occurrence of eutrophication in South Africa	6
Figure 1.2: Constructed wetland components and ecological processes	8
Figure 1.3: Free water surface constructed wetland with emergent plants	9
Figure 1.4: Longitudinal section of horizontal subsurface flow constructed wetland	11
Figure 1.5: Hybrid (vertical + horizontal subsurface flow) constructed wetland	12
Figure 1.6: Nitrogen transformation in wetland systems	20
Figure 1.7: Phosphorus cycle in constructed wetlands	24

Figure 2.1: Three dimensional structure of unplanted constructed wetland microcosm 47 with the above mansion measurements. Diagram (A) represents side view, B cross section and C top view.

Figure 2.2: pH variation (planted and reference section) from constructed wetland 51 microcosm in warm and cold seasons. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

Figure 2.3: Temperature variation (planted and reference section) from constructed 53 wetland microcosm in warm and cold seasons. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

Figure 2.4: Dissolved oxygen variation (planted and reference section) from 55 constructed wetland microcosm in warm and cold seasons. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

Figure 2.5: Chemical oxygen demand variation (planted and reference section) from 57 constructed wetland microcosm in warm and cold seasons. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

Figure 2.6: Ammonia removal efficiencies measured in constructed wetland 59 microcosms (planted and reference section) in warm and cold seasons. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

Figure 2.7: Nitrite removal efficiencies measured in constructed wetland microcosms 61 (planted and reference section) in warm and cold seasons. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

Figure 2.8: Nitrate removal efficiencies measured in constructed wetland microcosms 62 (planted and reference section) in warm and cold seasons. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

Figure 2.9: Phosphorus removal efficiencies measured in constructed wetland 64 microcosms (planted and reference section) in warm and cold seasons. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

Figure 2.10: The effect of pH on nutrient removal during warm season in the planted section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.

Figure 2.11: The effect of pH on nutrient removal during warm season in the 68 reference section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.

Figure 2.12: The effect of pH on nutrient removal during cold season in the planted 70 section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.

Figure 2.13: The effect of pH on nutrient removal during cold season in the reference 70 section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.

Figure 2.14: The effect of temperature on nutrient removal during warm season in 71 planted section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.

Figure 2.15: The effect of temperature on nutrient removal during warm season in 72 reference section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.

Figure 2.16: The effect of temperature on nutrient removal during cold season in 73 planted section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear

curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.

Figure 2.17: The effect of temperature on nutrient removal during cold season in 73 reference section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.

Figure 2.18: The effect of DO on nutrient removal during warm season in planted 74 section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.

Figure 2.19: The effect of DO on nutrient removal during warm season in reference 75 section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.

Figure 2.20: The effect of DO on nutrient removal during cold season in planted 75 section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.

Figure 2.21: The effect of DO on nutrient removal during cold season in reference 76 section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.

Figure 2.22: The effect of COD on nutrient removal during warm season in planted 77 section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.

Figure 2.23: The effect of COD on nutrient removal during warm season in reference 77 section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. The linear fits were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.

Figure 2.24: The effect of COD on nutrient removal during cold season in planted 78 section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.

Figure 2.25: The effect of COD on nutrient removal during cold season in reference 78 section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.

Figure 3.1: Agarose gel electrophoresis of the genomic DNA extracted from 93 wastewater sample in cold season. Lane M represent the DNA marker (10 000kb). Lane 1-3 represent the DNA extracted from the reference section while lane 4-6 represent the DNA extracted from the planted section.

Figure 3.2: Agarose gel electrophoresis of the genomic DNA extracted from 94 wastewater sample in warm season. Lane M represent the DNA marker (10 000kb). Lane 1-4 represent the DNA extracted from the reference section while lane 5-8 represent the DNA extracted from the planted section.

Figure 3.3: PCR product (amplicons) of nitrifying microorganisms in cold season. 94 Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.

Figure 3.4: PCR product (amplicons) of nitrifying microorganisms in warm season. 95 Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.

Figure 3.5: PCR product (amplicons) of denitrifying microorganisms in cold season. 95 Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.

Figure 3.6: PCR product (amplicons) of denitrifying microorganisms in warm season. 96 Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.

Figure 3.7: PCR product (amplicons) of phosphate accumulating microorganisms in 96 cold season. Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.

Figure 3.8: PCR product (amplicons) of phosphate accumulating microorganisms in 97 warm season. Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.

Figure 3.9: Microbial genus of nitrifying organisms identified in the planted and 99 reference sections of the microcosm in the warm and cold seasons.

Figure 3.10: Microbial genus of denitrifying organisms identified in the planted and 100 reference sections of the microcosm in the warm and cold seasons.

Figure 3.11: Microbial genus of phosphate accumulating organisms identified in the 102 planted and reference sections of the microcosm in the warm and cold seasons.

Table 1.1: Major roles of macrophytes characteristics in constructed wetlands.	15

Table 2.1: Physiochemical parameters obtained from the microcosm in warm and50cold season.

ABBREVIATIONS

AOB	: Ammonia oxidizing bacteria
Al	: Aluminum
Ca	: Calcium
COD	: Chemical oxygen demand
CWs	: Constructed wetlands
DO	: Dissolved oxygen
Fe	: Iron
FWS-CWs	: Free water surface constructed wetlands
HABS	: Harmful algal blooms
HSSF-CWs	: Horizontal subsurface flow constructed wetlands
HCWs	: Hybrid constructed wetlands
Mg	: Magnesium
NOB	: Nitrite oxidizing bacteria
Р	: Phosphorus
PAO	: Phosphate accumulating bacteria
PBS	: Phosphate buffer saline
PCR	: Polymerase chain reaction
pH	: Potential of hydrogen
SA	: South Africa
SSF-CWs	: Subsurface flow constructed wetlands
TDS	: Total dissolved solids
VSSF-CWs	: Vertical subsurface flow constructed wetlands

CONFERENCES ATTENDED

Papers Presented at Regional Conferences

 Zulu, NCT., Ndulini, SF., Sithole, GM., Basson, AK. and Mthembu, MS. 2018. Nutrient oxidizing microorganisms within a constructed wetland microcosm and the effect of seasonal variation on their functionality and population dynamics. *Faculty of Science and Agriculture Symposium.* 08 November 2018. University of Zululand, Science Centre, Richards bay (South Africa).

Papers Presented at International Conference

Zulu, NCT., Ndulini, SF., Basson, AK. and Mthembu, MS 2018. The effect of physiochemical parameters and seasonal variation on nutrient removal from municipal wastewater using a constructed wetland microcosm. 19th WaterNet/WAFSA/GWP-SA Symposium. 31st October – 02nd November 2018, Livingstone, Zambia.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Water is an essential universal element for every living organism. However, South Africa (SA) is currently facing a severe water scarcity. This is due to the fact that SA is a semi-arid country with low average rainfall of 450 mm per annum (Nkosi, 2015). Furthermore, this is also aggravated by global warming and the deterioration of natural water bodies such as dams, rivers and lakes (Mthembu, 2016). The deterioration of natural water sources is commonly triggered by anthropogenic activities such as the disposal of poorly treated nutrient-rich wastewater from households and institutions (Wise *et al.*, 2011; Zhou *et al.*, 2017).

The disposal of nutrient-rich wastewater is of serious concern because of the possible contribution to eutrophication which poses a major threat to the safety, quality, sustainability and ecological integrity of natural water bodies (Varol *et al.*, 2012). This is due to the fact that eutrophication can lead to anoxic or hypoxic conditions and subsequently eliminate aquatic life, impair the use of water for domestic purpose and completely destroy natural water bodies through oxygen depletion (Varol *et al.*, 2012; Huang *et al.*, 2017). Furthermore, eutrophication can lead to the occurrence of harmful algal blooms (HABs) that produce toxins such cyanotoxic, biotoxins, neurotoxins and cyclic peptide hepatotoxins (He *et al.*, 2016; Salamah, 2017).

Algal toxins are associated with a variety of deleterious human health conditions. These include cancer, diabetes, osteoporosis, spontaneous miscarriage, kidney failure, respiratory and neurological complications (Hampel, 2013; He *et al.*, 2016). Trevino-Garrison *et al.* (2015) reported that humans can be affected by these secondary metabolites through direct contact or consumption of food and water that is contaminated by HABs. Due to the public awareness of

these human and environmental health issues, major steps have been taken over the past two decades to reduce nutrient loading in natural water bodies (Thieu *et al.*, 2010; Liu *et al.*, 2017). This includes wastewater treatment prior to disposal into natural water bodies (Liu *et al.*, 2017).

Historically, conventional technologies such as wastewater treatment plants, activated sludge process, membrane bioreactor and membrane separation have been used globally for wastewater treatment (Wu *et al.*, 2015; Liu *et al.*, 2017; Zhou *et al.*, 2017). However, these technologies have received setbacks in many developing countries. This is due to the high financial investment associated with their construction, maintenance and skills necessary for their operation (Wu *et al.*, 2015). In addition, these technologies exhibit poor nutrient removal (Liu *et al.*, 2017). Poor nutrient removal in these systems is associated with nitrification and denitrification failure (Verhoeven *et al.*, 1999; Awolusi, 2016). Zhang *et al.* (2015) linked the failure of these microbial mediated process to the changes in environmental parameters such as seasonal temperature variation. Seasonal variation from warm to cold season interferes with microbial metabolism, thus inhibiting the activity of microbial enzymes responsible for nitrogen and phosphorus breakdown in conventional wastewater treatment systems (Awolusi, 2016). This affects the overall treatment efficiency of conventional technology and can results in the disposal of nutrient-rich effluent.

The aforementioned challenges have prompted the urgent need for an alternative technology for wastewater treatment. Ecological technologies such as constructed wetlands (CWs) are described as a convenient, emerging and innovative alternatives technologies for wastewater treatment (Wu *et al.*, 2015). This is due to their simple operation, cost effectiveness and environmentally friendly since they use biological processes to remove nutrient (Adrados *et al.*, 2014). Moreover, these systems have shown potential to produce great quality effluent (Zhang *et al.*, 2014). However, their treatment efficiencies differ from system to system.

According to Zhang *et al.* (2015) this variation is attributed to intricate combination of physical, chemical and biological processes brought about by the interaction between macrophytes, microorganisms and substrate.

Zhang *et al.* (2014) and Machado *et al.* (2017) further stated that the treatment efficiency of these systems is highly dependent on regional climatic conditions and latitude which makes them more efficient in warm compared to temperate climate conditions. This make CWs technology ideal for developing countries which mostly exhibit warm tropical and subtropical climatic conditions (Zhang *et al.*, 2014). Despite CWs showing a great potential as an alternative technology of wastewater treatment in developing countries like South Africa, their application is still limited. This is due to the limited research that has been conducted on these systems. This calls for more comprehensive research to be conducted about these systems especially in developing countries. This includes understanding the seasonal efficiency of these systems in nutrient removal.

1.2 AIM AND OBJECTIVES OF THE STUDY

The main aim of this study was to investigate the effect of seasonal variations on nutrient removal from municipal wastewater using a constructed wetland microcosm.

The specific objectives were:

- (a) To determine the effect of seasonal variation on nutrient removal in a constructed wetland microcosm.
- (b) To determine the physiochemical parameters (temperature, pH, dissolved oxygen and chemical oxygen demand) in a constructed wetland microcosm and relate them to nutrient removal.

(c) To identify and quantify microbial functional groups responsible for nutrient reduction in a wetland microcosm.

1.3 LITERATURE REVIEW

1.3.1 Introduction

Nutrient pollution in surface and ground water has become a global challenge and it is mostly propelled by anthropogenic activities (Xu *et al.*, 2012). Tan *el al.* (2017) reported that anthropogenic reactive nitrogen and phosphorus have increased drastically over the years from $\sim 15 \text{ Tg yr}^{-1}$ in 1860 to $\sim 187 \text{ Tg yr}^{-1}$ in 2005. This increase is caused by several human activities including disposing untreated and poorly treated municipal wastewater which can have a catastrophic effect on the health of humans and the environment (Xu *et al.*, 2012). Therefore, proper management and restraint of these nutrients has become a necessity to protect humans and the environment (Morris *et al.*, 2017).

Conventional technologies are globally used for nutrient removal in municipal wastewater. However, the application of these systems in rural settlements is still a challenge (Zhang *et al.*, 2015). These challenges are associated with their cost, susceptibility to environmental factors such as seasonal changes which affect microbial functionality for effective nitrogen and phosphate removal (Wu *et al.*, 2015). As a result, CWs are proposed as a potential costs effective biological alternative for nutrient removal in municipal wastewater (Wang *et al.*, 2017).

1.3.2 Overview and Water Scarcity in South Africa

Section 27 (b) of the constitution of the Republic of South Africa, ACT no.108 of 1996 stipulate that every citizen has a right to a sufficient supply of good quality water (Obrien, 2014). However, more than 12 million people in SA still lack access to safe drinking water especially

in the rural areas (Molobela *et al.*, 2011; Mulenga, 2017). This is due to the fact that SA is one of the driest countries in the world with limited and uneven distribution of natural sources of freshwater supply, hence previous studies have described SA as a water scarce country (Pindihama *et al.*, 2011; Thabethe, 2011; Olaniran *et al.*, 2012; Obrien, 2014; Mathembula, 2015; Ntshobeni, 2015).

Water scarcity in SA is also fuelled by unpredictable and erratic rainfall with an average of 465 mm annually which is below the global standard of 860 mm (Olaniran *et al.*, 2012). Furthermore, SA has high annual evaporation rate which is estimated to be four times higher than the annual average rainfall (Tabane, 2017). Mathembula (2015) reported that evaporation rate of 1500 mm has been recorded annually in south and eastern region of South Africa while 3000 mm has been recorded in the western region. These tremendously high evaporation rates lead to the depletion of surface water in the country (Mathembula, 2015).

The deterioration of natural water bodies and surface water is also fuelled by water pollution. Lai (2013) reported that surface water accounts for 77% of total water available in SA while ground and return flows water accounts for 14 and 9% respectively. However, despite surface water constituting the largest proportion of water in SA, a survey conducted by Swarts (2010) reported that only 30% of the surface water is clean and readily available for use. Adewumi *et al.* (2010) linked the high pollution of water in SA to urban and industrial activities that produce bulk amount of wastewater that is commonly discharged into natural water bodies untreated or partially treated. The disposal of untreated or partially treated wastewater effluent causes several types of water pollution such as nutrient pollution.

1.3.3 Wastewater Effluent as a Source of Nutrient Pollution

Nutrient enrichment in the environment continues to be a major threat affecting freshwater ecology through the process of eutrophication (Nhapi *et al.*, 2004; Hoffmann, 2006). Cai *et al.*,

(2013) reported that at least 48% of the lakes and reservoirs in North America, 54% in Asia, 53% in Europe, 41% in South America and 28% in Africa were eutrophic. These figures are expected to increase in the forthcoming decades especially in developing countries. This is due to the fact that more than 80% of wastewater in developing countries like South Africa is disposed into natural water sources untreated or without adequate treatment (Azizullah *et al.*, 2011; Nkosi, 2015; Mugagga *et al.*, 2016; Van der Merwe, 2016). The disposal of nutrient-rich wastewater in developing countries is caused by the shortage of infrastructure and poor operation and maintenance of wastewater treatment plants (WWTP) (Dos-santos *et al.*, 2017).



Figure 1.1: The occurrence of eutrophication in South Africa (Ndlela et al., 2016).

Mthembu *et al.* (2013) reported that SA comprises of 850 wastewater treatment plants. However, 10% of these systems are completely dysfunctional and 26% are incompetent. King (2014) denoted that these challenges were instigated by the chronic lack of capital investment from South African government to support, build and maintain the currently existing WWTPs, thus exposing natural water resources to nutrient pollution. As a result, the quality of natural surface water and natural water sources are deteriorating at an alarming rate due to eutrophication (Nyenje *et al.*, 2010). Matthews (2014) reported that 26% of the surface water in SA is hypertrophic, 34% is mesotrophic, 3% is oligotrophic and 37% is eutrophic due to eutrophication. The frequent occurrence of eutrophication has been reported throughout the country in natural water resources such as Nhlangazwan dam, Loskop dam, Lake Midmar, Hennops river, Vaal river and Berg river (Figure 1.1) (Nyenje *et al.*, 2010; Ndlela *et al.*, 2016). The high incidences of eutrophication, deterioration of natural water sources and incompetence of WWTP in South Africa indicates the urgent need for the development, implementation and establishment of new technologies to supplement the currently used technologies for wastewater treatment. These new technologies include the use of green technologies such as constructed wetlands.

1.3.4 Constructed Wetlands

Constructed wetlands also called treatment wetlands are artificially designed and engineered systems that are built to mimic natural wetlands that uses naturally occurring biological processes for wastewater treatment (Vymazal, 2014; Vymazal *et al.*, 2015; Li *et al.*, 2017). These systems make use of microorganisms, substrates and macrophytes to treat wastewater. The application of CWs in wastewater treatment started in Germany back in the late 1960's (Zhang, 2012; Vymazal, 2014). However, these systems have received international attention from both scientists and engineers in the past two decades as an alternative ecological technology for wastewater treatment. Developed countries like USA, China, Canada, Australia, and Spain are now relying on these systems for wastewater treatment (Wu *et al.*, 2014).

Initially, CWs were designed for municipal wastewater treatment. However, their application has expanded, hence they are now used for industrial, agricultural, storm water runoff and hospital wastewater treatment (Zhang *et al.*, 2014; Sehar *et al.*, 2015; Ilyas *et al.*, 2017). This is largely attributed to their simple construction and maintenance, high buffering capacity for hydraulic and organic load fluctuations, high robustness, process stability and low production

of sludge (Oon *et al.*, 2015; Ilyas *et al.*, 2017). In addition, the application of these systems is expanding due to their support to wildlife, providing aesthetic value and generating usable plant biomass (Sehar *et al.*, 2015).

Bioremediation of nitrogen and phosphorus in CWs is complex and dependent on several factors such as oxygen and carbon supply, hydraulic retention time, inlet nutrient concentration, microbial abundance, operational mode, vegetation type and physiochemical parameters (Zhang *et al.*, 2015; Machado *et al.*, 2017). Physiochemical parameters influence a variety of nutrient removal mechanisms such as plant uptake and nitrification (Figure 1.2) (Zhang *et al.*, 2015). This affect the overall treatment efficiency of these systems (Oon *et al.*, 2015). Furthermore, the treatment efficiency of these systems is influenced and highly reliant on the type of wastewater and type of CWs used (Vymazal *et al.*, 2015; Maucieri *et al.*, 2017).



Figure 1.2: Constructed wetland components and ecological processes (Mustafa, 2010).

1.3.5 Classification of Constructed Wetlands

Constructed wetlands are classified based on the dominant macrophytes (emergent, free floating, submerged), design and direction of water flow (Vymazal *et al.*, 2015). The predominantly used CWs are free water surface flow, subsurface flow and hybrid systems (Vymazal *et al.*, 2015; Maucieri *et al.*, 2017).

• Free water surface flow constructed wetlands

Free water surface flow constructed wetlands (FWS-CWs) (Figure 1.3) are described as wetland systems where water surface is exposed to the atmosphere. These systems closely resemble the natural wetlands in appearance and functionality (Westerhof *et al.*, 2014). Free water surface flow constructed wetlands are made of sequences of basins with 13-30 cm rooting soil or media that support the growth of macrophytes (Maucieri *et al.*, 2017).



Figure 1.3: Free water surface flow constructed wetland with emergent plants (Mustafa, 2010).

In free water surface flow constructed wetlands, as wastewater passes through the basins nutrients are removed by various biological and chemical processes (Mustafa, 2010). These processes include microbial biodegradation, nitrification and denitrification (Maucieri *et al.*, 2017). Previous studies mentioned that the removal efficiency of nitrogen and phosphorus in FWS-CWs can be up to 90% and above depending on numerous factors such dissolved oxygen concentration, plant species type and seasonal variation (Ibekwe *et al.*, 2007; Zhang *et al.*, 2014; Maucieri *et al.* 2017).

• Subsurface flow constructed wetland

Subsurface flow constructed wetlands (SSF-CWs) consists of gravel beds that are used to cultivate macrophytes. The removal of nutrient in SSF-CWs is dependent on several factors such as redox potential and dissolved oxygen (Corbella *et al.*, 2014). Subsurface flow constructed wetland are divided into two groups based on the flow direction of wastewater. These are:

(i) Horizontal subsurface flow constructed wetland

Horizontal subsurface flow constructed wetland (HSSF-CWs) (Figure 1.4) are constructed with gravel beds with an estimated depth of 0.6-0.8 m to permit the growth of macrophytes and allow the flow of wastewater from the inlet (Maucieri *et al.*, 2017). Water is then allowed to tardily flow through the porous medium beneath the bed surface until it reaches the outlet region in a horizontal path. The water is then collected and discharged into natural water bodies.

The removal of nutrients is facilitated by microbial degradation in anoxic or anaerobic condition because the beds are perennially saturated. Nitrogen is removed by denitrification. The efficiency of ammonium removal is inadequate because of the oxygen deficit in the filtration beds prompted by the permanent waterlogged conditions (Vymazal *et al.*, 2015; Maucieri *et al.*, 2017).



Figure 1.4: Longitudinal section of a horizontal subsurface flow constructed wetland (Mustafa, 2010).

(ii) Vertical subsurface flow constructed wetland

Vertical subsurface flow constructed wetlands (VSSF-CWs) are made of flat-bed graded gravel of 30-60 mm and sand of 60 mm that is used as a growth media for macrophytes. Vertical subsurface flow CWs are known to be very effective on the removal of total dissolved solids and organic compounds (Vymazal *et al.*, 2015; Maucieri *et al.*, 2017). According to Zhang *et al.* (2014) VSS-CWs can remove nitrogen and phosphorus up to 96 %.

