ENHANCING PHOSPHOROUS REMOVAL FROM WASTEWATER USING POLYPHOSPHATE ACCUMULATING ORGANISMS FROM DIFFERENT WASTE STREAMS

BY

SSEMPALA ANDREW BBUMBA

B. Civil and Building Eng. (KYU)

REG. NO: 17/U/14705/GMEW/PE

A DISSERTATION SUBMITTED TO KYAMBOGO UNIVERSITY DIRECTORATE OF RESEARCH AND GRADUATE TRAINING IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE IN WATER AND SANITATION ENGINEERING OF KYAMBOGO UNIVERSITY

AUGUST 2023

DECLARATION

I Ssempala Andrew Bbumba, hereby declare that this submission is my own work and that to the best of my knowledge and belief it contains no material previously published or written by another person nor material which has been accepted for the award of any other Degree of the university or any other institution of higher learning except where due acknowledgement has been made in the text and reference list.

Ssempala Andrew Bbumba

Reg. no: 17/U/14705/GMEW/PE

Sign: ----------

Date: ----------

APPROVAL

The undersigned approve that they have read and hereby recommend for acceptance for examination by Kyambogo University the research dissertation entitled Enhancing Phosphorous Removal from wastewater using Polyphosphate Accumulating Organisms in fulfilment of the requirements for the award of a Master of Science in Water and Sanitation Engineering Degree of Kyambogo University.

Dr. Eng. Anne Nakagiri (Main Supervisor)

Department of Civil and Environmental Engineering / Faculty of Engineering

Signature: ----------

Date: ----------

Dr. Eng. Charles Onyutha (Co-Supervisor)

Department of Civil and Environmental Engineering / Faculty of Engineering

Signature: ----------

Date: ----------

ACKNOWLEDGEMENT

First and foremost, I would like to thank the Almighty God for the good health and strength, may His holy name be glorified.

Appreciations go to my supervisor Dr Anne Nakagiri for the technical, professional guidance and co-operation towards this research.

Thanks also go to Kyambogo University and the Department Civil and Building Engineering for giving me an opportunity to study from Kyambogo University.

Last but not least, I thank all those who helped me in one way or the other contributing to the success of my research.

DEDICATION

This Research is dedicated to Prof. Senfuma John, Eng. Sonko Kiwanuka, my family and not forgetting my classmates for without these people it would not have been a success.

Dr Anne Nakagiri, you have guided me well, encouraged me to continue. I am so grateful for the support you have given me.

May the good Lord Almighty bless you all and reward you for your greatness.