• Hybrid constructed wetlands (HCWs)

Hybrid constructed wetland systems are derivatives of the original hybrid system developed in Germany by Siedel at Max Planck Institute in Krefeld. Hybrid constructed wetlands are a coalescence of FWS, HSSF and VSS that are arranged in series (Figure 1.5). The main goal of HCWs is to improve the total nitrogen removal since majority of these systems provide different redox conditions that enhance nitrification and denitrification. In general, HSSF CWs are known to offer adequate anaerobic conditions for the process of denitrification but lack the

feasibility to nitrify ammonia (Zhang *et al.*, 2015). On the other hand, VSSF systems have a high ability to remove ammonia-nitrogen because of the aerobic condition provided by these systems. As a result, combining these two systems enhance the performance of hybrid system in nutrient removal (Zhang *et al.*, 2015; Wang *et al.*, 2017). In a study conducted by Zhang *et al.* (2014), it was alluded that hybrid wetland systems can remove nitrogen and phosphorus up to 91% and 99% respectively.



Figure 1.5: Hybrid (vertical and horizontal subsurface flow) constructed wetland (Mustafa, 2010).

Experimentally, all the aforementioned wetland systems can be operated as either mesocosm or microcosm. Mesocosm experiments are described as any outdoor experimental system that examines the natural environment under a more controlled environment such as the green house. In most cases, these systems are medium to large size. Mesocosm experiments have some disadvantages, including their inability to adequately emulate the natural environment. Meanwhile, microcosm experiments are conducted outdoor under ambient uncontrolled environmental conditions. These systems are usually in small scales and have a great potential of imitating the natural environment. Their functionality is largely dependent on environmental parameters such as temperature which influence the activity of microorganisms and macrophytes in these systems.

1.3.6 Role of Macrophytes in Constructed Wetlands

Macrophytes are essential biological and structural components of CWs and play a staggering role as intermedium for wastewater treatment (Wu *et al.*, 2011; Fan *et al.*, 2016). This is through augmenting a variety of nutrient removing mechanisms such as uptake, absorption, retention and assimilation (Wu *et al.*, 2015; Zhou *et al.*, 2017). The ability of macrophytes to remove nutrient may vary according to retention time, loading rate, type of wastewater and system configuration (Wu *et al.*, 2015). Furthermore, the ability of macrophytes to uptake nutrients may vary amongst macrophytes species (Liang *et al.*, 2017). This is largely due to the fact that macrophytes have differences in growth, reproduction, morphological and physiological properties (Wu *et al.*, 2011; Zhang *et al.*, 2017). This signifies the importance of plant selection in wetland systems.

Jampeetong *et al.* (2012) alluded that phytoremediators to be used in wetland systems must be suitable for a particular type of wastewater. Suitable macrophytes must satisfy the following requirements:

- They must be ecologically acceptable and must not provide a threat or danger and disease risks to the ecological surroundings.
- They must be well suited to the surrounding climatic conditions and adapted to the wildlife.
- They must have high nutrient removal efficiency through different processes such as direct assimilation, store or improve nutrient removal through both direct and indirect microbial transformation (nitrification and denitrification).

- They must be able to tolerate the contaminants found in wastewater and hypertrophic waterlogged conditions.
- They must be rapidly established, propagated and fast growing.

In CWs, macrophytes alleviate surface beds, provides conducive conditions for physical filtration and provide a large surface area for biofilm attachments (Oon *et al.*, 2015; Machado *et al.*, 2017). Furthermore, macrophytes secrete organic exudates and translocate oxygen into the rhizosphere from the leaves and stems which stimulates aerobic degradation and nutrient uptake (Leung *et al.*, 2016; Zhang *et al.*, 2016; Liang *et al.*, 2017; Mechado *et al.*, 2017). Zhang *et al.* (2017) reported that nutrient uptake in CWs is greatly influenced by various environmental parameters such as photosynthetic rate of macrophytes, hydrological condition, amount of nutrients in water, radial oxygen loss (ROL) and seasonal dynamics (Zhou *et al.*, 2017).

Oon *et al.* (2015) reported that macrophytes propagate optimally in warm seasons while in cold seasons their functioning may be hindered due to frosting, decreased water temperature and macrophytes undergoing senescence. This affect the overall nutrient removal in CWs (Oon *et al.*, 2015). Furthermore, the process of plant nutrient uptake is influenced by the type of plant species (Zhou *et al.*, 2017). Zhang *et al.*, (2016) reported that each plant species possesses its own ability to function in different types of CWs based on their morphological adaptation and characteristics (Zhang *et al.*, 2016). The role of macrophyte characteristics are described in Table 1.1.

• Macrophytes used in this study

In the current study, *Amaranthus hybridus* and *Bidens pilosa L* were used as macrophytes of choice because they have high efficiency for nutrient removal and can grow rapidly (Bartolome *et al.*, 2013). *Amaranthus hybridus* is a garden weed that grows in temperate and tropical areas

in SA. These plants have also been found to grow in adverse environmental conditions (acidic, alkaline, saline, poor soil regions). They also have the ability to propagate in hot and dry weather conditions however, they propagate optimally in temperatures ranging between 18 and 25° C in well drained fertile soils and deeper soils with a pH of 6.4 (Stetter *et al.*, 2017). On the other hand, *Bidens pilosa L* is an annual forb that propagates up to 1.8 meters in height and belongs to the family of *Asteraceae*. This plant commonly grows in tropical and subtropical countries and propagates naturally in homestead gardens in developing countries like South Africa. These plants grow optimally at temperature and pH ranging between 25-30°C and 4-9 respectively (Bartolome *et al.*, 2013). Despite macrophytes being described as one of the key components of nutrient removal in CWs, microorganisms also play a substantial role in these wetland systems.

Macrophytes feature	Role in wastewater treatment process
	Decrease phytoplankton growth by reducing
Plant aerial part	light.
	Provide a suitable habitat for wildlife.
	Reduce resuspension by decreasing wind
	velocity and afford insulation in winter.
	Enhance formation of biofilm of microbes by
Immerse plant part in water	providing surface area.
	Nutrients uptake.
	Excretion of photosynthesis oxygen increase
	aerobic degradation.
	Enhance biodegradation and nitrification by
Roots and rhizomes in the sediment	releasing oxygen.
	Provide surface area for biofilm attachment.
	Release antibiotic.
	Uptake of nutrient.

Table 1.1: Major roles of macrophytes characteristics in constructed wetland treatment system(Dong 2013; Vymazal, 2009).

1.3.7 The Role of Microbes in Constructed Wetlands

Wastewater treatment systems are largely dominated by a barrage of microorganisms. These microorganisms instigate and control all biological and chemical processes occurring these systems (Srivastava *et al.*, 2016). In CWs, microorganisms form a complex community structures or biofilms in the surface of plant roots. These biofilms play an auxiliary role in all biogeochemical transformation and biodegradation of nutrients in wetland systems (Kivaisi, 2001; Iasur-Kruh *et al.*, 2010). The development of biofilms in CWs is largely dependent on environmental parameters such as hydraulic conditions and temperature (Calherios *et al.*, 2010). In addition, the development of biofilms is dependent on the concentration of nutrients and toxic compounds of wastewater as this can either stimulate or inhibit the growth, development and functioning of biofilms in treatment wetlands. This subsequently affects the overall biodegradation of nutrients in CWs (Calherios *et al.*, 2010).

A diverse microbial community structures of both aerobic and anaerobic microorganisms plays a vital role in the biodegradation and cycling of nutrients in wetland systems (Li *et al.*, 2010; Zhao *et al.*, 2010). They achieve this through a variety of mechanisms such as partial nitrification, bioaccumulation, biodegradation, nitrification, denitrification and microbial phosphorus removal (Button *et al.*, 2015; Rajasulochana *et al.*, 2016; Fu *et al.*, 2017). These mechanisms are stimulated by hefty quantities of organic exudates and oxygen found in the rhizosphere which promotes the activity of aerobic and anaerobic microorganisms (Zhao *et al.*, 2010; Mthembu *et al.*, 2013). Aerobic microbes function primarily next to the plant roots and attain their energy and nutritional source from symbiosis while anaerobic microbes oxidize organic matter in order to produce methane that is used as a source of energy and nutrition (Zhao *et al.*, 2010). The common nutrients oxidizing microorganisms in CWs are classified as nitrifiers, denitrifiers and phosphate accumulating organisms (PAO) based on their functionality (Zhang *et al.*, 2016). Nitrifying bacteria are subdivided into ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Deepnarain, 2014). Ammonia oxidizing bacteria transform ammonia into nitrite while NOB further oxidize nitrite to nitrate (Ramdhani, 2013). Denitrifying bacteria convert nitrate into dinitrogen (N₂) gas through the process of denitrification. Meanwhile, PAO feeds on the phosphorus available for their growth and proliferation. This result in the biotransformation of phosphorus in CWs (Deepnarain, 2014). Despite microorganisms playing an essential role in nutrient removal, their synergistic interaction with macrophytes elevate nutrient removal in treatment wetlands.

1.3.8 Macrophytes-Microbe's Interaction in Constructed Wetland

Plant-microbes' interaction is regarded as the main driving force of nutrient removal in wetlands systems and is influenced by three major factors. These are water chemistry (dissolved oxygen, dissolved organic matter, salt concentration, pH and electrical conductivity), redox conditions and nutrients availability (Srivastava *et al.*, 2016). Nonetheless, a comprehensive understanding of this interaction is still limited due to the fact that studies on the improvement of water quality have solely focused more on nutrient assimilation by either macrophytes or microorganisms (Srivastava *et al.*, 2016).

Despite the limited knowledge, it is a strong opinion that plants positively influence microorganisms in wetland systems. Macrophyte roots provides a surface area for benthic microbial community to rest and act as microbial niche, thereby providing constant oxygen, organic carbon and nutrients supply. This increases microbial abundance as well as their activity in the rhizosphere (Liu *et al.*, 2017). Meanwhile, microorganisms produce volatile organic compounds, minerals and nutrients that stimulate the growth of macrophytes.

In addition, microorganisms produce chemical compounds that stimulates systemic resistance genes in macrophytes, thus inducing defense mechanism and protective immunity against plant pathogens (Lareen *et al.*, 2016).

Zhao *et al.* (2010) reported that the rhizosphere is a distinct and active zone where all biochemical processes of nutrient removal occur. The rhizosphere offers both aerobic and anaerobic conditions for microbial activity. This enhance mineralization and biotransformation of nutrients in CWs (Pei *et al.*, 2010; Zhao *et al.*, 2010; Wang *et al.*, 2012). However, macrophytes have no influence on microbial community structures and diversity in wetland systems but intensify their activity. Furthermore, they play a substantial role in the establishment of macrophytes-microbe interrelation (Srivastava *et al.*, 2016).

Srivastava *et al.* (2016) mentioned that macrophytes and microorganisms forms two symbiotic relationships. The first one is endophyte relationship whereby microorganisms stay within the plant but cause no disease and colonize macrophytes internal tissues. These microorganisms consist of nitrogen (N_2) fixing diazotrophs and other nutrient assimilators. The second relationship is ectophytic, whereby microorganisms stay outside the macrophytes. These microorganisms include ammonia oxidizing bacteria and methanotrophic bacteria. This kind of symbiotic relationship includes interaction of roots and leaves and is vital because of the numerous biochemical interactions occurring in the interactive surface (Srivastava *et al.*, 2016). Hence, this interaction stimulates all the mechanisms responsible for nutrient removal, thus improving water quality (Srivastava *et al.*, 2016).

1.3.9 Mechanisms of Nitrogen Transformation in Constructed Wetlands

Nitrogen is a major pollutant in sewage wastewater that must be reduced prior to discharge into natural water ways in order to reduce the occurrence of eutrophication. In wastewater, nitrogen exists in the form of organic and inorganic nitrogen (Mustafa, 2010). Organic forms of nitrogen
are found in amino acid, uric acid, urea, purine and pyrimidines (Saeed *et al.*, 2012). Ammonia, nitrate, nitrite, nitrous oxide and nitrogen gas represent the inorganic form of nitrogen (Mustafa, 2010: Saeed *et al.*, 2012).

In constructed wetlands, nitrogen can be transformed and removed by several classical and newly discovered routes. The major nitrogen removal routes are presented in Figure 1.6. These routes include ammonia volatilization, ammonification, nitrification, denitrification, biodegradation-anammox routes, fixation and plant uptake (Saeed *et al.*, 2012). The overview of these biological pathway is given blow:

• Ammonia volatilization

Ammonia volatilization is described as a physiochemical process whereby volatile ammonium gas is removed from the water surface into the atmosphere (Saeed *et al.*, 2012). This process is profoundly influenced by the pH (Vymazal, 2007). Saeed *et al.* (2012) pointed out that this process is significant at pH ranging between 8.0 and 9.3. This is because within this pH range, the alkalinity of wastewater is augmented, thus converting ammonia ions into ammonia gas which is released into the atmosphere (Garcia *et al.*, 2010). In addition, photosynthetic macrophytes during the day increases the pH value of wastewater in CWs, thus enhancing ammonia volatilization is also influenced by temperature. Viero *et al.* (2014) reported that in warm season this process can remove ammonia ranging between 20-78%. However, in cold season like in winter the ammonium removal rate decrease.

• Ammonification

Ammonification is the initial process that produces ammonia through biological conversion of nitrogen (Lee *et al.*, 2009). It is an essential energy releasing catabolism in which amino acids

are subjected to numerous deamination reactions that produce ammonia. The deamination process can be represented by the following equation:

Amino acids \rightarrow Imino acids \rightarrow Keto acids \rightarrow NH₃

Kinetically, the process of ammonification in CWs occurs more frequently than nitrification (Vymazal, 2007; Lee *et al.*, 2009; Saeed *et al.*, 2012). Ammonification rate in wastewater is believed to occur rapidly in the upper zone where oxygen is in abundance while in the lower deoxygenated zone it is the opposite (Saeed *et al.*, 2012). According to Lee *et al.* (2009), ammonification rate is influenced by several physical and chemical parameters including seasonal changes, pH and nutrient availability. Dong (2013) and Saeed *et al.* (2012) reported that ammonification occurs optimally at temperature ranging between 40-60^oC.



Figure 1.6: Nitrogen transformation in wetland systems (Adhikari, 2012)

• Nitrification

Nitrification is the second stage of nitrogen removal in wastewater treatment. Ammonium nitrogen is oxidized into nitrate with nitrite functioning as an intermediate in this process

(Dong, 2013). Nitrification is microbial mediated and occurs in two steps (Mustafa, 2010). Initially, ammonium is transformed into nitrite by ammonia oxidizing bacteria (AOB) such *Nitrosomonas* followed by aerobic oxidation of nitrite into nitrate by nitrite oxidizing bacteria such as *nitrobacter* (Faulwetter *et al.*, 2009; Mustafa, 2010). In these two steps, chemolithoautotrophs uses ammonia or nitrite as a source of energy while oxygen is used as an electron acceptor and carbon dioxide is used as a source of carbon (Lee *et al.*, 2009). Lee *et al.* (2009) reported that nitrification requires bulk amount of oxygen to be utilized by microorganisms. The stoichiometry required for complete nitrification is 4.6 kg oxygen per kg NH4⁺-N and 1 mg/l of total dissolved oxygen (Faulwetter *et al.*, 2009). Previous studies have shown that nitrification rates are significantly influenced by physical and chemical parameters such as water alkalinity, dissolved oxygen and seasonal variation (Vymazal, 2007; Faulwetter *et al.*, 2009; Lee *et al.*, 2009; Lee *et al.*, 2009; Saeed *et al.*, 2012). Saeed et al. (2012) alluded that nitrification can remove nitrogen up to 90% in wetland systems.

• Denitrification

Denitrification is described as one of the preeminent pathways of nutrient removal in treatment wetlands (Wongkiew *et al.*, 2017). This process is mediated by microorganisms under anaerobic or anoxic conditions. It involves the biological oxidation of nitrogen compounds to produce nitrous oxide, nitric oxide and finally nitrogen gas (Saeed *et al.*, 2012). Facultative chemoheterotrophic microorganisms are responsible for nitrogen oxidation (Vymazal, 2007). These microorganisms include *Bacillus*, *Pseudomonas*, *Micrococcus*, *Spirillum* and *Enterobacter* species and uses organic compounds as electron donor and source of cellular carbon (Dong, 2013). In addition, these microorganisms use nitrogen oxide in an ion form and gas as an electron acceptor (Faulwetter *et al.*, 2009). Previously studies have reported that the process of denitrification can remove nitrogen ranging between 60-90% compared to 1-30%

transformed by macrophytes and algae (Wongkiew *et al.*, 2017). Hence, this process plays a vital role in nitrogen removal, thus enhancing water quality in constructed wetlands.

Anammox

Anaerobic ammonium oxidation (anammox) is a newly discovered biological process of nitrogen removal. This process converts nitrite and ammonia into nitrogen gas under anoxic condition (Vymazal, 2007; Saeed *et al.*, 2012). *Planctomycetes* bacteria are responsible for mediating this process (Mustafa, 2010). Saeed *et al.* (2012) pointed out that anammox process offers some advantages when compared to conversional nitrification and denitrification. These advantages include minimal requirement of oxygen, less energy consumption and requires no external carbon sources. Anammox bacteria are mostly autotrophs and they include *Candidatus brocadia anammoxidant*, *Thiomicrospira denitrificants* and *Paracoccus denitrificants*. All these autotrophic microorganisms do not require external carbon sources like heterotrophic denitrifiers which requires carbon as a source of energy. Therefore, this process reduces the requirement of organic carbon as an energy sources in wetland. Despite this, anammox process is still dependent on several factors such as the requirement of 1:32 ammonium to nitrite ratio for complete removal of ammonia. In terms of removal efficiency, this process is reported to remove total nitrogen ranging between 2.8-5.7 mg/l in constructed wetlands (Lee *et al.*, 2009; Vymazal, 2009; Saeed *et al.*, 2012).

Fixation

Nitrogen fixation is that process that convert gaseous nitrogen into ammonia. The process works in the presence of nitrogenase, sulfur and molybdenum containing enzyme complex that reduce substrate with triple covalent bonds such as nitrous oxide, acetylene and cyanides. In wetland media, the biological fixation of nitrogen may occur the soil surface, rhizosphere, surface of leaves and stems of the macrophytes.

Nitrogen is fixed by several microbes including asymbiotic blue-green algae, actinomycetes, autotrophic, heterotrophic bacteria and macrophytes (Mustafa, 2010). Other significant microorganisms responsible for nitrogen fixation are cyanobacteria associated with macrophytes roots. The removal efficiency of nitrogen in wetland systems by this process is still debatable. This is due to the fact that only a few studies have been conducted to evaluate the efficiency of this process in nitrogen removal (Vymazal, 2007; Mustafa, 2010). Therefore, more studies are still needed to evaluate the competence of nitrogen fixation in wetland systems.

• Plant uptake

Plant uptake is one of the major pathways of nitrogen removal in wetland. In this process, inorganic nitrogen is converted into organic compounds that function in the plant cells and act as tissue building blocks of macrophytes (Vymazal, 2007). Nitrogen is effectively assimilated by emergent macrophytes. The capability of emergent macrophytes to use nutrients from sediment may partly account for their bulk productivity compared to planktonic algae in wetland systems.

Nitrogen uptake is greatly influenced by both physical and chemical parameters such as seasonal temperature variation and total dissolved oxygen. In warm season macrophytes grows rapidly, thus augmenting nitrogen uptake. Nitrogen uptake and storage by aquatic phytoremediators is also dependent on nutrient concentration in their tissues. Therefore, macrophytes used for nitrogen removal in wetland systems must have expedient characteristics such as high tissue nutrient content, fast growth and the capacity to acquire a high-standing crop (Dong, 2013). Vymazal, (2007) pointed out that the rate of nitrogen assimilation by macrophytes is limited by the plant growth rates accompanied with nutrients concentration in the macrophytes tissue. Vymazal (2007) proposed that macrophytes with high productivity or

growth rates like *Amaranthus* and *Bidens pilosa* can remove bulk amount of nutrient as these macrophytes consume nitrogen for their growth.

1.3.10 Mechanisms of Phosphorus Transformation in Constructed Wetland

Phosphorus is one of the major constituent and pollutant in wastewater (Mustafa, 2010). Excess amount of phosphorus in the natural environment is of serious concern to both public and environmental health. This is because of the possible contribution to human illness and its toxic effect in the receiving water bodies (Withers *et al.*, 2008). This necessitates the removal of excess amount of phosphorus reaching natural water bodies. In wetland systems, phosphorus removal is achieved through different biological, physical and chemical processes (Mustafa, 2010). Figure 1.7 illustrates all the biological processes responsible for phosphorus removal. These biological processes include soil adsorption and precipitation, microbial uptake and plant assimilation (Kelly *et al.*, 2014).



Figure 1.7: Phosphorus assimilation in wetland systems (Dong, 2013).

• Soil adsorption and precipitation

Soil adsorption is the movement of soluble inorganic phosphorus from soil pore water to soil mineral surfaces. The soils ability to adsorb phosphorus normally increases with clay or

mineral components of that particular soil. The adsorption of inorganic phosphorus is related to high level of iron (Fe), calcium (Ca) and aluminum (Al) (Vymazal, 2007). Phosphorus sorption by the wetland media is controlled by the amount of phosphorus concentration in soil pore water and the capability of the solid phase to refill phosphorus into soil pore water.

The phosphorus sorption is divided into two-step process: the first process is phosphorus exchange amongst the wetland pore water and particles of the soil. The second process is that of absorption where phosphorus penetrates slowly into the solid phase (Kadlec, 2005). Similarly, phosphorus desorption takes place in a two-step process. One of the proposed mechanisms is the reductive dissolution of Fe (III) and Mn (IV) phosphorus minerals. Soils under anaerobic conditions releases more amount of phosphorus than aerobic condition. The difference in phosphorus behavior under both aerobic and anaerobic conditions is attributed to ferric oxyhydroxide changes brought by soil reduction (Vymazal, 2007).

In terms of precipitation, phosphorus ions react with metallic cations including Fe, Al, Ca and Mg, thus forming amorphous or poorly crystalline solids (Garcia *et al.*, 2010). This type of reaction commonly occurs at a higher phosphorus concentration. A diverse group of cations can precipitate phosphorus. In CWs, the essential precipitated minerals are apatite, hydroxylapatite and wavellite. In addition, phosphorus can also co-precipitate with other minerals including ferric oxyhydroxide and the carbonate minerals, thus enhancing the removal of phosphorus in wetland systems (Vymazal, 2007).

• Microbial transformation

Wetland systems are composed of microbial communities and algae that degrade phosphorus. Microbial uptake of phosphorus in wetland system is a rapid process because these organisms grow and multiply at an exponential rate in water temperatures, thus degrading phosphorus at a swift rate (Kadlec, 2005; Vymazal, 2007). Phosphorus is also sequestered by the algal component of CWs. However, only few studies have exploited the functionality of algae in wetland systems despite that the algae influence phosphorus cycling in wetland systems (Garcia *et al.*, 2010). Algae and algal assemblages can greatly influence phosphorus cycling directly via uptake or release. They also indirectly influence this cycle when photosynthesis induce changes in wetland water and water/soil interface parameters such as dissolved oxygen and pH (Vymazal, 2007). This influences microbial growth in water and soils, thus enhancing phosphorus removal in these systems.

Plant uptake

Macrophytes are well known to removes most phosphorus in wetland systems (Vymazal, 2007). However, the removal efficiency of phosphorus in these systems is dependent on the type of macrophytes and it characteristics. Emergent macrophytes have immense removal of phosphorus compared to submerged macrophytes. This is due to the fact that emergent macrophytes have more access to sunlight for photosynthesis and growth thus enhances phosphorus removal in these systems (Garcia *et al.*, 2010). Phosphorus is removed by macrophytes mostly in growing and humid seasons (spring, summer and autumn). This is because macrophytes are mostly active during warm seasons and accumulate phosphorus for their metabolic regulation and growth. The storage of phosphorus in macrophytes is dependent on phosphorus leaching from detrital tissue, phosphorus translocation from above to below ground biomass and rates of litter decomposition (Kadlec, 2005).

In the above ground biomass, phosphorus storage is short and released throughout decomposition of litter. In wetland systems, parts of macrophytes above the ground propagates and decay, thus releasing the absorbed phosphorus back to the wetland systems. Therefore, in CWs it is imperative to harvest macrophytes before they undergo decaying process. The amount of phosphorus uptake by macrophytes differs among sites of operation, plant type and

seasonal changes. Previously, it has been reported that macrophytes with high growth and productivity like *amaranthus* hybridus and *bidens pilosa* species can remove phosphorus up to 126 mg/l (Kadlec, 2005; Vymazal, 2007; Garcia *et al.*, 2010; Dong, 2013).

The removal efficiency of nitrogen and phosphorus in wetland systems is greatly influenced by several environmental factors. In order to maximize the removal efficiency of nitrogen and phosphorus, it is imperative to study and have extensive understanding of the effect of these environmental factors in nutrients removal (Cia *et al.*, 2013).

1.3.11 Factors Affecting Nutrient Transformation in Constructed Wetlands

The CWs performance is described as the ability of wetland to remove all forms of pollutant in wastewater. The treatment efficiency of CWs is influenced by several chemical and physical parameters. These parameters include temperature, pH, salinity, dissolved oxygen, electrical conductivity, turbidity and total dissolved solids.

• Potential of hydrogen

The potential of hydrogen (pH) influences overall functioning of the constructed wetland in nitrogen and phosphorus removal. This is because microorganisms have a specific pH that complement their growth and activity. Therefore, pH influences microbial communities responsible for nutrient degradation in constructed wetland. Variations in pH can be detrimental to microorganisms through disrupting their enzyme activity, plasma membrane and alters nutrient molecules ionization (Mthembu, 2016). This reduces microbial capacity of removing nutrient in wetland system, thus affecting the overall treatment efficiency of wetlands in nutrient removal (Wongkiew *et al.*, 2017).

In extremely high or low pH levels, wastewater become basic or acidic, thus hindering the growth of macrophytes and microorganism responsible for nutrient transformation and removal

in wetland systems (Wongkiew *et al.*, 2017). Potential of hydrogen ranging between 6.5 and 8.5 promotes the occurrence of ammonification, nitrification and denitrification which are essential processes for nutrient removal in constructed wetland (Vymazal, 2007). This favors the treatment of wastewater such as municipal wastewater since it has a neutral pH (6.0-8.7) that favors the growth of microbes and macrophytes, thus enhancing the removal efficiency of nutrients (Lee *et al.*, 2009).

• Salinity

Inorganic salts enhance microbial growth (He *et al.*, 2012). However, high salinity in wastewater can result in:

- ✓ Cell plasmolysis because of the intense increase in osmotic pressure and alterations of microbial metabolism (Cortés-Lorenzo *et al.*, 2014).
- ✓ Reduce microbial degradation of pollutant because of the lethal effect of sodium content on microbial communities not adapted to high saline concentration (He *et al.*, 2012).
- ✓ Reduction of microbial hydrolytic rates.
- ✓ The density of water might increase in high salinity environment. This can result in the outflow of microorganisms in large numbers with turbid effluent (He *et al.*, 2012).
- ✓ Reduction of COD/BOD₅ (Mthembu, 2016).

The high salinity inhibits microbial growth and activities in wetland systems and subsequently affects the removal of nutrients. Cortes-lorenzo *et al.* (2014) reported that the process of nitrification is more susceptible to high salinity. Even though AOB and NOB respond differently to varying environmental conditions, AOB are more sensitive to high concentrations of salt than NOB. This reduces the rate of nitrification. The levels of salinity beyond the average

range (15 ppt) causes stress to microbial communities and affect nutrient available for plants. This affects the overall treatment efficiency of wetland systems (Cortes-lorenzo *et al.*, 2014).

• Electrical conductivity

Electrical conductivity is the ability of wastewater to conduct ionic activity and electrical current. The high level of electrical conductivity indicates colossal quantities of dissolved salts in wastewater. Denitrification process is adversely affected by high electrical conductivity. This reduces nitrogen removal rates in wetlands. The high levels of salt content from organic waste in wastewater deters the removal efficiency of constructed wetlands. This is because high salt content hinders macrophytes and microbial growth, thus affecting the water quality (Mthembu, 2016).

• Dissolved oxygen

The concentration of dissolved oxygen (DO) is one of the essential parameters in biological wastewater treatment and its distribution in treatment wetland has a direct impact in nutrient assimilation (Shi *et al.*, 2018). Dissolved oxygen in wastewater is reduced by the presence of organic matter which results in the reduction of nutrient breakdown by microorganisms. The high concentration of microbial biofilm is directly proportional to the oxygen demand for nutrient biodegradation. In wastewater treatment, DO concentration ranging between 1.5-2.0 mg/l is regarded as the optimum (Ramdhani, 2013). High levels of DO indicate that nutrient concentration in water is low, whereas low amount of DO indicate that the concentration of pollutants in water is relatively high. High levels of oxygen in constructed wetlands is essential since it enhances the process of nitrification (Mthembu, 2016).

• Hydraulic retention time / hydraulic load

Hydraulic retention time and hydraulic load are essential factors that control the removal of nutrients in treatment wetlands. Increasing hydraulic load permits water to pass through wetland substrate rapidly. This reduces contact time with the substrate, thus decreasing the removal of nutrients (Shi *et al.*, 2018). Meanwhile, increasing hydraulic retention time increases the contact period between nutrients, macrophytes, substrate and microbial biofilm. This leads to high removal of nutrients in constructed wetland systems (Shi *et al.*, 2018).

• Turbidity

Turbidity is the degree at which water loses its transparency because of the suspended particulates. Turbidity is influenced by several factors such as re-suspended sediments from the bottom, sediments from erosion and temperature (Shadrack *et al.*, 2015). Shadrack *et al.* (2015) reported that cloudy water indicates more suspended solids present in wastewater. This may prevent sunlight from reaching macrophytes resulting in limited aquatic plant growth which reduces nutrient removal. In addition, pollutants may adhere to suspended solids and precipitates at the bottom of the wetland system and become more concentrated resulting in clogging, thus reducing the performance of wetland systems in removing the nutrients (Shadrack *et al.*, 2015).

• Total dissolved solids (TDS)

Total dissolved solids (TDS) are described as all the molecules of salts, minerals and metals that are completely dissolved in water. They are composed of inorganic salts such as calcium, potassium, sodium, magnesium, sulfates, bicarbonates and chlorides. In drinking water and natural water bodies, TDS originate from urban run-off, wastewater, nature of piping or hardware utilized in plumbing and the chemical compounds used during the treatment processes. Total dissolved solids influence diffusion of oxygen required by microbes to degrade nutrients in constructed wetlands. Total dissolved solids are also regarded as one of the important indicators of pollutants amount in water. Therefore, it must always be monitored, and the satisfactory values of TDS range is between 500-750 mg/l (Mthembu, 2016).

• Seasonal temperature variation

The overall functioning of constructed wetlands is predominantly influenced by seasonal temperature variation (El-Refaie, 2010; Pei *et al.*, 2010). Seasonal variation influences the temperatures of water in constructed wetland, thus affecting microbial and macrophytes growth and functionality (El-Refaie, 2010). This has a profound impact on all the biological, chemical and physical processes responsible for nitrogen and phosphorus removal in wetlands systems (Mthembu, 2016).

In seasons with warm temperatures, microorganisms and macrophytes grows optimally. This enhances their functioning in nutrient removal in wetland systems. However, in cold temperatures (winter) it is reported that nutrient removal by constructed wetland declines due to the fact that cold temperatures inhibit microbial and macrophytes growth and activity (Feng *et al.*, 2012: Mancilla-Villalobos *et al.*, 2013; Zhang *et al.*, 2017). In addition, macrophytes in cold seasons easily wither and undergo dormancy which affect their functioning in nutrient removal (Gao *et al.*, 2014). Furthermore, in winter the production of essential dissolved oxygen by macrophytes is reduced which affects the activity of aerobic microorganisms in nutrient assimilation. (Feng *et al.*, 2012). The decrease in oxygen production by macrophytes in cold season is attributed to weak plants metabolism caused by plant senescence, thus affecting biological processes responsible for nutrient degradation (Zhang *et al.*, 2017).

Saeed *et al.* (2012) and Zhang *et al.* (2017) alluded that essential biological processes such as nitrification and denitrification in treatment wetland systems occur optimally at temperatures

ranging between 20 and 40^oC. Normally, these temperatures normal occurs in spring, summer and autumn. In these warm seasons, nitrogen and phosphorus can be reduced to 90% (Saeed *et al.*, 2012). However, Pei *et al.* (2010) noted that these nutrients may still be assimilated in low temperatures ranging between 10 and 20^oC. However, the removal in these low temperatures occurs at a slow rate. This is because nitrifying and denitrifying bacterial grow steadily at these temperatures. Zhang *et al.* (2017) reported that in cold temperatures nitrogen and phosphorus removal can be decreased by 45% and 16% respectively.

Zhang *et al.* (2014) proposed that constructed wetlands are more efficient in developing countries like South Africa because of their warm tropical and subtropical climatic conditions. This is because warm temperatures are conducive for macrophytes growth and microbial activities responsible for nutrient removal in CWs. Therefore, this supports the use of constructed wetland for wastewater treatment in countries like South Africa. In addition, South African Weather Services reported that the climate in Zululand region (Empangeni) where this study was conducted is subtropical with an average temperature of 29.4°C. In winter the average temperature is 14.5°C during the day and may drop to 11.3°C at night. These temperatures are suitable for important biological processes like nitrification and denitrification. Therefore, this supports the use of constructed wetland for wastewater treatment in this region.

1.4 CONCLUSION

Developing a green technology for wastewater treatment is of significant importance in order to protect the natural water bodies that serve as sources of drinking water and other domestic and industrial purposes. Despite CWs showing a great removal efficiency of nutrients, their removal efficiency under different seasons of the year is not well understood. Therefore, studying the effectiveness of these systems seasonally is important since this knowledge and understanding may help in optimizing these systems for wastewater treatment. This can lead to the complete removal of nutrients in wastewater prior to discharge, thus decreasing the frequent occurrence of eutrophication. This will further improve public and environmental health.

1.5 REFERENCES

Adewumi, J.R., Ilemobade, A.A. and Van Zyl, J.E., 2010. Treated wastewater reuse in South Africa: Overview, potential and challenges. *Resources, Conservation and Recycling*, 55, pp.221-231.

Adhikari, U., 2012. Treatment of Agricultural Wastewater with Constructed wetlands. Michigan State University (Doctoral dissertation).

Adrados, B.Á.R.B.A.R.A., Sánchez, O.L.G.A., Arias, C.A., Becares, E.L.O.Y., Garrido, L.A.U.R.A., Mas, J.O.R.D.I., Brix, H.A.N.S. and Morató, J.O.R.D.I., 2014. Microbial communities from different types of natural wastewater treatment systems: vertical and horizontal flow constructed wetlands and biofilters. *Water Research*, *55*, pp.304-312.

Awolusi, O.O., 2016. Evaluation of seasonal impacts on nitrifiers and nitrification performance of a full-scale activated sludge system. Durban University of technology (Doctoral dissertation).

Azizullah, A., Khattak, M.N.K., Richter, P. and Häder, D.P., 2011. Water pollution in Pakistan and its impact on public health—a review. *Environment International*, *37*, pp.479-497.

Bartolome, A.P., Villaseñor, I.M. and Yang, W.C., 2013. "Bidens pilosa L. (Asteraceae): Botanical Properties, Traditional Uses, Phytochemistry, and Pharmacology," Evidence-Based Complementary and Alternative Medicine, 2013, pp. 51.

Button, M., Nivala, J., Weber, K.P., Aubron, T. and Müller, R.A., 2015. Microbial community metabolic function in subsurface flow constructed wetlands of different designs. *Ecological Engineering*, *80*, pp.162-171.

Cai, T., Park, S.Y. and Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. *Renewable and Sustainable Energy Reviews*, *19*, pp.360-369.

Calheiros, C.S.C., Teixeira, A., Pires, C., Franco, A.R., Duque, A.F., Crispim, L.F.C., Moura, S.C. and Castro, P.M.L., 2010. Bacterial community dynamics in horizontal flow constructed wetlands with different plants for high salinity industrial wastewater polishing. *Water Research*, *44*, pp.5032-5038.

Corbella, C., Garfí, M. and Puigagut, J., 2014. Vertical redox profiles in treatment wetlands as function of hydraulic regime and macrophytes presence: surveying the optimal scenario for microbial fuel cell implementation. *Science of the Total Environment*, 470, pp.754-758.

Cortés-Lorenzo, C., Sipkema, D., Rodríguez-Díaz, M., Fuentes, S., Juárez-Jiménez, B., Rodelas, B., Smidt, H. and González-López, J., 2014. Microbial community dynamics in a submerged fixed bed bioreactor during biological treatment of saline urban wastewater. *Ecological Engineering*, *71*, pp.126-132.

Deepnarain, N., 2014. Development and optimization of remedial measures to control filamentous bacteria in a full-scale biological nutrient removal plant. Durban University of Technology (Doctoral dissertation).

Dong, Y., 2013. Application of integrated constructed wetlands for contaminant treatment and diffusion. University of Edinburgh (Doctoral dissertation).

Dos Santos, S., Adams, E.A., Neville, G., Wada, Y., De Sherbinin, A., Bernhardt, E.M. and Adamo, S.B., 2017. Urban growth and water access in sub-Saharan Africa: Progress, challenges, and emerging research directions. *Science of the Total Environment*, 607, pp.497-508.

El-Refaie, G., 2010. Temperature impact on operation and performance of Lake Manzala Engineered Wetland, Egypt. *Ain Shams Engineering Journal*, *1*, pp.1-9.

Fan, J., Zhang, J., Ngo, H.H., Guo, W. and Yin, X., 2016. Improving low-temperature performance of surface flow constructed wetlands using Potamogeton crispus L. plant. *Bioresource Technology*, *218*, pp.1257-1260.

Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J., Camper, A.K. and Stein, O.R., 2009. Microbial processes influencing performance of treatment wetlands: a review. *Ecological Engineering*, *35*, pp.987-1004.

Feng, W., Liu, Y., Ma, Y., Wu, X. and Yang, H., 2012. Characterization of nitrification and microbial community in a shallow moss constructed wetland at cold temperatures. *Ecological Engineering*, *42*, pp.124-129.

Fu, G., Huangshen, L., Guo, Z., Zhou, Q. and Wu, Z., 2017. Effect of plant-based carbon sources on denitrifying microorganisms in a vertical flow constructed wetland. *Bioresource Technology*, 224, pp.214-221.

Gao, J., Wang, W., Guo, X., Zhu, S., Chen, S. and Zhang, R., 2014. Nutrient removal capability and growth characteristics of Iris sibirica in subsurface vertical flow constructed wetlands in winter. *Ecological Engineering*, *70*, pp.351-361.

Garcia, J., Rousseau, D.P., Morato, J., Lesage, E.L.S., Matamoros, V. and Bayona, J.M., 2010. Contaminant removal processes in subsurface-flow constructed wetlands: a review. *Critical Reviews in Environmental Science and Technology*, *40*, pp.561-661.

Hampel, K., 2013. The Characterization of Algae Grown on Nutrient Removal Systems and Evaluation of Potential Uses for the Resulting Biomass. Western Michigan University (Doctoral dissertation).

He, X., Liu, Y.L., Conklin, A., Westrick, J., Weavers, L.K., Dionysiou, D.D., Lenhart, J.J., Mouser, P.J., Szlag, D. and Walker, H.W., 2016. Toxic cyanobacteria and drinking water: impacts, detection, and treatment. *Harmful Algae*, *54*, pp.174-193.

He, Y., Tao, W., Wang, Z. and Shayya, W., 2012. Effects of pH and seasonal temperature variation on simultaneous partial nitrification and anammox in free-water surface wetlands. *Journal of Environmental Management*, *110*, pp.103-109.

Hoffmann, S. and Boyd, J., 2006. Environmental Fees: can incentives help solve the Chesapeake's nutrient pollution problems. *Resources for the Future*, 2006, pp. 06-38.

Huang, J., Xu, C.C., Ridoutt, B.G., Wang, X.C. and Ren, P.A., 2017. Nitrogen and phosphorus losses and eutrophication potential associated with fertilizer application to cropland in China. *Journal of Cleaner Production*, *159*, pp.171-179.

Iasur-Kruh, L., Hadar, Y., Milstein, D., Gasith, A. and Minz, D., 2010. Microbial population and activity in wetland microcosms constructed for improving treated municipal wastewater. *Microbial Ecology*, *59*, pp.700-709.

Ibekwe, A.M., Lyon, S.R., Leddy, M. and Jacobson-Meyers, M., 2007. Impact of plant density and microbial composition on water quality from a free water surface constructed wetland. *Journal of Applied Microbiology*, *102*, pp.921-936.

Ilyas, H. and Masih, I., 2017. The performance of the intensified constructed wetlands for organic matter and nitrogen removal: A review. *Journal of Environmental Management*, *198*, pp.372-383.

Jampeetong, A., Brix, H. and Kantawanichkul, S., 2012. Effects of inorganic nitrogen forms on growth, morphology, nitrogen uptake capacity and nutrient allocation of four tropical aquatic macrophytes (Salvinia cucullata, Ipomoea aquatica, Cyperus involucratus and Vetiveria zizanioides). *Aquatic Botany*, *97*, pp.10-16.

Kadlec, R.H., 2005. Phosphorus removal in emergent free surface wetlands. *Journal of Environmental Science and Health*, 40, pp.1293-1306.

Kelly, P.T. and He, Z., 2014. Nutrients removal and recovery in bioelectrochemical systems: a review. *Bioresource Technology*, *153*, pp.351-360.

Kings, S. 2014. South Africa may need to turn to private sector to solve its water crisis. http://mg.co.za/article/2014-07-29-sa-may-need-to-turn-to-private-sector-to-solve-its-water-crisis. Accessed 14 November 2018.

Kivaisi, A.K. 2001. The potential for constructed wetlands for wastewater treatment and reuse in developing countries. *Ecological Engineering*. *16*, pp. 45-560.

Lai, J., 2013. Impact of anthropogenic pollution on selected biota in Loskop Dam. University of Pretoria (Doctoral dissertation).

Lareen, A., Burton, F. and Schäfer, P., 2016. Plant root-microbe communication in shaping root microbiomes. *Plant Molecular Biology*, *90*, pp.575-587.

Lee, C.G., Fletcher, T.D. and Sun, G., 2009. Nitrogen removal in constructed wetland systems. *Engineering in Life Sciences*, *9*, pp.11-22.

Leung, J.Y., Cai, Q. and Tam, N.F., 2016. Comparing subsurface flow constructed wetlands with mangrove plants and freshwater wetland plants for removing nutrients and toxic pollutants. *Ecological Engineering*, *95*, pp.129-137.

Li, M., Zhou, Q., Tao, M., Wang, Y., Jiang, L. and Wu, Z., 2017. Comparative study of microbial community structure in different filter media of constructed wetland. *Journal of Environmental Sciences*, 22, pp.127-133

Liang, Y., Zhu, H., Bañuelos, G., Yan, B., Zhou, Q., Yu, X. and Cheng, X., 2017. Constructed wetlands for saline wastewater treatment: A review. *Ecological Engineering*, *98*, pp.275-285.

Liu, J., Wu, Y., Wu, C., Muylaert, K., Vyverman, W., Yu, H.Q., Muñoz, R. and Rittmann, B., 2017. Advanced nutrient removal from surface water by a consortium of attached microalgae and bacteria: a review. *Bioresource Technology*, *241*, pp.1127-1137.

Machado, A.I., Beretta, M., Fragoso, R. and Duarte, E., 2017. Overview of the state of the art of constructed wetlands for decentralized wastewater management in Brazil. *Journal of Environmental Management*, *187*, pp.560-570.

Mancilla-Villalobos, R., Zúñiga, J., Salgado, E., Schiappacasse, M.C. and Chamy Maggi, R., 2013. Constructed wetlands for domestic wastewater treatment in a Mediterranean climate region in Chile. *Electronic Journal of Biotechnology*, *16*, pp.5-5.

Mathebbula, 2015. Assessment of surface water quality of the main rivers feeding the Katse Dam, Lesotho. University of Pretoria (Masters dissertation).

Matthews, M.W., 2014. Eutrophication and cyanobacterial blooms in South African inland waters: 10 years of MERIS observations. *Remote Sensing of Environment*, *155*, pp.161-177.

Maucieri, C., Barbera, A.C., Vymazal, J. and Borin, M., 2017. A review on the main affecting factors of greenhouse gases emission in constructed wetlands. *Agricultural and Forest Meteorology*, 236, pp.175-193.

Molobela, I.P. and Sinha, P., 2011. Management of water resources in South Africa: A review. *African Journal of Environmental Science and Technology*, *5*, pp.993-1002.

Morris, L., Colombo, V., Hassell, K., Kellar, C., Leahy, P., Long, S.M., Myers, J.H. and Pettigrove, V., 2017. Municipal wastewater effluent licensing: A global perspective and recommendations for best practice. *Science of The Total Environment*, *580*, pp.1327-1339.

Mthembu, M.S., 2016. Removal of organic and inorganic nutrients in a constructed rhizofiltration system using macrophytes and microbial biofilms. Durban University of Technology (Doctoral dissertation).

Mthembu, M.S., Odinga, C.A., Swalaha, F.M. and Bux, F., 2013. Constructed wetlands: A future alternative wastewater treatment technology. *African Journal of Biotechnology*, *12*.

Mugagga, F. and Nabaasa, B.B., 2016. The centrality of water resources to the realization of Sustainable Development Goals (SDG). A review of potentials and constraints on the African continent. *International Soil and Water Conservation Research*, *4*, pp.215-223.

Mulenga, K., 2017. Challenges of water management at local government municipal level in the Eastern Cape of South Africa. University of Witwatersrand (Doctoral dissertation).

Mustafa, A., 2010. Nutrient removal with integrated constructed wetlands: microbial ecology and treatment performance evaluation of full-scale integrated constructed wetlands. University of Edinburgh. (Doctoral dissertation).

Ndlela, L.L., Oberholster, P.J., Van Wyk, J.H. and Cheng, P.H., 2016. An overview of cyanobacterial bloom occurrences and research in Africa over the last decade. *Harmful Algae*, 60, pp.11-26.

Nhapi, I. and Tirivarombo, S., 2004. Sewage discharges and nutrient levels in Marimba River, Zimbabwe. *Water SA*, *30*, pp.107-113.

Nkosi, B.R., 2015. Analysis of water pollution control laws in South Africa: a comparative analysis of South Africa, India and the United Kingdom. University of Limpopo (Doctoral dissertation).

Ntshobeni, N.B., 2015. Antibiogram and virulence determinants of pseudomonas and *Legionella* Spp. recovered from treated wastewater effluents and receiving surface water in Durban. University of KwaZulu-Natal (Doctoral dissertation).

Nyenje, P.M., Foppen, J.W., Uhlenbrook, S., Kulabako, R. and Muwanga, A., 2010. Eutrophication and nutrient release in urban areas of sub-Saharan Africa—a review. *Science of the Total Environment*, *408*, pp.447-455.

O'Brien, M., 2014. The worth of water: A look at the water scarcity crisis and the perceptions of the basic need of water in South Africa. Trinity College. (Masters dissertation).

Ochieng, G.M., Seanego, E.S. and Nkwonta, O.I., 2010. Impacts of mining on water resources in South Africa: A review. *Scientific Research and Essays*, *5*, pp.3351-3357.

Olaniran, A.O., Naidoo, S. and Pillay, B., 2012. Surveillance of invasive bacterial pathogens and human enteric viruses in wastewater final effluents and receiving water bodies–a case study from Durban, South Africa. *CLEAN–Soil, Air, Water, 40*, pp.681-691.

Oon, Y.L., Ong, S.A., Ho, L.N., Wong, Y.S., Oon, Y.S., Lehl, H.K. and Thung, W.E., 2015. Hybrid system up-flow constructed wetland integrated with microbial fuel cell for simultaneous wastewater treatment and electricity generation. *Bioresource Technology*, *186*, pp.270-275.

Pei, Y., Yang, Z. and Tian, B., 2010. Nitrate removal by microbial enhancement in a riparian wetland. *Bioresource technology*, *101*, pp.5712-5718.

Pindihama, G.K., Gumbo, J.R. and Oberholster, P.J., 2011. Evaluation of a low cost technology to manage algal toxins in rural water supplies. *African Journal of Biotechnology*, *10*, pp. 19883-19889

Rajasulochana, P. and Preethy, V., 2016. Comparison on efficiency of various techniques in treatment of waste and sewage water–A comprehensive review. *Resource-Efficient Technologies*, 2, 175-184.

Ramdhani, N., 2013. Detection and quantification of nitrifying bacteria from South African biological nutrient removal plants. Durban University of Technology (Doctoral dissertation).

Saeed, T. and Sun, G., 2012. A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. *Journal of Environmental Management*, *112*, pp.429-448.

Salamah, S., 2017. Biological nutrient removal (BNR) process optimization and recovery of embedded energy using biodiesel by-product. University of Central Florida (Doctoral dissertation).

Sehar, S., Naeem, S., Perveen, I., Ali, N. and Ahmed, S., 2015. A comparative study of macrophytes influence on wastewater treatment through subsurface flow hybrid constructed wetland. *Ecological Engineering*, *81*, pp.62-69.

Shadrack, M., Charles, N., Kamau, G., Gilbert, O., Phanice, W., Joyce, K. and Dennis, C., 2015. Physical Parameters of Tropical Wetlands in Lake Victoria Basin: A Case Study of Kigwal/Kimondi, Nyando and Nzoia Wetlands. *Journal of Environmental & Analytical Toxicology*, *5*, p.1.

Shi, W., Li, H. and Li, A., 2018. Mechanism and influencing factors of nitrogen removal in subsurface flow constructed wetland. *Applied Chemical Engineering*, *1*(1).

Srivastava, J.K., Chandra, H., Kalra, S.J., Mishra, P., Khan, H. and Yadav, P., 2016. Plantmicrobe interaction in aquatic system and their role in the management of water quality: a review. *Applied Water Science*, *7*, pp.1079-1090. Stetter, M.G. and Schmid, K.J., 2017. Analysis of phylogenetic relationships and genome size evolution of the Amaranthus genus using GBS indicates the ancestors of an ancient crop. *Molecular Phylogenetics and Evolution*, *109*, pp.80-92.

Swarts, R.J., 2010. An investigation into a treatment strategy for the Berg River at the Voelvlei wastewater treatment plant. University of Pretoria (Doctoral dissertation).

Tabane, L.I., 2017. The effects of water scarcity on rural livelihoods: a case study of Borakalalo village in Lehurutshe (North West Province). University of Witwatersrand (Doctoral dissertation).

Tan, E., Hsu, T.C., Huang, X., Lin, H.J. and Kao, S.J., 2017. Nitrogen transformations and removal efficiency enhancement of a constructed wetland in subtropical Taiwan. *The Science of the Total Environment*, *601*, p.1378.

Thabethe, P.D., 2011. An evaluation of water and sanitation provision by Uthukela Water in selected districts of KwaZulu-Natal. University of KwaZulu-Natal (Doctoral dissertation).

Thieu, V., Garnier, J. and Billen, G., 2010. Assessing the effect of nutrient mitigation measures in the watersheds of the Southern Bight of the North Sea. *Science of the Total Environment*, 408, pp.1245-1255.

Trevino-Garrison, I., DeMent, J., Ahmed, F.S., Haines-Lieber, P., Langer, T., Ménager, H., Neff, J., Van Der Merwe, D. and Carney, E., 2015. Human illnesses and animal deaths associated with freshwater harmful algal blooms—Kansas. *Toxins*, *7*, pp.353-366.

Van der Merwe, I.S.W., 2016. An evaluation of the eutrophication prevention potential of high rate Algae Ponds through the development of a deterministic design model. Stellenbosch University. (Doctoral dissertation).

Varol, M. and Şen, B., 2012. Assessment of nutrient and heavy metal contamination in surface water and sediments of the upper Tigris River, Turkey. *Catena*, *92*, pp.1-10.

Verhoeven, J.T. and Meuleman, A.F., 1999. Wetlands for wastewater treatment: opportunities and limitations. *Ecological Engineering*, *12*, pp.5-12.

Viero, F., Bayer, C., Fontoura, S.M.V. and Moraes, R.P.D., 2014. Ammonia volatilization from nitrogen fertilizers in no-till wheat and maize in southern Brazil. *Revista Brasileira de Ciência do Solo*, *38*, pp.1515-1525.

Vymazal, J. and Březinová, T., 2015. The use of constructed wetlands for removal of pesticides from agricultural runoff and drainage: a review. *Environment International*, 75, pp.11-20.

Vymazal, J., 2007. Removal of nutrients in various types of constructed wetlands. *Science of the Total Environment*, *380*, pp.48-65.

Vymazal, J., 2009. The use constructed wetlands with horizontal sub-surface flow for various types of wastewater. *Ecological Engineering*, *35*, pp.1-17.

Vymazal, J., 2014. Constructed wetlands for treatment of industrial wastewaters: a review. *Ecological Engineering*, 73, pp.724-751.

Wang, M., Zhang, D.Q., Dong, J.W. and Tan, S.K., 2017. Constructed wetlands for wastewater treatment in cold climate—A review. *Journal of Environmental Sciences*, *57*, pp.293-311.

Wang, R., Baldy, V., Périssol, C. and Korboulewsky, N., 2012. Influence of plants on microbial activity in a vertical-downflow wetland system treating waste activated sludge with high organic matter concentrations. *Journal of Environmental Management*, *95*, pp.158-S164.

Westerhoff, P., Sharif, F., Halden, R., Herckes, P., and Rosa Krajmalnik-Brown., 2014. Constructed Wetlands for Treatment of Organic and Engineered Nanomaterial Contaminants of Emerging Concerns. *Water Research Foundation*. (WaterRF Report 4334)

Wise, D.R. and Johnson, H.M., 2011. Surface-water nutrient conditions and sources in the United States Pacific Northwest. *JAWRA Journal of the American Water Resources Association*, 47, pp.1110-1135.

Withers, P.J.A. and Jarvie, H.P., 2008. Delivery and cycling of phosphorus in rivers: a review. *Science of the Total Environment*, 400, pp.379-395.

Wongkiew, S., Hu, Z., Chandran, K., Lee, J.W. and Khanal, S.K., 2017. Nitrogen transformations in aquaponic systems: A review. *Aquacultural Engineering*, *76*, pp.9-19.

Wu, H., Zhang, J., Li, P., Zhang, J., Xie, H. and Zhang, B., 2011. Nutrient removal in constructed microcosm wetlands for treating polluted river water in northern China. *Ecological Engineering*, *37*, pp.560-568.

Wu, H., Zhang, J., Ngo, H.H., Guo, W., Hu, Z., Liang, S., Fan, J. and Liu, H., 2015. A review on the sustainability of constructed wetlands for wastewater treatment: design and operation. *Bioresource Technology*, *175*, pp.594-601.

Wu, S., Kuschk, P., Brix, H., Vymazal, J. and Dong, R., 2014. Development of constructed wetlands in performance intensifications for wastewater treatment: a nitrogen and organic matter targeted review. *Water Research*, *57*, pp.40-55.

Xu, P., Zeng, G.M., Huang, D.L., Feng, C.L., Hu, S., Zhao, M.H., Lai, C., Wei, Z., Huang, C., Xie, G.X. and Liu, Z.F., 2012. Use of iron oxide nanomaterials in wastewater treatment: a review. *Science of the Total Environment*, *424*, pp.1-10.

Zhang, D.Q., Jinadasa, K.B.S.N., Gersberg, R.M., Liu, Y., Ng, W.J. and Tan, S.K., 2014. Application of constructed wetlands for wastewater treatment in developing countries–a review of recent developments (2000–2013). *Journal of Environmental Management*, *141*, pp.116-131.

Zhang, D.Q., Jinadasa, K.B.S.N., Gersberg, R.M., Liu, Y., Tan, S.K. and Ng, W.J., 2015. Application of constructed wetlands for wastewater treatment in tropical and subtropical regions (2000–2013). *Journal of Environmental Sciences*, *30*, pp. 30-46.

Zhang, J., Sun, H., Wang, W., Hu, Z., Yin, X., Ngo, H.H., Guo, W. and Fan, J., 2017. Enhancement of surface flow constructed wetlands performance at low temperature through seasonal plant collocation. *Bioresource Technology*, 224, pp.222-228.

Zhang, S., Xiao, R., Liu, F., Zhou, J., Li, H. and Wu, J., 2016. Effect of vegetation on nitrogen removal and ammonia volatilization from wetland microcosms. *Ecological Engineering*, *97*, pp.363-369.

Zhang, Y., 2012. Design of a Constructed Wetland for Wastewater Treatment and Reuse in Mount Pleasant, Utah. Utah State University (Masters dissertation).

Zhao, Y., Liu, B., Zhang, W., Hu, C. and An, S., 2010. Effects of plant and influent C: N: P ratio on microbial diversity in pilot-scale constructed wetlands. *Ecological Engineering*, *36*, pp.441-449.

Zhou, X., He, Z., Jones, K.D., Li, L. and Stoffella, P.J., 2017. Dominating aquatic macrophytes for the removal of nutrients from waterways of the Indian River Lagoon basin, South Florida, USA. *Ecological Engineering*, *101*, pp.107-119.

CHAPTER 2: DETERMINATION OF NUTRIENT REMOVAL AND THE SEASONAL EFFECT OF PHYSIOCHEMICAL PARAMETERS ON NUTRIENT REMOVAL IN A MICROCOSMIC WETLAND

2.1 INTRODUCTION

Eutrophication caused by the disposal of nutrient-rich effluents continues to be a prevalent, economic and pervasive environmental problem affecting the quality and integrity of surface water (Nyenje *et al.*, 2010; Kerr, 2014). This has a negative effect on human health, habitat quality and biodiversity (Sutherland *et al.*, 2017). These challenges have impelled the use of green technologies such as CWs which is described as an ideal alternative technology for nutrient removal in wastewater (Vymazal, 2010).

The application of these new innovative technologies has evolved enormously. This is due to these technologies being eco-friendly, cost effective and possess high oxidation capacity of nutrients (Yan *et al.*, 2014). Furthermore, these systems are reported to assimilate nutrients up to 90% through different mechanisms such as nitrification, denitrification and plant uptake (Zhang *et al.*, 2014). Previous studies conducted by Wu *et al.* (2011) and Cui *et al.* (2013) reported that nutrient removal in these systems is highly dependent on physiochemical parameters such as dissolved oxygen, total dissolved solids, temperature and pH amongst other (Cui *et al.*, 2013). Therefore, the treatment efficiency of these systems can be optimized through comprehensive understanding the influence dynamics of these physiochemical parameters on nutrient removal.

Leung *et al.* (2016) reported that pH is not a limiting factor while temperatures which are influenced by seasonal changes are the main driving force of nutrient assimilation in wetland systems. This is because temperatures can negatively or positively affect the activity of microorganisms and macrophytes in wetlands depending on the operational season (Zhao *et*

al., 2015). Mancilla-Villalobos *et al.* (2013) reported that warm seasons provided favourable conditions for microbial and macrophytes growth and activity. This enhanced bioaccumulation of nutrient through the processes of nitrification, denitrification and phytoremediation. However, these processes may be impeded in cold season, hence nutrient removal might be reduced in cold seasons (Gao *et al.*, 2014).

Gao et al. (2014) reported that cold seasons interfered with metabolism and enzymatic activity of microorganism in constructed wetland. Previous studies also reported that macrophytes undergoes senescence, insulation and dormancy in cold season (Meng et al., 2014; Yan et al., 2014; Fan et al. 2016). Furthermore, Fan et al. (2016) reported that plants photosynthetic rate was reduced in cold season. This subsequently limited oxygen diffusion into the sediments, rhizosphere and water surface which is imperative for the growth and proliferation of nitrifiers and denitrifiers. In turn, this affected the overall treatment efficiency of constructed wetland microcosm. In contempt of these challenges, Yan et al. (2014) reported that diverse phytoremediators composition may enhance microbial diversity and activity in CWs and enhance nutrient removal more effectively than a monoculture of macrophytes. This is due to the fact that different macrophytes exhibit different oxygen transfer rates (Meng et al., 2014). This can subsequently enhance the biological processes such as nitrification and denitrification in constructed wetland systems. Therefore, in this study Amaranthus hybridus and Bidens pilosa plants were used as macrophytes to evaluate nutrient removal and the effects of pH, temperature and dissolved oxygen and COD on nutrient removal from constructed wetland microcosms.

2.2 AIM AND OBJECTIVES

The aim of this chapter was to determine nutrient removal and the seasonal effect of physiochemical parameters on nutrient removal in a microcosmic wetland.

The specific objectives of this chapter were:

- (a) To measure pH, DO, COD and temperature in the wetland microcosm over warm and cold seasons.
- (b) To determine seasonal nutrient removal in wetland microcosm.
- (c) To determine the relationship between physiochemical parameters and nutrient removal in wetland microcosm.

2.3 METHODOLOGY

2.3.1 Constructed Wetland Microcosm Setup

Two free water surface flow wetland microcosms (Figure 2.1) were setup using polyethylene bath tubs of the same size with a height of 60 cm and 74 cm width. The bath tubs were labelled A and B. Both bath tubs were filled up to quarter with sand soil (20 cm) and gravel (5 cm). The sand soil served as a growth medium for macrophytes while gravel served as a substrate for biofilm attachment. Bath tub A was used as an experimental/planted section while bath tub B was used as reference section. The experimental section was planted with *Amarunthus hybridus* and *Bidens pilosa* seeds harvested in the vicinity of the University of Zululand, KwaDlangezwa Campus. The seeds were randomly sowed by hand at roughly equal depth (5 cm) and irrigated with water until germination. Then the plants were irrigated with municipal wastewater collected at Vulindlela Wastewaters Treatment Plant every 2 days. This was done to facilitate their growth and adaptability to wastewater. Meanwhile, the reference section was unplanted and only filled with gravel and soil. The planted section was filled with 5 litres of municipal wastewater and allowed to circulate for a week. This was done to enhance macrophytes adaptability to high water levels of wastewater. After a week, the previously added wastewater

was drained and new 25 litres of municipal wastewater was introduced in both wetlands. The initial water samples were than collected for analyses prior treatment.



Figure 2.1: Three dimensional structure of unplanted constructed wetland microcosm with the above mentioned measurements. Diagram (A) represents side view, B cross section and C top view.

2.3.2 Water Sampling and Physiochemical Parameter Analysis

Water samples were collected aseptically at 8am in morning from both experimental and reference section using autoclaved 250 ml Schott bottles. The samples were collected at 4 dayintervals for a duration of one month. The samples were then analysed on site immediately after collection for temperature, pH and dissolved oxygen using Ino_Lab IDS 9310 multiparameter probe. The samples were then transported on ice to the laboratory for the analysis of organic matter.

2.3.3 Organic Matter Analysis

The collected water samples were analysed for ammonia, nitrite, nitrate, phosphate and chemical oxygen demand in triplicate for statistical analyses. Organic matter was analysed by the spectrophotometric method using Spectroquant® Pharo 100. The spectrophotometer was

used with cell and reagent test kits obtained at Merck following manufacturer's protocol as explained bellow:

• Ammonia

The ammonium test kits 000683 (Merck) was used for the analysis of ammonia following manufactures protocol and the pH of the samples was maintained within 4-13 using sulphuric acid. Five millilitres of NH₄-1 was pipetted into 10 ml test tube followed by pipetting 200 μ l of the wastewater using a micropipette. The content was vigorously mixed and 1 microspoon of reagent NH₄-2 was added and then mixed using a hand until the mixture was completely dissolved. The content was allowed to react for 15 minutes and transferred into 10 mm cuvette, then measured with a Spectroquant[®] Pharo 100 (Merck) and the results were recorded.

• Nitrite

The test kits 14776 (Merck) was used following manufacturers protocol and the pH of the samples was maintained within 2-10 using sulphuric acid. Five millilitres of the sample was transferred into the test tube using a micropipette and 1 microspoon of reagent NO₂-1 was added. The content was mixed until completely dissolved, then allowed to react for 10 minutes and transferred into a 10 mm cuvette. The concentration was measured using a Spectroquant® Pharo 100 (Merck) and the results were recorded.

• Nitrate

The cell test kits 14776 (Merck) was used following manufacturers protocol. One microspoon of reagent NO₃-1 was transferred into an empty dry test tube and 5 millilitres of NO₃-2 was pipetted into the test tubes. The content was mixed until completely dissolved and 1.5 μ l of the sample was pipetted into the test tube. The content was mixed and allowed to react for 10

minutes. The content was then transferred to 10 mm cuvette and the concentration was measured using a Spectroquant® Pharo 100 (Merck) and the results were recorded.

Phosphorus

The cell test kits 14729 (Merck) was used following manufactures protocol and the pH was within the specified range (0-10). One millilitre of the sample was transferred into the reaction cell and 1 dose of P-1K was added. The content was mixed and heated in an aqualytic thermoreactor ET108 (Merck) at 120^oC for 30 minutes. The cells were then removed from the thermoreactor and allowed to cool at room temperature. Five drops of P-2K were added and mixed until completely dissolved. One dose of P-3K was added using a blue-dose-metering cap. The content was mixed and allowed to react for 15 minutes. The concentration was measured using Spectroquant® Pharo 100 (Merck) and the results were recorded.

• Chemical oxygen demand

The cell tests were carefully held on the neck and swirled to suspend the bottom sediments. The sample (3 ml) was pipetted into the reaction cell and the screw cap was tightly closed. The content was vigorously mixed and the cell tests were heated for 120 minutes at 148^oC in an aqualytic thermoreactor ET108 (Merck). After 2 hours of heating, the cells were removed from the thermoreactor and placed in a test-tube rack to cool at room temperature for 10 minutes. The cell tests were then swirled and placed back on the test-tube rack for complete cooling. The concentration was measured using a Spectroquant® Pharo 100 (Merck) and the results were recorded.

2.3.4 Statistical Analysis

The data obtained was analysed using inferential statistics, where the *t-test* (paired) was carried out to compare nutrient removal during wastewater treatment before and after treatment. The

removal was considered significant at $p \le 0.05$. Pearson's coefficient (*r*) was used to correlate physiochemical parameters with nutrient removals.

2.4 RESULTS AND DISCUSSION

The result of physiochemical parameters are presented in Table 2.1. The results indicated that the physiochemical parameters were influenced by seasonal variation.

2.4.1 Physiochemical Parameters Analysis

Physiochemical parameters are important parameters in treatment wetland because of their potential to directly or indirectly influence bioremediation of nutrients. These parameters influence microbial and macrophytes activities, thus affecting the overall nutrient removal in the wetland systems.

Warm season		Cold season		Wastewater discharged
Planted	Reference	Planted	Reference	limits
28-39	27-37	14-28	15-26	No range
5.3-10	4.2-9.0	5.7-9.0	4.1-8	4.0-11
5.4-8.5	5.2-7.8	6.3-7.4	5.0-6.6	5.0-9.5
0.3-14*	$0.4 - 9.7^{*}$	3.4-12*	7.5-12*	7
0.1-0.98	0.3-0.9	0.1-0.7	0.1-0.8	15
0.1-14	0.4-14.4	2.5-6.8	4.7-6.8	15
0.3-10	0.4-8.0	3.7-7.3	5.2-7.1	10
94-314*	122-300*	95-250 [*]	120-240*	75
	Warm Planted 28-39 5.3-10 5.4-8.5 0.3-14* 0.1-0.98 0.1-14 0.3-10 94-314*	Warm seasonPlantedReference28-3927-375.3-104.2-9.05.4-8.55.2-7.80.3-14*0.4-9.7*0.1-0.980.3-0.90.1-140.4-14.40.3-100.4-8.094-314*122-300*	Warm season Cold served Planted Reference Planted 28-39 27-37 14-28 5.3-10 4.2-9.0 5.7-9.0 5.4-8.5 5.2-7.8 6.3-7.4 0.3-14* 0.4-9.7* 3.4-12* 0.1-0.98 0.3-0.9 0.1-0.7 0.1-14 0.4-14.4 2.5-6.8 0.3-10 0.4-8.0 3.7-7.3 94-314* 122-300* 95-250*	Warm seasonCold seasonPlantedReferencePlantedReference28-3927-3714-2815-265.3-104.2-9.05.7-9.04.1-85.4-8.55.2-7.86.3-7.45.0-6.60.3-14*0.4-9.7*3.4-12*7.5-12*0.1-0.980.3-0.90.1-0.70.1-0.80.1-140.4-14.42.5-6.84.7-6.80.3-100.4-8.03.7-7.35.2-7.194-314*122-300*95-250*120-240*

Table 2.1: Physiochemical parameters obtained from the microcosm in warm and cold seasons.

* Values above discharged standards

2.4.1.1 Potential of hydrogen (pH)

The results of pH in warm and cold seasons are represented in Figure 2.2. In warm season, the initial mean value of pH was 5.4 and 5.2 in the planted and reference section while in cold

season, it was 7 in the planted and 6.5 in reference section respectively. An increase in pH values from acidic to basic was observed in warm season in both planted and reference sections.



Planted section (Warm season)
Reference section (Warm season)
Planted section (Cold season)
Reference section (Cold season)

Figure 2.2: pH variation (planted and reference section) from constructed wetland microcosm in warm and cold season. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

The mean pH values obtained in warm season ranged between 6.2-8.5 and 6.5-7.8 in planted and reference sections with fluctuating patterns observed at different hydraulic retention time. These pH ranges were within discharged limits of treated wastewater into natural waster bodies as stipulated by the Department of Water and Sanitation (SA). In a study conducted by Sehar *et al.* (2013), similar results were obtained which also indicated that the wetland systems normal pH should range between 6.5 and 8.5 in warm seasons. Lu *et al.* (2014) noted that pH values ranging between 6.5 and 8.7 are conducive for the optimal growth of nutrient reducing bacteria and macrophytes. This enhanced the occurrence of nitrification and denitrification which are responsible for nutrient removal in wetland systems. Based on the study conducted by Lu *et al.* (2014), it can be deduced that the wetland microcosms used in this study provided favourable environmental conditions for the growth of microorganisms and macrophytes, thus enhanced nutrient removal. This was supported by the high oxidation of ammonia (Figure 2.6), nitrite (Figure 2.7), nitrate (Figure 2.8) and phosphorus (Figure 2.9) in warm season. Meanwhile, in cold season the mean values of pH initially decreased in the first 4 days of treatment in both systems (planted and reference section) reaching 6.3 planted and 6.2 in reference section. This can be explained by the decrease in temperature which might have affected the activity of microorganism and macrophytes. This notable decrease in pH coincided with low removal of ammonia (Figure 2.6) and nitrite (Figure 2.7) respectively. After 4 days of treatment, the planted section demonstrated sudden increase in pH concentration reaching the highest mean value of 7.4 while in the reference section the continual decrease was observed until the 16th day where the pH was 5. The sudden increase in the pH values in the planted section after 4 days was attributed to adaptability and activity of macrophytes in transferring sufficient oxygen and remediating nutrients in the systems. The observed continual decrease in mean pH values from the reference section might be attributed to organic acids that were produced by the decay of organic matter (Cortes-Lorenzo *et al.*, 2014).

In a survey conducted by Zhang *et al.* (2010), a similar trend was observed in cold season where the pH values initially decreased prior to increasing. This emphasized that seasonal changes from warm to cold season affects the growth and activity of microorganisms and macrophytes, thus affected nutrient removal. Despite the initial decrease in pH value in cold season, the pH values were within the discharged limit in both warm and cold season. However, the planted sections demonstrated the highest pH values than reference section. This indicated that the presence of macrophytes increases the pH value of wastewater in wetland microcosm. Based on these results it can be concluded that the wetland microcosms have a potential to keep pH values of treated wastewater at acceptable levels.

2.4.1.2 Temperature

Water temperature determines the suitability of water for use or survival of organisms and the functionality of aquatic systems (Mthembu, 2016). Figure 2.3 shows the temperature results

obtained in warm and cold seasons. Warm season had the highest temperature than cold season. In warm season, the temperature ranged between 28-39^oC and 27-37^oC in planted and reference sections respectively.

These results were conducive for nutrient transformation. This was supported by previous studies which alluded that temperature ranging between $20-40^{\circ}$ C enhanced nutrient removal in wetland systems (Wu *et al.*, 2011; Yan *et al.*, 2014; Mesquita *et al.*, 2017). This was due to the fact that these temperatures enhanced the processes of nitrification, denitrification and phytoremediation in the microcosms (Fan *et al.*, 2016). Furthermore, this was supported by the current study as the highest removal of ammonia (Figure 2.6), nitrite (Figure 2.7), nitrate (Figure 2.8) and phosphorus (Figure 2.8) was obtained in warm season.



Planted section (Warm season)
Reference section (Warm season)
Planted section (Cold season)
Reference section (Cold season)

Figure 2.3: Temperature variation (planted and reference section) from constructed wetland microcosm in warm and cold season. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

In cold the season, the temperature significantly declined and ranged between $14-28^{\circ}$ C and $15-26^{\circ}$ C in planted and reference sections respectively. Yan *et al.* (2014) reported that decreasing temperature inhibited plant metabolism. This led to insufficient oxygen transfer in the rhizome which supressed the growth and activity of microorganisms, thus affecting nutrient biotransformation (Fan *et al.*, 2016). In the present study, nutrients were still removed optimally despite the decrease in temperature observed in the cold season. This was supported

by the removals of ammonia (Figure 2.6), nitrite (Figure 2.7), nitrate (Figure 2.8) and phosphorus (Figure 2.9) which were all above 50%. These findings suggested that macrophytes provided oxygen and carbon source for microorganisms despite the decrease in temperature. This enhanced microbial activity and subsequently enhanced nutrient reduction. El-sheik *et al.* (2010), Meng *et al.* (2014) and Yan *et al.* (2014) reported that nitrification and denitrification may still occur at slow rate in temperatures ranging between 10 and 20^oC. This could be the reason for the significant removal of nutrients in the cold season in this study.

2.4.1.3 Dissolved oxygen (DO)

Dissolved oxygen is one of the significant parameters in the assessment of water quality, and it reflects the biological and physical processes occurring in natural water bodies (Sehar *et al.*, 2013). Figure 2.4 present the DO results obtained in warm and cold seasons in microcosms. Initially, the mean concentration of DO was 8.2 mg/l in the planted, and 7.5 mg/l in the reference section in warm season while in cold season it was 9 and 8 mg/l in planted and reference sections respectively. However, a substantial decrease was observed in the first 8 days of treatment, followed by an increase from both season in planted and reference sections. This indicated that there was a high concentration of pollutant in the first 8 days in the microcosms. Furthermore, this could suggest that macrophytes were still adapting to wastewater with high amount of nutrients. In cold season (Figure 2.3).


Planted section (Warm season) Reference section (Warm season) Planted section (Cold season) Reference section (Cold season)

Figure 2.4: Dissolved oxygen variation (planted and reference section) from constructed wetland microcosm in warm and cold season. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

After 8 days of treatment, DO concentrations increased in both seasons with warm season showing the highest increase of 10 and 9 mg/l in planted and reference sections respectively while in cold season it was 9 and 8 mg/l in planted and reference sections. These results were in line with the studies by Stefanakis *et al.* (2012) and Bosak *et al.* (2016) who reported that DO concentrations were high in warm seasons than in cold seasons. The high concentration of DO in warm seasons is associated with an increase in temperature which provided a complimentary condition for the growth and activity of photosynthetic macrophytes (Khisa *et al.*, 2014). This subsequently enhanced DO transfer into the rhizosphere and water bodies. Meanwhile, Mesquita *et al.* (2017) reported that low DO concentrations in cold seasons indicated that the growth and photosynthesis of macrophytes is hindered by cold season, thus less oxygen is transferred into the system. This was supported by the current study as warm season showed high concentration of DO than cold season (Figure 2.4). Despite DO concentrations demonstrating seasonality, the obtained mean values in the current study were within the discharged limits set by the Department of Water and Sanitation.

The obtained DO concentrations in warm and cold seasons enhanced the occurrence of nitrification and denitrification in the microcosms. This was supported by the studies conducted

by El-Sheik *et al.* (2010) and Hsueh *et al.* (2014) who reported the DO above 1.5 mg/l stimulates the occurrence of nitrification and denitrification. Based on these findings, it was concluded that the wetland microcosms have a potential of supplying the sufficient oxygen to the level of sustaining, as well as reducing nutrients and contamination in a microcosm.

2.4.1.4 Chemical oxygen demand (COD)

Chemical oxygen demand is defined as the measure of the capacity of water to utilize oxygen during the oxidation of organic matter (Xhu *et al.*, 2010). Figures 2.5 present the COD results obtained in both warm and cold seasons. Primarily, COD was high in both seasons reaching above 200 mg/l. There was a statistical significant difference (p< 0.05) when the planted section in warm season was compared with the planted section in cold season with warm season retaining more removal efficiency. The removal ranged between 30-74% and 15-59% in planted and reference sections. In cold season, the removal ranged between 20-64% and 5-50% in planted and reference sections respectively. These results were similar to the results obtained by Mancilla-Villalobos *et al.*, (2013), Gunes *et al.*, (2012) and Taylor *et al.* (2011) who reported that COD removal was high in warm seasons and decrease in cold seasons. Furthermore, Taylor *et al.* (2011) noted that planted sections demonstrated the highest removal than unplanted section. Similar observations were demonstrated by the present study in both seasons where the planted sections had high removal efficiency of COD than reference sections.



Planted section (Warm season)
Reference section (Warm season)
Planted section (Cold season)
Reference section (Cold season)

Figure 2.5: Chemical oxygen demand variation (planted and reference section) from constructed wetland microcosm in warm and cold season. The mean value of water samples are shown for each day. Whiskers represent standard errors of means.

The high removal of COD in warm season was attributed to inclination of temperatures. Fan *et al.* (2016) reported that high temperature promoted the activity of heterotrophic bacteria and phytoremediators, therefore stimulating the removal of COD in wetland systems. Gagnon *et al.* (2010) and Mancilla-Villalobos *et al.* (2013) reported that COD removal in cold season decreased due to low temperature which subsequently led to the decaying of macrophyte biomass, thus hindered COD removal. This could be the reason for the decrease in COD removal observed in cold season in this study.

Zhu *et al.* (2014) reported that increasing HRT enhanced COD removal in wetland systems. This was supported by the current study where a strong and a very strong positive correlation was observed in planted (r=0.83) and reference (r=0.87) sections in warm season respectively. In cold season, a strong positive correlation was observed in planted section (r=0.69) while a fair positive correlation was observed in the reference section (r=0.46). This indicated that elongated HRT influenced COD removal in the microcosm. However, in the current study COD removal did not meet the discharged standards despite microcosms showing high removal efficiency of COD.

2.4.2 Nutrient Removal

The removal of nutrients in wetland system is highly complex and is depended on numerous processes instigated by microbial and macrophytes activities. In this study, the removal of ammonia, nitrate, nitrate and phosphorus was investigated.

2.4.2.1 Ammonia

Ammonia (NH₃) is one of the main contaminants present in the wastewater. The results of ammonia removal in the microcosms are presented in Figure 2.6. The results did not meet the discharge limit in both seasons despite the planted section showing high removal efficiency compared to the reference section. There was a significant difference (p < 0.05) when the planted section in warm season was compared to the planted section in cold season, with warm season having a significantly high removal of 97% than 69% obtained in the cold season.

A similar trend was observed in the reference sections where the warm season demonstrated the highest removal of 60% than 42% obtained in cold season. These observations could be associated with the seasonal temperature variations (Meng *et al.*, 2014) and these observations were similar to the results obtained by Dong *et al.* (2011), Wu *et al.* (2013), Fan *et al.* (2016) and Mesquita *et al.* (2017). In aforementioned studies, it was argued that the highest ammonium removal in warm season was due to increasing temperature which enhanced oxidation of ammonia via the process of nitrification. Furthermore, Pei *et al.* (2010) and Zhang *et al.* (2017) noted that the oxidation of ammonia via nitrification occurred at stagnant rate in low temperatures such as in cold season. This could be the reason behind the observed decline in ammonia assimilation in cold season of the current study.



Planted section (Warm season)
Reference section (Warm season)
Planted section (Cold season)
Reference section (Cold season)

Figure 2.6: Ammonia removal efficiencies measured in constructed wetland microcosms (planted and reference section) in warm and cold season. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

The high removal of ammonia observed in warm season could also be attributed to the process of phytoremediation. Stefanakis *et al.* (2012) reported that temperatures in warm seasons increase growth, metabolism and activity of phytoremediators. This enhanced bioaccumulation of ammonia in wetlands systems. Zhang *et al.* (2017) further reported that phytoremediation account for more than 21% of total removal of ammonia in warm seasons whereas the phytoremediators undergo senescence and dormancy in cold season which decreases the removal of ammonia. This could be the reason for the differences in ammonia removals obtained in the current study in warm and cold seasons.

In addition to plant uptake, Khisa *et al.* (2014) reported that macrophytes significantly increased the concentration of DO in microcosmic system in warm season compared to cold season. This was due to increasing photosynthetic rate, thus releasing abundant amount of DO into the systems. This increases the growth and activity of nitrifying microorganism such as *Nitrosomonas* species in warm seasons than in cold seasons (Akinbile *et al.*, 2016). In addition, previous studies alluded that the oxidation of ammonia via nitrification improved by 35% in high amount of DO (3.75-10 mg/l) and pH (6-8.5) (El-Sheik *et al.*, 2010; Fan *et al.*, 2016; Zhang *et al.*, 2017). In the present study, the concentrations of DO and pH were within these

reported ranges in both season. Therefore, the oxidation of ammonia in both seasons in the current study may be attributed to plant uptake and nitrification. However, the fluctuations in removal patterns could be accredited to seasonal temperature variation which enhanced microbial and macrophytes metabolic breakdown of ammonia in warm season compared to cold season. The results obtained in this study shows that the microcosm lacked the capacity of reducing ammonia into accepted levels. Furthermore, these results showed that the planted sections were more efficient in ammonium removal compared to unplanted section in both seasons.

2.4.2.2 Nitrite

Nitrite is another form of organic nitrogen found in municipal wastewater. It can be seen from Figure 2.7 that the planted sections had more removal efficiency than reference sections in both seasons. The nitrite reduction was seasonal, with warm season showing high reduction efficacy of 95% compared to 73% obtained in cold season. In the reference sections, similar patterns were observed where warm season showed high removal efficiency of 69% while cold season had 64%. These results indicated that nitrite reduction was seasonal and increasing temperature was directly proportional to nitrite reduction. Despite the demonstrated effect of seasonal variation in nitrate reduction, the results were within the disposal limits.

The highest nitrite reduction in warm season coincided with an increasing pH and temperature. This indicated that warm season provided a favourable environmental conditions for nitrite biotransformation through different mechanisms such plant uptake and denitrification (He *et al.*, 2012). These findings were similar to the studies conducted by He *et al.* (2012) and Mabhena (2012) who reported that nitrite reduction was high in warm seasons compared to cold seasons.



Figure 2.7: Nitrite removal efficiencies measured in constructed wetland microcosms (planted and reference section) in warm and cold season. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

A slight decline in nitrite assimilation observed in cold season was similar to the results of Mabhena (2012). Mabhena (2012) pointed out that the bioaccumulation of nitrite in surface flow constructed wetland in cold season can be reduced by 30% in planted sections and 60% in unplanted (reference) sections. This was due to the decrease in temperature which affected microbial activity and plant photosynthesis, thus affecting biodegradation and phytoextraction of nitrite. This could be the reason for the decline in nitrite reduction observed in cold season in the current study.

In addition to phytoremediators and microbial activity, Wu *et al.* (2011) reported that nitrite reduction was also attributed to HRT. Elongated HRT contributed enormously to nitrite assimilation in the current study as warm season showed a strong positive correlation in the planted (r= 0.79) and reference (r=0.72) sections. Meanwhile, in cold season a very strong positive correlation was observed in both planted (r= 0.93) and reference (r= 0.90) sections. Furthermore, the coefficient of determination (\mathbb{R}^2) indicated that in warm season, HRT contributed 62% and 51% of nitrite assimilation in planted and reference sections respectively. In cold season, HRT contributed 87% and 81% of nitrite oxidation in planted and reference section respectively. These findings demonstrated that HRT played a significant role in nitrite removal in the microcosms. In addition, the overall nitrite reduction results obtained in this study indicated that the microcosms have a capacity of transforming nitrite into acceptable discharge limits. Furthermore, these findings demonstrated that macrophytes played a major role in nitrite assimilation through plant uptake and oxygen transfer, hence a highest nitrite oxidation was obtained in the planted sections than in the reference sections.

2.4.2.3 Nitrate

Nitrate is described as another form of inorganic nitrogen that is essential for microbial and macrophytes cellular growth. Mthembu (2016) pointed out that in surface water, nitrate should be less than 1 mg/l. The results of nitrate are presented in Figure 2.8. In warm season, nitrate concentration ranged between 0.1-14.0 mg/l and 0.40-14.4 mg/l in planted and reference sections while in cold season it ranged between 2.5-6.85 and 4.7-6.8 mg/l in planted section and reference section respectively. Statistical significant differences were obtained when *t*-test was performed to compare the reference sections of warm and cold seasons (p < 0.05).



Planted section (Warm season) Reference section (Warm season) Planted section (Cold season) Reference section (Cold season)

Figure 2.8: Nitrate removal efficiencies measured in constructed wetland microcosms (planted and reference section) in warm and cold season. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

Initially, nitrate concentration was high in both seasons. However, a significant decrease was observed in warm season than in cold season. As a result, the effluent in warm season had less nitrate concentration than the effluent in the cold season. Chang *et al.* (2012) reported that significantly low nitrate concentration in the effluent demonstrate that a complete denitrification process was achieved or nearly attained. Therefore, the low nitrate concentration obtained in the effluent of the current study in warm season might conjecture that complete denitrification was achieved.

Figure 2.8 showed that warm season had the highest removal of 90% and 82% in planted and reference sections, while in cold season it was 65% and 50% in planted and reference sections respectively. These results are similar to the results obtained in a study conducted by Datta *et al.* (2016) where a removal of 6% was obtained in cold season while a removal of 45% was obtained in warm season. These findings emphasised that the occurrence of a major biological processes such as denitrification and plant uptake were enhanced in warm season while in cold season they occurred slowly. This was due to warm season providing favourable environmental conditions for the activity of phytoaccumulators and denitrifying microorganisms (Gagnon *et al.*, 2010; Datta *et al.*, 2016). In a similar study conducted by Gagnon *et al.* (2010) the highest removal in cold season. The high removal in a study by Gagnon *et al.* (2010) in cold season was attributed to the carbon sources (glucose and fructose) that were added to enhance denitrification. Meanwhile, in the current study macrophytes were the only source of carbon. This could be the reason for the less removal if nitrate in this study compared to the results obtained to the results obtained in this study compared to the results obtained by Gagnon *et al.* (2010).

Wu *et al.* (2011) pointed out that reduction nitrate in cold season can be conceivably overcome by elongated HRT. This was in line with the results obtained in the present study as a moderate

positive correlation in planted (r= 0.57) and reference (r= 0.59) sections were observed. Furthermore, the coefficient of determination (\mathbb{R}^2) demonstrated that HRT contributed to 33% and 77% of nitrate removal in planted and reference sections respectively. Therefore, in the current study it was concluded that elongated HRT augment nitrate removal in constructed wetland microcosms. In addition, the overall findings demonstrated that nitrate assimilation was seasonal with more nitrate removal occurring in warm season. It was also observed that the planted sections were more efficient in nitrate removal than the reference sections. These findings demonstrated that microcosms could be used as an alternative green technology for nitrate removal since the results were within the discharge limits.

2.4.2.4 Phosphorus

Phosphorus reduction trend showed high variation in warm and cold seasons (Figure 2.9). During the treatment period, phosphorus concentration in warm season ranged between 0.3-10 and 0.4-8.0 mg/l in planted and reference sections, while in cold season it ranged between 3.7-7.3 mg/l and 5.2-7.1 mg/l in planted and reference sections respectively. These result were within the discharge limit.



Planted section (Warm season) Reference section (Warm season) Planted section (Cold season) Reference section (Cold season)

Figure 2.9: Phosphorus removal efficiencies measured in constructed wetland microcosms (planted and reference section) in warm and cold season. The mean values of three readings are represented by each point with whiskers representing standard errors of means.

The warm season had the highest removal efficiency of 70% and 57% in planted and reference sections respectively. Similar trends were observed in the cold season where the planted section had higher removal of 68% while the reference section had 46%. These results were similar to the results obtained by Yan et al. (2014), where it was reported that phosphorus removal was up to 68.9% in warm season, while in cold season it was 47%. In the present study, the removal was imputed to the substrate used in the microcosms. Akinbile et al. (2016) reported that substrate enhanced phosphorus removal in wetland systems through the processes of adsorption. Rai et al. (2015) reported that the removal of phosphorus could be attributed to phytoremediation which is reported to accumulate phosphorus up to 60%. Furthermore, previous studies alluded that phosphate accumulating microorganisms proliferating in the plant roots can assimilate phosphorus ranging between 31 and 70% (Wu et al., 2011; Rai et al., 2015; Akinbile et al., 2016; Zhang et al., 2017). Keizer-Vlek et al. (2014) and Zhang et al. (2017) reported that the decrease in phosphorus removal in cold season was due to the reduced temperature which interfered with enzymatic activities of microorganisms and macrophytes, thus affecting phosphorus assimilation. Therefore, phosphorus reduction and the noticeable seasonal variations in phosphorus removal in our system could be attributed to the same reasons.

Moreover, the increase in phosphorus removal in warm season coincided with increasing pH DO and temperature while in cold season the pH remained between acidic and neutral while DO and temperature decreased. Previous studies reported that alkaline conditions, high temperatures and DO enhanced microbial activities. This subsequently enhanced phosphorus removal in wetland systems (Lee *et al.*, 2009; Rai *et al.*, 2015; Akinbile *et al.*, 2016; Wongkiew *et al.*, 2017). Therefore, the findings in this study indicated that warm season offered a conditions that promoted the growth and activity of macrophytes and microbial biofilms, thus enhanced phosphorus removal. Meanwhile, in cold season the environmental conditions were

unfavourable for the growth and activity of microorganisms and macrophytes, thus hindered phosphorus removal. The decrease in phosphorus reduction in cold season coincided with the decrease in DO concentration. This emphasised that there was insufficient oxygen available for microbial metabolism and activity which affected their ability to oxidise the available phosphorus in the wetland microcosm.

Rai *et al.* (2015) further reported that the highest removal of phosphorus in constructed wetlands was due to increasing HRT. In the current study, the elongated HRT contributed enormously in phosphorus removal in both warm and cold seasons. The coefficient of determinant (R^2) in warm season was 0.85 in planted section and 0.81 in reference section, while in cold season it was 0.52 and 0.69 in planted and reference sections respectively. These findings indicated that HRT contributed in the overall phosphorus removal in both seasons in the wetland microcosm.

2.4.3 The Effect of Physiochemical Parameters on Nutrient Removal in the Microcosms

The effect of seasonal variation and physiochemical parameters on nutrient removal was determined using linear and nonlinear regression model. This model was chosen based on the physical observation of the coordinates in the graphs. Furthermore, these models offers better correlation results.

2.4.3.1 Effect of pH on nutrient removal

The effect of pH on nutrient removal in wetland microcosm was determined by correlating pH with the removal efficiencies of ammonia, nitrite, nitrate and phosphorus in warm and cold seasons. The effect of pH on nutrient removal in warm season is shown in Figures 2.10 (planted section) and 2.11 (reference section) while the effect of pH on nutrient removal in cold season is shown in Figures 2.12 (planted section) and 2.13 (reference).



Figure 2.10: The effect of pH on nutrient removal during warm season in the planted section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.

It can be seen from the figures that the planted sections in both warm and cold seasons had a positive correlation between pH and nutrient removal efficiencies except for phosphorus removal in cold season (Figure 2.12) where a negative correlation was obtained. The planted section in warm season (Figure 2.10) demonstrated a weak, fair, strong and a very strong positive correlation between pH and the removal efficiencies of ammonia (r= 0.44), nitrate (r= 0.58), phosphorus (r= 0.67) and nitrite (r= 0.94) respectively. While the planted section in cold season (Figure 2.12) showed a fair positive correlation for nitrite (r= 0.62) and nitrate (r= 0.43) while a strong positive and a weak negative correlations was observed for ammonia (r= 0.71) and phosphorus (r= -0.30). In warm season, the planted section (Figure 2.10) had the highest pH value of 8.5 and it was at this pH where phosphorus was removed at a highest level reaching 70%. However, nitrogen removal was highest at pH below 8.5 with optimum removal obtained at pH ranging between 7.2 and 8.0.



Figure 2.11: The effect of pH on nutrient removal during warm season in the reference section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.

These findings suggested that pH above 8.0 decreased the process of nitrification and denitrification. These results were also supported by the survey of Yin *et al.* (2016) which noted that pH above 8.0 hindered the processes of nitrification and denitrification in constructed wetland microcosms. In the planted section of cold season (Figure 2.12), the highest pH value was 7.4. At this pH, nitrogen removal was satisfactory as the removal of ammonia, nitrite and nitrate were 60%, 73% and 60% respectively. However, the removal of phosphorus at this pH was 45%. Mthembu (2016) reported that phosphorus removal increases in acidic condition due to phosphorus binding to aluminium and iron while in neutral and alkaline condition phosphorus forms complexes with calcium and magnesium which decreases the available phosphorus for plant uptake. This could substantiate the low reduction of phosphorus in the present study at the high pH values (neutral and alkaline).

In contrast to planted sections, the reference sections had no correlation and a negative correlation in both seasons. In warm season, the reference section (Figure 2.11) had no correlation for nitrite (r= 0.01) and nitrate (r= 0.02) while a very weak and weak negative correlation was observed for phosphorus (r= -0.17) and ammonia (r= -0.32) respectively. In

the cold season, the reference section (Figure 2.13) had no correlation for ammonia (r= -0.05) and nitrate (r= -0.04) while a weak and a fair negative correlation was obtained for nitrite (r= -0.30) and phosphorus (r= -0.54). This indicated that nutrient removal was inversely proportional to increase in pH in the absence of macrophytes.

Furthermore, the highest mean value of pH in reference section (Figure 2.11) of warm season was 7.8. At this pH, the removal of nitrogen and phosphorus were low with ammonia, nitrite, nitrate and phosphorus showing the removals of 40%, 50%, 47% and 36% respectively. This indicated that more alkaline condition hindered nutrient removal in reference section. Meanwhile, in cold season the highest pH value in reference section (Figure 2.13) was 6.6. It was at this pH where the highest removal of ammonia (42%) and nitrite (64%) were obtained while the highest removals of nitrite (50%) and phosphorus (46%) were obtained at pH below 6.6. Yin *et al.* (2016) stated that high pH affects microbial intracellular metabolic activities, thus affecting biodegradation of nutrient. This was supported by the present study as increasing pH negatively affected the biodegradation of nutrients in the microcosms. Based on the findings of this study, it can be deduced that increasing pH in the reference section negatively affected nitrogen and phosphorus biotransformation in the microcosms. Furthermore, it can be concluded that the presence of macrophytes enhanced microbial activity and nutrient removal in microcosms through the process of plant uptake.



Figure 2.12: The effect of pH on nutrient removal during cold season in the planted section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.



Figure 2.13: The effect of pH on nutrient removal during cold season in the reference section of the wetland microcosm. The mean values of pH were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between pH and nutrient removal efficiencies in the system.

2.4.3.2 Effect of temperature on nutrient removal

The effect of temperature on nutrient removal in the wetland microcosm systems is shown in Figures 2.14 to 2.17. In warm season, the planted section (Figure 2.14) showed a fair positive correlation for ammonia (r= 0.47), nitrite (r= 0.63) and nitrate (r= 0.57) while a phosphorus oxidation had a very strong positive correlation with temperature (r= 0.86). Meanwhile, the planted section in cold season (Figure 2.16) showed positive correlation between temperature and nutrient removal efficiencies. A very strong positive correlation was observed for ammonia

(r=0.95) and nitrite (r=0.93) while a very weak and weak positive correlation was observed for phosphorus (r=0.16) and nitrate (r=0.35).



Figure 2.14: The effect of temperature on nutrient removal during warm season in planted section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.

A similar pattern was observed in the reference section where a positive correlation was obtained between temperature and nutrient removals. In warm season (Figure 2.15), a weak positive correlation was observed for ammonia (r= 0.31) and nitrite (r= 0.39) while a fair positive correlation was shown for nitrate (r= 0.58) and phosphorus (r= 0.61) respectively. In cold season (Figure 2.17), a strong positive correlation was observed for ammonia (r= 0.82) and nitrite (r= 0.78) while a weak positive correlation was observed for nitrate (r= 0.30) and phosphorus (r= 0.39). These results indicated that nutrient removal in the microcosms were reliant on high temperatures. Furthermore, these findings were supported by Yuan *et al.* (2013) who noted that nutrient oxidation via the processes of plant uptake, ammonification, nitrification and denitrification was enhanced by high temperatures.



Figure 2.15: The effect of temperature on nutrient removal during warm season in reference section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.

Lee *et al.* (2009) reported that the process of nitrification and denitrification occurs optimally at temperatures ranging between 20^oC and 40^oC. This was also observed in the present study where the highest temperatures were 39^oC for the planted section in warm season (Figure 2.14) while in planted section in cold season (Figure 2.16) it was 28^oC. At these temperatures, nutrient removal was satisfactory as nitrogen and phosphorus had removals of more than 60%. Similar observations were obtained in the reference sections except in cold season where the removal efficiencies of nitrate and phosphorus were 31 and 32% at a highest temperature. These results were consistent with the studies of Stefanakis *et al.* (2012) and Papaevangelou *et al.* (2016) who reported that increasing temperature enhanced nutrient oxidation. Furthermore, these results were supported by the study of Stefanakis *et al.* (2012) who reported that increased temperature do not only enhance microbial reactions, nitrification and denitrification but also favours the phytoremediation of nutrient in CWs. This was supported by the highest removals obtained in the planted section than in the reference section in both seasons.



Figure 2.16: The effect of temperature on nutrient removal during cold season in planted section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.



Figure 2.17: The effect of temperature on nutrient removal during cold season in reference section of the wetland microcosm. The mean values of temperature were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between temperature and nutrient removal efficiencies in the system.

2.4.3.3 Effect of dissolved oxygen on nutrient removal

The removal of nitrogen and phosphorus was dependent on the levels of DO concentration except in cold season where a negative correlation was demonstrated in the reference section. The planted sections in warm (Figure 2.18) and cold (Figure 2.20) seasons showed a positive

correlation between DO and the removal of ammonia, nitrite, nitrate and phosphorus. Similar results were obtained in the reference section in warm season (Figure 2.19).



Figure 2.18: The effect of DO on nutrient removal during warm season in planted section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.

Based on these findings it can be deduced that DO concentration was seasonal and that increasing DO levels enhanced nutrient removal. These findings were also supported by Stefanakis *et al.* (2012) who reported that DO was seasonal and increasing DO concentration enhanced nitrogen and phosphorus. Mthembu (2016) reported that upsurge DO concentration create aerobic conditions that enhanced microbial enzymatic activities. In addition, Quan *et al.* (2012) alluded that increasing DO did not only support microbial growth and respiration in the wetland systems but also created a breeding ground for microorganisms for fast growth. This justifies the direct proportionality between increasing DO and increasing nutrient removal in this study.

The highest DO concentration in the planted sections was 10 and 9 mg/l in warm (Figure 2.18) and cold (Figure 2.20) seasons respectively. At this level of DO, nutrient removal was above 50% except for ammonia in cold season (Figure 2.19) which showed 41% removal. The reference section in warm (Figure 2.19) and cold (Figure 2.21) seasons had 9 and 8 mg/l as the

highest DO concentrations. At this DO levels, nitrogen and phosphorus oxidation were above 50% in warm season. However, in cold season (Figure 2.21) nutrient removal was reduced at highest DO concentration with ammonia, nitrite, nitrate and phosphorus reduced by 12%, 24%, 11% and 19% respectively. Based on these finding it can be concluded that increasing DO concentration enhanced the removal of organic matter in the planted sections and reference section in warm season.



Figure 2.19: The effect of DO on nutrient removal during warm season in reference section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.



Figure 2.20: The effect of DO on nutrient removal during cold season in planted section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.



Figure 2.21: The effect of DO on nutrient removal during cold season in reference section of the wetland microcosm. The mean values of DO were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between DO and nutrient removal efficiencies in the system.

2.4.3.4 Effect of COD on nutrient removal

The effect of COD on nutrient oxidation is shown in Figures 2.22 to 2.25. Nutrient transformation increased with increasing COD removal, except for nitrate in reference section in cold season (Figure 2.25). In warm season, the planted section (Figure 2.22) had a very strong positive correlation for nitrite (r= 0.86) while ammonia, nitrate and phosphorus showed a strong positive correlation. The planted section in cold season (Figure 2.24) had a very weak and weak positive correlation for nitrate and phosphorus, a fair positive correlation was obtained for ammonia (r= 0.52) and nitrite (r= 0.62).

Similarly, a fair positive correlation was observed for ammonia (r= 0.52) while a strong positive correlation was observed for nitrite (r= 0.89), nitrate (r= 0.84) and phosphorus (r= 0.68) in reference section of warm season (Figure 2.23). In contrast, the reference section in cold season (Figure 2.25) had a very weak negative correlation for nitrate (r= -0.12). Ammonia and phosphorus had a weak positive correlation while nitrite demonstrated a fair positive correlation (r= 0.49). Based on these findings, it can be concluded that the removal of COD was directly proportional to nutrient removal. This could be due to the fact that COD is another contaminant found in wastewater that is biodegraded by microorganisms and macrophytes

(Mthembu. 2016). These findings were supported by the study of Lu *et al.* (2014) who noted that nutrient removal was directly proportional to COD removal.



Figure 2.22: The effect of COD on nutrient removal during warm season in planted section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.



Figure 2.23: The effect of COD on nutrient removal during warm season in reference section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. Linear fits were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.



Figure 2.24: The effect of COD on nutrient removal during cold season in planted section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.



Figure 2.25: The effect of COD on nutrient removal during cold season in reference section of the wetland microcosm. The mean values of COD were model against the corresponding nutrient removal efficiencies. Linear and non-linear curve fit were used to demonstrate the relationship between COD and nutrient removal efficiencies in the system.

2.5 CONCLUSION

Based on the results, free water surface flow constructed wetland microcosm's planted with *Amarunthus hybridus* and *Bidens pilosa* have a great potential of reducing nutrients. The major findings were:

• The performance of microcosms was influenced by seasonal variation with highest removal efficiency obtained in warm season.

- Nutrient removal from the systems was dependent on the presence of macrophytes with ammonia, nitrite and nitrate showing the highest removal efficiency in warm season than in the cold season.
- Physiochemical parameters (DO and pH) were found to be within the discharged limit except for chemical oxygen demand which was above discharged limit after treatment.
- Increasing temperature and DO was directly proportional to nutrient removal in planted and reference section in both warm and cold seasons.
- Increasing COD positively influenced nutrient removal in both seasons except for nitrate in cold season (reference section)
- Increasing pH concentration positively influenced nutrient removal in planted sections of both warm and cold seasons except for phosphorus in cold season.
- Alkaline conditions coupled with increasing temperatures and dissolved oxygen was directly proportional to nutrient removal in warm season.
- Nutrient (nitrite, nitrate and phosphorus) removal met the discharged limit except for ammonia which was found to be above the limit after treatment.

2.6 RECOMMENDATION

Nutrient removal in constructed wetland microcosms is influenced by several factors such as physiochemical parameters, oxygen availability, nutrient concentration and other factors. Therefore, to increase nutrient removal it is recommended that:

- Physiochemical parameters (temperature, pH, DO) are optimized.
- Hydraulic retention time is elongated to increase the biodegradation period of nutrients.
- There's artificial aeration to ensure sufficient supply of oxygen for microbial activities.

These recommendations will not only advance nutrient removal from domestic wastewater but will also secure the safety of the public and environment.

2.7 REFERENCES

Akinbile, C.O., Ogunrinde, T.A., Man, H.C. and Aziz, H.A., 2016. Phytoremediation of domestic wastewaters in free water surface constructed wetlands using Azolla pinnata. *International Journal of Phytoremediation*, *18*, pp.54-61.

Bosak, V., Vander Zaag, A., Crolla, A., Kinsley, C. and Gordon, R., 2016. Performance of a constructed wetland and pretreatment system receiving potato farm wash water. *Water*, *8*, p.183.

Chang, J.J., Wu, S.Q., Dai, Y.R., Liang, W. and Wu, Z.B., 2012. Treatment performance of integrated vertical-flow constructed wetland plots for domestic wastewater. *Ecological Engineering*, *44*, pp.152-159.

Cortés-Lorenzo, C., Sipkema, D., Rodríguez-Díaz, M., Fuentes, S., Juárez-Jiménez, B., Rodelas, B., Smidt, H. and González-López, J., 2014. Microbial community dynamics in a submerged fixed bed bioreactor during biological treatment of saline urban wastewater. *Ecological Engineering*, *71*, pp.126-132.

Cui, L., Ouyang, Y., Gu, W., Yang, W. and Xu, Q., 2013. Evaluation of nutrient removal efficiency and microbial enzyme activity in a baffled subsurface-flow constructed wetland system. *Bioresource Technology*, *146*, pp.656-662.

Datta, A., Wani, S.P., Patil, M.D. and Tilak, A.S., 2016. Field scale evaluation of seasonal wastewater treatment efficiencies of free surface-constructed wetlands in ICRISAT, India. *Current Science*, *110*, pp.1756-1763.

Dong, X. and Reddy, G.B., 2010. Soil bacterial communities in constructed wetlands treated with swine wastewater using PCR-DGGE technique. *Bioresource Technology*, *101*, pp.1175-1182.

El-Sheikh, M.A., Saleh, H.I., El-Quosy, D.E. and Mahmoud, A.A., 2010. Improving water quality in polluted drains with free water surface constructed wetlands. *Ecological Engineering*, *36*, pp.1478-1484.

Fan, J., Zhang, J., Ngo, H.H., Guo, W. and Yin, X., 2016. Improving low-temperature performance of surface flow constructed wetlands using Potamogeton crispus L. plant. *Bioresource Technology*, *218*, pp.1257-1260.

80

Gagnon, V., Maltais-Landry, G., Puigagut, J., Chazarenc, F. and Brisson, J., 2010. Treatment of hydroponics wastewater using constructed wetlands in winter conditions. *Water, Air, & Soil Pollution*, *212*, pp.483-490.

Gao, J., Wang, W., Guo, X., Zhu, S., Chen, S. and Zhang, R., 2014. Nutrient removal capability and growth characteristics of Iris sibirica in subsurface vertical flow constructed wetlands in winter. *Ecological Engineering*, *70*, pp.351-361.

Gunes, K., Tuncsiper, B., Ayaz, S. and Drizo, A., 2012. The ability of free water surface constructed wetland system to treat high strength domestic wastewater: a case study for the Mediterranean. *Ecological Engineering*, *44*, pp.278-284.

He, Y., Tao, W., Wang, Z. and Shayya, W., 2012. Effects of pH and seasonal temperature variation on simultaneous partial nitrification and anammox in free-water surface wetlands. *Journal of Environmental Management*, *110*, pp.103-109.

Hsueh, M.L., Yang, L., Hsieh, L.Y. and Lin, H.J., 2014. Nitrogen removal along the treatment cells of a free-water surface constructed wetland in subtropical Taiwan. *Ecological Engineering*, *73*, pp.579-587.

Keizer-Vlek, H.E., Verdonschot, P.F., Verdonschot, R.C. and Dekkers, D., 2014. The contribution of plant uptake to nutrient removal by floating treatment wetlands. *Ecological Engineering*, 73, pp.684-690.

Kerr, L., 2014. Compelling a nutrient pollution solution: how nutrient pollution litigation is redefining cooperative federalism under the clean water act. *Environmental Law*, *15*, pp.1219-1254.

Khisa, M.K., Tole, M., Obiero, S.A. and Mwangi, S.W., 2014. The Efficacy of a Tropical Constructed Wetland for Treating Wastewater during the Wet Season: The Kenyan Experience. *Journal of Environment and Earth Science*, *4*.

Lee, C.G., Fletcher, T.D. and Sun, G., 2009. Nitrogen removal in constructed wetland systems. *Engineering in Life Sciences*, *9*, pp.11-22.

Leung, J.Y., Cai, Q. and Tam, N.F., 2016. Comparing subsurface flow constructed wetlands with mangrove plants and freshwater wetland plants for removing nutrients and toxic pollutants. *Ecological Engineering*, *95*, pp.129-137.

Lu, H., Chandran, K. and Stensel, D., 2014. Microbial ecology of denitrification in biological wastewater treatment. *Water Research*, *64*, pp.237-254.

Mabhena, B., 2015. Monitoring of selected contaminants (physico-chemical and bacteriological parameters) in wetland filters: A case study of a 10-year old Johannesburg zoo constructed wetland. University of Johannesburg (Doctoral dissertation).

Mancilla Villalobos, R., Zúñiga, J., Salgado, E., Schiappacasse, M.C. and Chamy Maggi, R., 2013. Constructed wetlands for domestic wastewater treatment in a Mediterranean climate region in Chile. *Electronic Journal of Biotechnology*, *16*, pp.5-5.

Meng, P., Pei, H., Hu, W., Shao, Y. and Li, Z., 2014. How to increase microbial degradation in constructed wetlands: influencing factors and improvement measures. *Bioresource Technology*, *157*, pp.316-326

Mesquita, M.C., Albuquerque, A., Amaral, L. and Nogueira, R., 2017. Seasonal variation of nutrient removal in a full-scale horizontal constructed wetland. *Energy Procedia*, *136*, pp.225-232.

Mthembu, M.S., 2016. Removal of organic and inorganic nutrients in a constructed rhizofiltration system using macrophytes and microbial biofilms. Durban University of Technology (Doctoral dissertation).

Nyenje, P.M., Foppen, J.W., Uhlenbrook, S., Kulabako, R. and Muwanga, A., 2010. Eutrophication and nutrient release in urban areas of sub-Saharan Africa—a review. *Science of the Total Environment*, 408, pp.447-455.

Papaevangelou, V., Gikas, G.D. and Tsihrintzis, V.A., 2016. Effect of operational and design parameters on performance of pilot-scale horizontal subsurface flow constructed wetlands treating university campus wastewater. *Environmental Science and Pollution Research*, 23, pp.19504-19519.

Pei, Y., Yang, Z. and Tian, B., 2010. Nitrate removal by microbial enhancement in a riparian wetland. *Bioresource technology*, *101*, pp.5712-5718.

Quan, Y., Han, H. and Zheng, S., 2012. Effect of dissolved oxygen concentration (microaerobic and aerobic) on selective enrichment culture for bioaugmentation of acidic industrial wastewater. *Bioresource Technology*, *120*, pp.1-5.

Rai, U.N., Upadhyay, A.K., Singh, N.K., Dwivedi, S. and Tripathi, R.D., 2015. Seasonal applicability of horizontal sub-surface flow constructed wetland for trace elements and nutrient removal from urban wastes to conserve Ganga River water quality at Haridwar, India. *Ecological Engineering*, *81*, pp.115-122.

Sehar, S., Aamir, R., Naz, I., Ali, N. and Ahmed, S., 2013. Reduction of contaminants (physical, chemical, and microbial) in domestic wastewater through hybrid constructed wetland. *ISRN Microbiology*, 2013. pp. 9.

Stefanakis, A.I. and Tsihrintzis, V.A., 2012. Effects of loading, resting period, temperature, porous media, vegetation and aeration on performance of pilot-scale vertical flow constructed wetlands. *Chemical Engineering Journal*, *181*, pp.416-430.

Sutherland, D.L. and Craggs, R.J., 2017. Utilising periphytic algae as nutrient removal systems for the treatment of diffuse nutrient pollution in waterways. *Algal Research*, *25*, pp.496-506.

Taylor, C.R., Hook, P.B., Stein, O.R. and Zabinski, C.A., 2011. Seasonal effects of 19 plant species on COD removal in subsurface treatment wetland microcosms. *Ecological Engineering*, *37*, pp.703-710.

Vymazal, J., 2010. Constructed wetlands for wastewater treatment. Water, 2, pp.530-549.

Wongkiew, S., Hu, Z., Chandran, K., Lee, J.W. and Khanal, S.K., 2017. Nitrogen transformations in aquaponic systems: A review. *Aquacultural Engineering*, *76*, pp.9-19.

Wu, H., Zhang, J., Li, P., Zhang, J., Xie, H. and Zhang, B., 2011. Nutrient removal in constructed microcosm wetlands for treating polluted river water in northern China. *Ecological Engineering*, *37*, pp.560-568.

Wu, H., Zhang, J., Wei, R., Liang, S., Li, C. and Xie, H., 2013. Nitrogen transformations and balance in constructed wetlands for slightly polluted river water treatment using different macrophytes. *Environmental Science and Pollution Research*, *20*, pp.443-451.

Xu, J., Zhang, J., Xie, H., Li, C., Bao, N., Zhang, C. and Shi, Q., 2010. Physiological responses of Phragmites australis to wastewater with different chemical oxygen demands. *Ecological Engineering*, *36*, pp.1341-1347.

Yan, Y. and Xu, J., 2014. Improving winter performance of constructed wetlands for wastewater treatment in northern China: a review. *Wetlands*, *34*, pp.243-253.

Yin, X., Zhang, J., Hu, Z., Xie, H., Guo, W., Wang, Q., Ngo, H.H., Liang, S., Lu, S. and Wu, W., 2016. Effect of photosynthetically elevated pH on performance of surface flow-constructed wetland planted with Phragmites australis. *Environmental Science and Pollution Research*, *23*, pp.15524-15531.

Yuan, H., Nie, J., Zhu, N., Miao, C. and Lu, N., 2013. Effect of temperature on the wastewater treatment of a novel anti-clogging soil infiltration system. *Ecological Engineering*, *57*, pp.375-379.

Zhang, B.Y., Zheng, J.S. and Sharp, R.G., 2010. Phytoremediation in engineered wetlands: mechanisms and applications. *Procedia Environmental Sciences*, *2*, pp.1315-1325.

Zhang, D.Q., Jinadasa, K.B.S.N., Gersberg, R.M., Liu, Y., Ng, W.J. and Tan, S.K., 2014. Application of constructed wetlands for wastewater treatment in developing countries–a review of recent developments (2000–2013). *Journal of Environmental Management*, *141*, pp.116-131.

Zhang, J., Sun, H., Wang, W., Hu, Z., Yin, X., Ngo, H.H., Guo, W. and Fan, J., 2017. Enhancement of surface flow constructed wetlands performance at low temperature through seasonal plant collocation. *Bioresource Technology*, 224, pp.222-228.

Zhao, X., Wei, Z., Zhao, Y., Xi, B., Wang, X., Zhao, T., Zhang, X. and Wei, Y., 2015. Environmental factors influencing the distribution of ammonifying and denitrifying bacteria and water qualities in 10 lakes and reservoirs of the Northeast, China. *Microbial Biotechnology*, 8, pp.541-548.

Zhu, H., Yan, B., Xu, Y., Guan, J. and Liu, S., 2014. Removal of nitrogen and COD in horizontal subsurface flow constructed wetlands under different influent C/N ratios. *Ecological Engineering*, *63*, pp.58-63.

CHAPTER 3: MICROORGANISMS, POPULATION SHIFT AND THEIR ROLE IN NUTRIENT TRANSFORMATION IN THE MICROCOSM

3.1 INTRODUCTION

Microorganisms are reported to be the main drivers of nutrient assimilation in wetland systems (Ligi *et al.*, 2014). They use nutrient constituents for their growth, anabolism, catabolism and subsequently leading to nutrient removal in wastewater (Weber, 2016). This indicates the importance of comprehensive studying and understanding of microbial community structures, population shift and the effect of seasonal variation on microbial population dynamics in these systems. This will further improve the design, performance and success of these technologies in wastewater treatment.

Fernandes *et al.* (2015) reported that microorganisms use different metabolic reactions to oxidise nutrients in CWs. These include biodegradation, bioaccumulation, nitrogen fixation, nitrification and denitrification. These mechanisms are highly dependent on the relative abundance of microorganisms, microbial community structures and their population shift (Arroyo *et al.*, 2010; Dong *et al.*, 2010; Zielinska *et al.*, 2016; Cao *et al.*, 2017). Microbial population structures, diversity and their role in wetlands are significantly influenced by the presence of macrophytes (Sims *et al.*, 2012). This is attributed to the aerobic and anaerobic conditions provided by macrophytes in wetland systems (Arroyo *et al.*, 2010).

Furthermore, Sims *et al.* (2012) reported that the role of microorganisms and population dynamics in wetlands are considerably affected by environmental and operational parameters such as pH, substrate, dissolved oxygen and seasonal variations. Lee *et al.* (2009) and Chen *et al.* (2017) reported that temperature and seasonal variations are the central components that fuel microbial functionality and community structures in wetlands systems. This is because

temperature influences the concentration of carbon and dissolved oxygen in treatment wetlands through photosynthetic macrophytes. Lee *et al.* (2009) reported that warm seasons enhance the process of photosynthesis, thus enhancing oxygen diffusion in wetlands while cold seasons result in low oxygen transfer into the rhizosphere. Mthembu (2016) further stipulated that high dissolved oxygen enhanced the activity, growth and abundance of nitrifying, denitrifying and phosphate accumulating bacteria. This subsequently enhanced the process of nitrification, denitrification and phosphate biotransformation in wetland systems. Furthermore, various studies have reported that seasonal fluctuation affect microbial abundance and community structures with cold seasons demonstrating a high microbial population shift (Sims *et al.*, 2012; Awolusi, 2016; Ibekwe *et al.*, 2016; Chen *et al.*, 2017). Severe microbial population shift in wetland systems have been reported in countries with extremely low or high temperatures while there is still a limited information about microbial abundance and population shift in countries with temperate conditions (Ibekwe *et al.*, 2016). This is due to the limited studies that have been conducted to evaluate the effect of seasonal variation on microbial abundance, community structures and population shift in countries like South Africa (Awolusi, 2016).

Despite various studies reporting the significance of microorganisms in nutrient biodegradation in treatment wetlands, microbial ecology in these systems has not been adequately studied (Arroyo *et al.*, 2013). Previously, traditional technologies such as plate count, selective media and most probable number count were used to identify microbial abundance, community structures and population shift (Kim *et al.*, 2013). However, these techniques received a lot of drawbacks and impediments due to the fact that 99% of environmental microorganisms cannot be cultured in the laboratory (Lu *et al.*, 2014). Therefore, various studies argued that culture based techniques provided a limited information about microbial population dynamics (Dong *et al.*, 2010; Kim *et al.*, 2013; Lu *et al.*, 2014). This has led to the application of more rapid, selective, sensitive and reliable molecular based technologies for analysing microbial ecology in treatment wetland (Adrados *et al.*, 2014).

Therefore, this chapter was aimed at determining microbial community structures, population shift and the role of microorganisms in nutrient biotransformation during seasonal change in a constructed wetland microcosm using molecular techniques. The knowledge obtained in this study will broaden scientific understanding of microorganisms, microbial community structures, population shift and their role in seasonal removal of nutrients in wetland systems.

3.2. AIM AND OBJECTIVES

The aim of this chapter was to determine microbial community structures of nutrient reducing microorganisms, their seasonal distribution and role in nutrient removal in the microcosm systems.

The objectives of this chapter were:

- a) To identify nutrient oxidizing microorganism in the system.
- b) To determine the seasonal microbial population shift and community structures in the microcosm.
- c) To determine the effect of seasonal variations on microbial population dynamics in the microcosm.

3.3 METHODOLOGY

The following methods were used in order to study microorganisms and their population shift in the wetland microcosms:

3.3.1 Sample Collection

Water samples were collected using autoclaved 500 ml Schott bottles from both planted and reference sections in the cold (May-September) and warm (October-April) seasons. The samples were collected and transported on ice cold cooler bags and analysed within 2 hours after collection. The samples were filtered using membrane filtration using Whatman's filter papers (Sigma-Aldrich). This was done to concentrate microorganisms in the samples. This resulted in the extraction of high quality DNA. After filtration, the filter papers were cut into small squares using a sterile scissor. These small squares were then used to extract the DNA following the manufacturer's protocol.

3.2.2 DNA Extraction

The DNA from water samples was extracted using Zymo Research (ZR) Fungal/Bacterial DNA mini-Prep kit (Inqaba Biotech) following the manufacturers protocol. The procedure was as follows: Small pieces of the filter papers were introduced to 5 ml bashing bead lysis tubes. Two hundred microlitres of phosphate buffered saline (PBS) (137 mM NaCl, 27 mM KCl, 4.3 mM NaHPO₄ and 2.8 mM KH₂PO₄) was added, followed by the addition of 750 μ l of the lysis solution. The sample was vortexed for 10 minutes and centrifuged (Eppendorf 5804 R) at 10 000 xg for a minute. After centrifugation, 400 μ l of supernatant was transferred into the Zymo-Spin IV tube inside the collector tube and centrifuged (Eppendorf 5804 R) again for one minute at 7 000 xg. One thousand two hundred microlitres of DNA binding mixture was transferred into the Zymo III column inside a new collecting tube and centrifuged (Eppendorf 5804 R) at 1 000 xg for one minute. After centrifugation, the flow through in a collection tube was discarded and 800 μ l of a DNA binding mixture was added into the same Zymo III column and centrifuged again for one minute at the same speed as above. Two hundred microlitres of DNA

pre-wash buffer was then added to the Zymo-Spin column in a collection tube and centrifuged (Eppendorf 5804 R) at 10 000 xg for a minute. After centrifugation, 500 μ l of Fungal/Bacterial DNA wash buffer was added to the Zymo-Spin IIC column and centrifuged (Eppendorf 5804 R) for one minute at 1 000 xg. The Zymo- Spin IIC column was then transferred into 1.5 ml Eppendorf tubes and 30 μ l of DNA elution buffer was added and centrifuged for 30 seconds. The quality and quantity of the extracted DNA was then stored at -40°C before use in order to prevent degradation.

3.2.3 DNA Analysis

The extracted DNA was visualized on 1% agarose in the presence of ethidium bromide. The ethidium bromide was use for the proper visualisation of the DNA bands. The agarose was suspended in 0.5 x TAE buffer (2 mM Tris base, 10 mM glacial acetic acid, 5 mM EDTA, pH 8.0). It was then boiled for approximately 3 minutes to dissolve and then cooled, and 15 µl of ethidium bromide was added to make a final concentration of 5 µl. The agarose was poured into a casting tank in the presence of a 20 well-comb for making the gel wells. The comb was then removed after the gel had set. The gel was placed into electrophoresis tank and 0.5 X TAE buffer was added to a level sufficient to cover the gel. The electrophoresis tank was connected to a power source to ensure that the DNA moved toward the anode. The voltage was adjusted to 100 volts and the gel was allowed to run for 45 minutes. The DNA was run along with the GeneRuler 1kb DNA ladder (Inqaba Biotec) for size determination of the extracted DNA. The gel was visualized using a Vilber smart imaging BioVision (Inqaba Biotec). The gel images were used to estimate the seasonal occurrence of microorganisms in the wetland microcosm based on the intensity of the DNA bands obtained from samples collected from warm and cold seasons. To study community structures, population shift and the effect of seasonal variation

on the relative abundance of microorganisms, the extracted DNA was amplified, purified and sequenced as described below.

3.2.4 DNA Amplification

The extracted DNA was amplified using a Thermal cycler (Applied Biosystems) with genus specific primers targeting 16S rRNA genes of nitrifiers, denitrifiers and phosphate accumulating bacteria as explained below:

• Nitrifying microorganisms

Nitrifying microorganisms were amplified using the following primer set: the forward primer EUB8F 5-AGAGTTTGATCMTGGCTCAG-3' and reverse primer UNIV1392R 5-ACGGGCGGTGTGTRC-3'. The polymerase chain reaction mixture had an overall volume of 30 μ l. The reaction mixture contained 3 μ l of 10X Taq buffer (MBI, Fermentas, USA), 0.2 μ l Taq polymerase, 3 μ l dNTP's, 0.2 μ M forward and reverse primers, 100 μ g DNA and 5 μ g of nuclease-free water. The conditions were set up as follows: Initial denaturation cycle was 95°C for 7 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and final extension was at 72°C for 10 minutes.

• Denitrifying microorganisms

Denitrifying microorganisms were amplified using the following primer set: forward primer NIRS832F 5-TACCACCCCGAGCCGGCGCT-3' and reverse primer NIRS3R 5'-GCCGCCGTCRTGVAGGAA-3'. The polymerase chain reaction mixture had an overall volume of 20 μ l. The reaction mixture contained 3 μ l of 10X Taq buffer (MBI, Fermentas, USA), 2.5 units Taq polymerase, 200 μ M dNTP's, 12.5 μ M forward and reverse primers, 100 ng DNA and 8 μ g of nuclease-free water. The conditions were set up as follows: Initial denaturation cycle was 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for
25 seconds, annealing at 65° C for 30 seconds, extension at 72° C for 25 seconds and final extension was at 72° C for 10 minutes.

• Phosphate accumulating bacteria (POA)

Phosphate accumulating microorganisms were amplified using the following primer set: forward primer 27F 5-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1492R 5-GGTTACCTTGTTACGACTT-3'. The polymerase chain reaction mixture had an overall volume of 50 μ l. The reaction mixture contained 2 mM of 10X Taq buffer (MBI, Fermentas, USA), 2.5 unit Taq polymerase, 0.2 mM dNTP's, 0.1 μ M forward and reverse primers, 10 ng DNA and 5 μ g of nuclease-free water. The conditions were set up as follows: Initial denaturation cycle was 94°C for 5 minutes, followed by 27 cycles of denaturation at 94°C for 4 seconds, annealing at 56°C for 4 seconds, extension at 70°C for 1 minute and final extension was at 72°C for 10 minutes.

The PCR products of nitrifiers, denitrifiers and PAO were electrophoresed on a 1% agarose gel as explained in section 3.2.3 and then purified.

3.2.5 DNA Purification from Agarose Gel and Sequencing

The DNA was purified using a Zymoclean gel DNA recovery kits (Inqaba Biotech) following the manufacturer's instructions as follows: The bands from the gel were excised using a sterile razor blade in order to remove the fragments of DNA. The excised DNA fragments were transferred into 1.5 ml microcentrifuge tubes followed by the addition of 150 μ l of agarose dissolving gel (ADG) solution. The samples were then incubated for 10 minutes at 37^oC up until the gel was completely dissolved. This was followed by transferring the dissolved agarose solution into a Zymo-spin I column in a 5 ml collection tube and centrifuged (Eppendorf 5804 R) at 1 000 xg for one minute. The flow through in the collection tube was discarded and 200 μ l of wash buffer was added into the column. This step was repeated twice. The DNA was then

eluted with 10 µl of nuclease free water which was directly added into the column matrix. The purified PCR products were sequenced using the Big DyeTM terminator cycle sequencing kits and the sequences were analysed on an ABI 3730 genetic analyser capillary instrument (Applied Biosystems). The obtained sequences were compared with the sequences from the GeneBank using Basic Local Alignment Search Tool (BLAST) search algorithm from the National Centre for Biotechnology Information (<u>www.ncbi.nim.gov</u>). All bacterial isolates were classified at a genus level.

3.4 RESULTS AND DISCUSSION

The results of the genomic DNA, amplicons and microorganisms identified in the wetland microcosms are presented below.

3.4.1 DNA in Water Samples

Figures 3.1 and 3.2 present the gel electrophoresis results of the DNA isolated from the planted and reference sections of a microcosm in warm and cold seasons. In warm season (Figure 3.1), the planted section (lane 4-6) showed a slightly high quantity of genomic DNA than the reference section (lane 1-3). Similar results were also obtained in cold season (Figure 3.2) where the planted section (lane 4-6) had high quantities of the DNA than the reference section (lane 1-3). The high quantities of DNA in the planted sections indicated a high abundance of microorganisms. DNA marker (lane M) was used to determine the size of the extracted DNA. The size of the isolated genomic DNA was around 10 000 kb in both warm and cold seasons.

Figures 3.3 to 3.8 are results of the amplicons obtained after DNA amplification. Figures 3.3 and 3.4 present the results of genomic amplicons targeting nitrifying microorganisms in cold and warm seasons while Figures 3.5 and 3.6 are the amplicon results of denitrifying bacteria in cold and warm seasons. Figures 3.7 and 3.8 are the genomic amplicons of phosphate

accumulating microorganisms. In all the amplicons, lanes 1-4 present the results of the reference sections while lanes 5-8 are the results of the planted sections. The amplicons of the planted sections (lane 5-8) demonstrated a marginally high amplification than the reference sections in both seasons. However, all the planted sections in cold season had less amplification (DNA producing amplicons) than the planted section in warm season. Based on the differences in the intensity of the isolated genomic DNA and amplicons between the planted and reference sections in both warm and cold seasons, it can be deduced that microbial population dynamics were influenced by seasonal variations. Also, it can be deduced that warm season had high microbial abundance than the cold season. Furthermore, it can be concluded that there was a high microbial abundance and diversity in the planted sections of the microcosms than in the reference sections.



Figure 3.1: Agarose gel electrophoresis of the genomic DNA extracted from wastewater sample in cold season. Lane M represent the DNA marker. Lane 1-3 represent the DNA extracted from the reference section while lane 4-6 represent the DNA extracted from the planted section.



Figure 3.2: Agarose gel electrophoresis of the genomic DNA extracted from wastewater sample in warm season. Lane M represent the DNA marker. Lane 1-3 represent the DNA extracted from the reference section while lane 4-6 represent the DNA extracted from the planted section.



Figure 3.3: PCR product (amplicons) of nitrifying microorganisms in cold season. Lane M is the DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.



Figure 3.4: PCR product (amplicons) of nitrifying microorganisms in warm season. Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.



Figure 3.5: PCR product (amplicons) of denitrifying microorganisms in cold season. Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.



Figure 3.6: PCR product (amplicons) of denitrifying microorganisms in warm season. Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.



Figure 3.7: PCR product (amplicons) of phosphate reducing microorganisms in cold season. Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.



Figure 3.8: PCR product (amplicons) of phosphate reducing microorganisms in warm season. Lane M is a DNA marker. Lane 1-4 represent the amplicons of the reference section while 5-8 represent the amplicons of the planted section.

3.4.2 Microorganisms Identified in Water Samples

Figures 3.9 to 3.11 present the genus of nitrifying, denitrifying and phosphate accumulating microorganisms that were identified in the microcosms. The results indicated that there was a modest fraction of microbial genus in the microcosm with denitrifiers (Figure 3.10) and phosphate accumulating microorganisms (Figure 3.11) showing the highest number of genera. Wang *at al.* (2016) reported that microbial communities in wetland can be fully grown and stabilized after 2-3 months of operation. Meanwhile in the current study, the microcosms were operated for one month in both seasons. This could be the reason for the few genera that were identified. This may suggest that microbial community structures were not fully established in the systems.

Despite the results showing that microorganisms may have not fully developed in the current study, *Nitrosomonas* and *Nitrobacter* species were the most abundant AOB and NOB in warm and cold seasons respectively (Figure 3.9). However, the planted section in both seasons had high microbial population structures and diversity than the reference section. These results

were similar to results that were obtained in the studies conducted by Wang *et al.* (2016) and Sun *et al.* (2017). These studies found that macrophytes significantly enhanced the growth and establishment of AOB and NOB. Furthermore, Sun *et al.* (2017) pointed out that AOB have high ammonia affinity and are adapted to low concentration of ammonia, thus the low level of ammonia in planted microcosms stimulate the growth of AOB. This was supported by the current study as AOB were more abundant in planted section with low ammonium concentration than the reference section.

Nitrosomonas spp. which are known AOB, accounted for 37% in the planted and 22% in the reference section, while in cold season Nitrosomonas accounted for 15% and 10% in the planted and reference sections respectively. Analogous results were obtained for Nitrobacter spp. as well, where in warm season it accounted for 29% and 15% in the planted and reference sections. Meanwhile, in cold season Nitrobacter accounted for 13% in the planted and 8% in the reference section. Other genera such as Nitrosospira, Nitrosococcus, Bacillus, Nitrospira and Nitrosobulus were amongst the other groups of nitrifying organisms that were identified in the wetland microcosms. These genera were more abundant in warm season than in the cold season. Wang et al. (2016) reported that macrophytes in cold season undergo senescence and produce a variety of organic compounds such as volatile fatty acids and amino acids which are labile to nitrifiers. This could be the reason for the nitrifying communities to be less abundant in the cold season in the current study. The decrease in the abundance of nitrifying microorganisms in the cold season was directly proportional to the decrease in the nitrification process. This was supported by the decrease in ammonium (Figure 2.6) and nitrite (Figure 2.7) removal in this study. The aforementioned results imply that Nitrosomonas, Nitrosospira, Nitrosococcus and Nitrosolobus shared the responsibility of oxidizing ammonia into nitrite while only Nitrobacter, Nitrospira and Nitrococcus were responsible for the oxidation of nitrite into nitrate in the microcosm.



Planted section (Warm season)
Reference section (Warm season)
Planted section (Cold season)
Reference section (Cold season)

Figure 3.9: Microbial genera of nitrifying organisms identified in the planted and reference sections of the microcosm in the warm and cold seasons.

In CWs, the process of nitrification is followed by denitrification which is a microbial mediated process. In this process heterotrophs and autotrophic microorganisms reduce inorganic nitrogen into nitrogen gas (Lee *et al.*, 2009). In the current study, a diverse group of denitrifying microorganism were identified (Figures 3.10). This was despite Wang *et al.* (2016) reporting that denitrifying bacteria were commonly established after 75 days of wetland operation. The dominant denitrifying organisms were identified in the planted sections. Gagnon *et al.* (2007) and Fernandes (2014) noted that denitrifiers were significantly stimulated by the presence of macrophytes. This is due to the anaerobic and aerobic conditions provided by the macrophytes. Furthermore, this is attributed to the root exudates which are rich in carbon, enzymes and nutrients that enhanced the occurrence, establishment and stability of denitrifying organisms in a microcosm. Wang *et al.* (2016) and Sun *et al.* (2017) further alluded that denitrifiers were also enhanced by the use of different growth and physiological properties, thus providing different quantities of carbon and exudates in microcosms. This could be the reason for the occurrence of a variety of denitrifying bacteria in the planted section of the current study.

Thauera, Pseudomonas and *Acidovorax* were the most prevalent denitrifying microorganisms in both seasons in the planted and reference sections respectively. The relative abundance of these microbial genera accounted for more than 15% in the planted sections in both seasons. However, their abundance was reduced in the reference sections. Other microbial genera such as *Paracoccus, Bacillus, Rhizobium, Azospira, Clostridium, Rhodoplane, Aeromonas, Rhodobacter, Brazyrhizobium* and *Azoarcus* were also identified in the planted section in warm season. However, *Brazyrhizobium* and *Azoarcus* were not identified in the reference section in warm season. In cold season, similar microbial genera were identified in small quantities. However, genera such as *Rhodoplane, Aeromonas* and *Rhodobacter* were only identified in the planted section while *Brazyrhizobium* and *Azoarcus* were not present in both planted and reference sections. The relatively high abundance of denitrifiers in warm season indicated that the warm season was conducive for the occurrence and development of denitrifiers and subsequently enhancing the process of denitrification in the microcosm. This was supported by the high removal of nitrate in the warm season than in the cold season (Figure 2.8).



Figure 3.10: Microbial genus of denitrifying organisms identified in the planted and reference sections of the microcosm in the warm and cold seasons.

Phosphate accumulating organisms were also identified in the wetland microcosms. Most PAO were identified in warm season than in cold season (Figure 3.11). The observed high abundance

of PAO in warm season coincided with the high reduction of phosphorus in the microcosm in the warm season (Figure 2.9). Most of the PAO identified in the microcosms were gram negative microorganisms. Mthembu (2016) noted that gram negative microorganisms have enzyme phosphatase that enables PAO to degrade and accumulate phosphate into their cell. Furthermore, these microorganisms are capable of oxidizing phosphorus into orthophosphate that is readily available for plant uptake.

Accumulibacter was the most dominant genus in warm season and accounted for 32 and 15% in the planted and reference sections respectively. In a study conducted by Mthembu (2016) similar results were obtained where *Accumulibacter* was the most preeminent microorganism and can accumulate phosphorus up to 48% in wetlands and aquaponic systems. Despite *Accumulibacter* being the most dominant PAO in warm season, its abundance was significantly influenced by seasonal changes from warm to cold season. It accounted for 14 and 6% in the planted and reference sections respectively during cold season. *Bacillus* spp. was the most dominant genus in cold season and accounted for 19 and 9% in the planted and reference sections respectively. The abundance of *Bacillus* spp. in the reference section can be attributed to the ability of these microorganisms to form spores that enables them to thrive under unfavourable conditions (Ibekwe *et al.*, 2017).

Other PAO's such as *Pseudomonas*, *Acinetobacter*, *Citrobacter*, *Burkholderia*, *Azorhizobacter* and *Alcaligenes* were also identified in both seasons except for *Alcaligenes* which were not found in the cold season. Furthermore, *Burkholderia* and *Azorhizobacter* spp were only found in the planted section in cold season. *Pseudomonas* species were also amongst the dominant PAO in both seasons despite being the abundant denitrifier. Mthembu (2016) reported that *Pseudomonas* species are able to oxidize a diverse group of nutrients in wastewater. It is capable of oxidizing both phosphorus and nitrogen in wetlands. Based on these results it can

be deduced that the population structure and diversity of PAO is influenced by the presence of macrophytes and seasonal dynamics.



Figure 3.11: Microbial genera of phosphate accumulating organisms identified in the planted and reference sections of the microcosm in the warm and cold seasons.

3.5 CONCLUSION

Based on these results, it can be concluded that:

- Microorganism were more abundant in warm season than cold season. This indicated that seasonal changes negatively affected microbial population dynamics.
- The planted sections had high microbial abundance and diversity than the reference section. This indicated that macrophytes positively influenced microbial population structure and diversity in the microcosm.
- Nutrient removal in the microcosms was a results of microbial activities and their abundance which varied seasonally. This was supported by the high nitrogen and phosphorus removal in warm season.

3.6 RECOMMENDATIONS

It is recommended that the hydraulic retention time and the operation period of the microcosm is elongated. This will ensure the occurrence, development and stability of microorganisms in the systems. This is because microorganism takes longer to develop and stabilize in a microcosm wetland. Increasing the operation periods of the system can further provide a comprehensive insight of microbial population shift.

3.6 REFERENCES

Adrados, B.Á.R.B.A.R.A., Sánchez, O.L.G.A., Arias, C.A., Becares, E.L.O.Y., Garrido, L.A.U.R.A., Mas, J.O.R.D.I., Brix, H.A.N.S. and Morató, J.O.R.D.I., 2014. Microbial communities from different types of natural wastewater treatment systems: vertical and horizontal flow constructed wetlands and biofilters. *Water Research*, *55*, pp.304-312.

Akinbile, C.O., Ogunrinde, T.A., Man, H.C. and Aziz, H.A., 2016. Phytoremediation of domestic wastewaters in free water surface constructed wetlands using Azolla pinnata. *International journal of Phytoremediation*, *18*, pp.54-61.

Arroyo, P., Ansola, G. and de Miera, L.E.S., 2013. Effects of substrate, vegetation and flow on arsenic and zinc removal efficiency and microbial diversity in constructed wetlands. *Ecological Engineering*, *51*, pp.95-103.

Arroyo, P., Ansola, G., Blanco, I., Molleda, P., Calabuing, E.D. and Saenzde-Mieara, L.E. 2010. Comparative analysis of the composition of bacterial communities from two constructed wetlands for municipal and swine wastewater treatment. *Water and Health*, *8*, 147-157.

Awolusi, O.O., 2016. Evaluation of seasonal impacts on nitrifiers and nitrification performance of a full-scale activated sludge system. Durban University of Technology (Doctoral dissertation).

Cao, Q., Wang, H., Chen, X., Wang, R. and Liu, J., 2017. Composition and distribution of microbial communities in natural river wetlands and corresponding constructed wetlands. *Ecological Engineering*, *98*, pp.40-48.

Chen, Y., Lan, S., Wang, L., Dong, S., Zhou, H., Tan, Z. and Li, X., 2017. A review: driving factors and regulation strategies of microbial community structure and dynamics in wastewater treatment systems. *Chemosphere*, *174*, pp.173-182.

Dong, X. and Reddy, G.B., 2010. Soil bacterial communities in constructed wetlands treated with swine wastewater using PCR-DGGE technique. *Bioresource Technology*, *101*, pp.1175-1182.

Fernandes, J.P., 2014. Response of microorganisms from natural and constructed wetlands to veterinary drugs.

Fernandes, J.P., Almeida, C.M.R., Pereira, A.C., Ribeiro, I.L., Reis, I., Carvalho, P., Basto, M.C.P. and Mucha, A.P., 2015. Microbial community dynamics associated with veterinary antibiotics removal in constructed wetlands microcosms. *Bioresource Technology*, *182*, pp.26-33.

Gagnon, V., Chazarenc, F., Comeau, Y. and Brisson, J., 2007. Influence of macrophyte species on microbial density and activity in constructed wetlands. *Water Science and Technology*, *55*, pp.249-254.

Ibekwe, A.M., Ma, J., Murinda, S. and Reddy, G.B., 2016. Bacterial community dynamics in surface flow constructed wetlands for the treatment of swine waste. *Science of the Total Environment*, 544, pp.68-76.

Ibekwe, A.M., Ma, J., Murinda, S. and Reddy, G.B., 2017. Microbial Diversity in Continuous Flow Constructed a Wetland for the Treatment of Swine Waste. *Hydrol Current Res*, 8, pp.2.

Kim, J., Lim, J. and Lee, C., 2013. Quantitative real-time PCR approaches for microbial community studies in wastewater treatment systems: applications and considerations. *Biotechnology Advances*, *31*, pp.1358-1373.

Lee, C.G., Fletcher, T.D. and Sun, G., 2009. Nitrogen removal in constructed wetland systems. *Engineering in Life Sciences*, *9*, pp.11-22.

Ligi, T., Oopkaup, K., Truu, M., Preem, J.K., Nõlvak, H., Mitsch, W.J., Mander, Ü. and Truu, J., 2014. Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16S rRNA amplicon sequencing. *Ecological Engineering*, *72*, pp.56-66.

Lu, H., Chandran, K. and Stensel, D., 2014. Microbial ecology of denitrification in biological wastewater treatment. *Water Research*, *64*, pp.237-254.

Mthembu, M.S., 2016. Removal of organic and inorganic nutrients in a constructed rhizofiltration system using macrophytes and microbial biofilms (Doctoral dissertation).

Sims, A., Gajaraj, S. and Hu, Z., 2012. Seasonal population changes of ammonia-oxidizing organisms and their relationship to water quality in a constructed wetland. *Ecological Engineering*, *40*, pp.100-107.

Sun, H., Liu, F., Xu, S., Wu, S., Zhuang, G., Deng, Y., Wu, J. and Zhuang, X., 2017. Myriophyllum aquaticum constructed wetland effectively removes nitrogen in swine wastewater. *Frontiers in Microbiology*, 8, p.1932.

Wang, Q., Xie, H., Ngo, H.H., Guo, W., Zhang, J., Liu, C., Liang, S., Hu, Z., Yang, Z. and Zhao, C., 2016. Microbial abundance and community in subsurface flow constructed wetland microcosms: role of plant presence. *Environmental Science and Pollution Research*, 23, pp.4036-4045.

Weber, K., 2016. Microbial community assessment in wetlands for water pollution control: Past, present, and future outlook. *Water*, *8*, p.503.

Zielińska, M., Rusanowska, P., Jarząbek, J. and Nielsen, J.L., 2016. Community dynamics of denitrifying bacteria in full-scale wastewater treatment plants. *Environmental Technology*, *37*, pp.2358-2367.

CHAPTER 4: EXECUTIVE DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 INTRODUCTION

Water pollution associated with the disposal of nutrient-rich wastewater effluent continues to be a major public and environmental concern especially in developing countries like South Africa. This is due to the possible contribution to eutrophication in natural water sources (Dossantos *et al.*, 2017). The occurrence of eutrophication can lead to the destruction of aquatic ecosystem and chronic poisoning of people (Azzizulah *et al.*, 2011). This indicates the importance of searching for an alternate technology for wastewater treatment to reduce the amount of nutrient reaching natural water bodies.

Constructed wetlands are an ideal alternative green technology for wastewater treatment. This is due to their high nutrient removal, low capital investment requirement, produce high quality effluent, ease of operation and maintenance (Saeed *et al.*, 2012; Wang *et al.*, 2017). The removal of organic and inorganic nutrients in these systems ensues as a result of complex interaction between macrophytes, substrate and microbial biofilms (Vymazal *et al.*, 2014; Li *et al.*, 2017).

4.2 NUTRIENT REMOVAL

Nutrients were removed in both seasons through the combined activity of microorganisms and macrophytes. Nitrogen in the form of ammonia, nitrite and nitrate were removed up to 97%, 95%, and 90% while the removal of phosphorus was up to 70%. The highest removals were obtained in the planted section of the warm season. This indicated that seasonal changes influenced nutrient removal and warm season provided conducive environment for nutrient biotransformation in the microcosms. Furthermore, the presence of macrophytes enhanced the

removal of nutrients through the process of plant uptake and provision of oxygen for microorganisms in the rhizosphere. Lee *et al.* (2009) and Mthembu (2016) reported that macrophytes can remove nitrogen ranging between 20-30% in surface flow CWs through the process of uptake. This could be the reason for the highest removal of nutrients in the planted sections than in the reference section.

The removal of nutrients in the current study was also attributed to microbial mediated processes such as nitrification, denitrification and phosphorus degradation. Saeed *et al.* (2012) reported that the process of nitrification can remove nitrogen up to 90% while other numerous studies have reported that denitrification can remove nitrogen ranging between 60-90% in CWs (Vymazal, 2007; Wongkiew *et al.*, 2017). Phosphorus was attributed to the process of adsorption, precipitation, microbial degradation and plant uptake (Akinbile *et al.*, 2016; Zhang *et al.* 2017). The removal of nitrogen and phosphorus varied with varying environmental parameters such as pH, dissolved oxygen and seasonal changes. These parameters positively and negatively influenced nitrogen and phosphorus removal.

4.3 THE EFFECT OF SEASONAL CHANGES AND PHYSIOCHEMICAL PARAMETERS ON NUTRIENT REMOVAL

The study investigated the effect of seasonal temperature variation, pH, dissolved oxygen and chemical oxygen demand on ammonia, nitrite, nitrate and phosphorus removal in a wetland microcosm. The microcosms were situated in an open environment at KwaDlangezwa (University of Zululand) and operated in warm and cold season.

The high treatment efficiency was obtained in warm season with nutrient and phosphorus showing high reduction in microcosms. Warm season provided suitable environmental conditions for nutrient removal. This resulted in the highest removal of nitrogen and phosphorus in warm season while in cold season nutrient removal was decreased. Other parameters such as pH positively influenced nutrient removal in the planted sections in both warm and cold seasons while in the reference sections increasing pH negatively affected microbial activity. This led to a decrease in nutrient removal in both seasons. As for COD, the increase in removal efficiency of COD was directly proportional to the nutrient removal in both seasons except for nitrate in the reference section of the cold season. Based on the results, it can be deduced that physiochemical parameters positively influenced nutrient removal in the planted section in warm season than in the cold season. It was further deduced that seasonal temperature variation from warm season to cold season negatively influenced nutrient removal in the microcosms.

4.4 THE ROLE OF MICROORGSNISM IN NUTRIENT REMOVAL

Microbial community structure and diversity were more dominant in warm season than in the cold season. The abundance of these microorganisms in warm seasons resulted in high removal of nitrogen and phosphorus in warm season. When the planted section was compared with the reference section, the planted section had high microbial abundance and resulted in high nutrient removal. This indicated that the abundance and diversity of nutrient oxidising microorganisms and their activities were positively influenced by the presence of macrophytes in microcosms.

Pseudomonas and *Bacillus* were found in high abundance as denitrifiers and phosphate accumulators from both seasons in the planted and reference sections. This was because these microorganisms are able to function in both aerobic and anaerobic conditions. These microorganisms were capable of using nitrate as a source of electrons during the process of denitrification. In addition, these microorganisms have enzymes which enabled them to breakdown phosphorus in a microcosm.

4.5 CONCLUSION

The microcosm showed great potential of removing nutrients in both seasons. This was supported by the great quality effluent obtained in a study which met discharge limits, except for ammonia and COD. Despite the system not meeting the discharge limit for ammonia and COD, there was still a high removal of these contaminants. Seasonal temperature variation from warm to cold season negatively influenced nutrient removal in the system. Other physiochemical parameters such as pH, DO and COD positively influenced nutrient removal in the planted sections in both seasons. Hence, a high removal was obtained in the planted section through the synergistic activity of macrophytes and microorganisms. However, these physiochemical parameters had negative effect in the reference section in both warm and cold seasons.

4.6 RECOMMENDATIONS

The practical application of this technology is new in developing countries such as South Africa. However, these technologies are already applied with great success in many developed countries such as China. It is therefore, recommended that these technologies are also applied in developing countries. It is also recommended that a continual research on these systems is conducted. This include increasing the hydraulic retention time of these system especially in cold season, their application in industrial and agriculture wastewater treatment. Furthermore, research on microbial activity, community structure, population shift and the effect of physiochemical parameters on pollutant removal in these systems. This will help in the optimization and application of these green technologies in wastewater treatment.

4.7 REFERENCES

Akinbile, C.O., Ogunrinde, T.A., Man, H.C. and Aziz, H.A., 2016. Phytoremediation of domestic wastewaters in free water surface constructed wetlands using Azolla pinnata. *International journal of Phytoremediation*, *18*, pp.54-61.

Azizullah, A., Khattak, M.N.K., Richter, P. and Häder, D.P., 2011. Water pollution in Pakistan and its impact on public health—a review. *Environment International*, *37*, pp.479-497.

Dos Santos, S., Adams, E.A., Neville, G., Wada, Y., De Sherbinin, A., Bernhardt, E.M. and Adamo, S.B., 2017. Urban growth and water access in sub-Saharan Africa: Progress, challenges, and emerging research directions. *Science of the Total Environment*, 607, pp.497-508.

Lee, C.G., Fletcher, T.D. and Sun, G., 2009. Nitrogen removal in constructed wetland systems. *Engineering in Life Sciences*, *9*, pp.11-22.

Li, Y.D., Chen, Y., Wang, L., Yao, L., Pan, X.M. and Lee, D.J., 2017. Pollution tolerant protozoa in polluted wetland. *Bioresource Technology*, 240, pp.115-122.

Mthembu, M.S., 2016. Removal of organic and inorganic nutrients in a constructed rhizofiltration system using macrophytes and microbial biofilms (Doctoral dissertation).

Saeed, T. and Sun, G., 2012. A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. *Journal of Environmental Management*, *112*, pp.429-448.

Vymazal, J., 2007. Removal of nutrients in various types of constructed wetlands. *Science of the Total Environment*, *380*, pp.48-65.

Vymazal, J., 2014. Constructed wetlands for treatment of industrial wastewaters: a review. *Ecological Engineering*, 73, pp.724-751.

Wang, M., Zhang, D.Q., Dong, J.W. and Tan, S.K., 2017. Constructed wetlands for wastewater treatment in cold climate—A review. *Journal of Environmental Sciences*, *57*, pp.293-311.

Wongkiew, S., Hu, Z., Chandran, K., Lee, J.W. and Khanal, S.K., 2017. Nitrogen transformations in aquaponic systems: A review. *Aquacultural Engineering*, *76*, pp.9-19.

Zhang, J., Sun, H., Wang, W., Hu, Z., Yin, X., Ngo, H.H., Guo, W. and Fan, J., 2017. Enhancement of surface flow constructed wetlands performance at low temperature through seasonal plant collocation. *Bioresource Technology*, 224, pp.222-228.

APPENDICES

Appendix 1: Physiochemical parameters in the wetland microcosm during wastewater treatment

Sampling	Warm season		Cold season	
Period	Planted	Reference	Planted	Reference
0	5.4	5.2	7.0	6.5
4	6.2	6.9	6.3	6.2
8	7.8	7.1	7.0	6.3
12	7.0	7.8	7.4	6.0
16	7.2	6.5	6.6	5.0
20	8.5	7.3	7.4	6.6
24	7.8	6.8	7.3	6.0
28	8.0	7.2	6.8	5.1

Potential of hydrogen

Temperature	(⁰ (C)
-------------	------------------	----

Sampling	Warm season		Cold season	
Period	Planted	Reference	Planted	Reference
0	28	27	19	19
4	32	30	14	15
8	34	31	20	19
12	36	35	27	25
16	35	35	25	23
20	39	37	28	26
24	35	32	26	23
28	37	36	26	22

Sampling	Warm season		ason Cold season	
Period	Planted	Reference	Planted	Reference
0	8.2	7.5	9.0	8.0
4	6.3	4.2	7.5	7.0
8	5.3	4.4	7.0	6.5
12	8.8	7.1	9.0	8.0
16	10	9.0	7.8	6.0
20	8.0	7.5	5.7	4.1
24	6.0	5.7	7.1	6.0
28	7.5	6.2	6.5	5.8

Dissolved oxygen (mg/l)

COD (mg/l)

Sampling	Warm season		Cold season	
Period	Planted	Reference	Planted	Reference
0	314	300	250	240
4	220	225	127	144
8	115	175	113	120
12	158	182	200	127
16	94	155	125	139
20	80	122	90	120
24	100	150	100	146
28	94	138	95	144