Table of Contents

DECLARATION ii

APPROVAL iii

ACKNOWLEDGEMENT iv

DEDICATION v

List of Tables ix

List of Figures x

ACRONYMS xi

ABSTRACT xii

CHAPTER ONE: INTRODUCTION 1

1.1 Background 1

1.2 Problem Statement 3

1.3 Objectives 4

1.3.1 Main Objective 4

1.3.2 Specific Objective 4

1.4 Research Questions 4

1.5 Justification 4

1.6 Significance/ Importance/ contribution 5

1.7 Scope of the study 6

1.7.1 General Scope 6

1.7.2 Content Scope 6

1.8 Time Scope 6

1.9 Conceptual Framework 6

1.10 Outline of the dissertation 7

CHAPTER TWO: LITERATURE REVIEW 8

2.1 Introduction 8
2.2 Concentration of Phosphorous in waste water and storm water 8
2.3 Concentration of Phosphorous in waste water receiving bodies 8
2.4 Phosphorous implication on public health and environmental pollution 8
2.5 The use of PAO in Phosphorous removal 9
2.5.1 Principal 9
2.5.2 Case studies where it has been applied 10
2.5.3 Operating conditions 10
2.6 Limitations 13
2.7 Work on modelling removal 14
2.7.1 Biochemical Model 15
2.8 Knowledge gaps 16
CHAPTER THREE: METHODOLOGY 17
3.1 Description of the study area 17
3.2 Study Design 18
3.3 Research Approach 18
3.3.1 Quantification of Polyphosphate Accumulating Organisms present in different waste streams 18
3.3.1.1 Sample Collection 18
3.3.1.2 Sample preparation 21
3.3.2 Effect on inoculation of Polyphosphate Accumulating Organisms (PAOs) 27
3.3.3 Optimising the working conditions for effective phosphorous removal by Polyphosphate Accumulating Organisms (PAOs) 29
CHAPTER FOUR: RESULTS AND DISCUSSION 32
4.1 Polyphosphate Accumulating Organisms present in different waste streams 32
4.2 Effect on inoculation of Polyphosphate Accumulating Organisms (PAOs) 38

4.3 Optimal working conditions for effective phosphorous removal by Polyphosphate Accumulating Organisms (PAOs). 40

CHAPTER 5: CONCLUSION AND RECOMMENDATION 44

5.1 Conclusion....44

5.2 Recommendation....44

REFERENCES 45

APPENDICES 53

APPENDEX I: Sequential Analysis 54

APPENDIX II: Inoculation results of critical parameters 56

APPENDIX III: Letter of acceptance for sample collection at NWSC Bugolobi 58

APPENDIX IV: Introductory Letter to Makerere University....59

APPENDIX V: Letter of shipment of PCR product samples for sequencing analysis....60

APPENDIX VI: Letter of Authorisation from Macrogen Europe 61

APPENDIX VII: Clearance letter from Kyambogo University 62

List of Tables

TABLE 3-1: COMPOSITION OF SELECTIVE MEDIA WITH PH=7.2 23
TABLE 3-2: COMPOSITION OF SELECTIVE ACIDIC MEDIA WITH PH=3.5 23
TABLE 3-3: REACTION COMPONENTS 26
TABLE 3-4: PCR THERMO CYCLING PROGRAM (35 CYCLES) 26
TABLE 4-1: ISOLATION OF ORGANISMS, GRAM AND METHYLENE BLUE STAINING (CHEMICAL AND MORPHOLOGICAL CHARACTERISATION) 33
TABLE 4-2: EFFECT OF INOCULATION ON CRITICAL PARAMETERS 39
TABLE 4-3: PHOSPHORUS LEVELS ACROSS DIFFERENT ENHANCEMENTS, ORIGINAL AND A NEGATIVE CONTROL 41
TABLE A-1: SEQUENCE ANALYSIS 54
TABLE A-2: EFFECT OF INOCULATION ON CRITICAL PARAMETERS 56
TABLE A-3: EFFECT OF VARYING CONDITIONS ON PHOSPHORUS REMOVAL 56

List of Figures

FIGURE 1.1: CONCEPTUAL FRAMEWORK 7
FIGURE 2.1: EXPERIMENTAL SETUP 15
FIGURE 3.1: BUGOLOBI WASTE WATER TREATMENT PLANT 17
FIGURE 3.2: SAMPLE COLLECTION POINTS FROM BUGOLOBI WASTE WATER TREATMENT PLANT. 19
FIGURE 3.3: SAMPLE STORAGE IN A REFRIGERATOR 20
FIGURE 3.4: SAMPLE COLLECTION POINTS FROM UGANDA BREWERIES LIMITED 21
FIGURE 3.5: REFRIGERATION OF SAMPLES 21
FIGURE 3.6: CENTRIFUGE OF SAMPLES 22
FIGURE 3.7: GRAM STAINING 25
FIGURE 3.8: PCR 26
FIGURE 3.9: COLLECTION OF WASTEWATER FROM THE KAMPALA CITY ABATTOIR AND A REACTOR SET UP 28
FIGURE 3.10: EXPERIMENTAL SETUP 30
FIGURE 4.1: GRAM STAINING 34
FIGURE 4.2: GEL ELECTROPHORESIS OF THE PCR AMPLICONS 35
FIGURE 4.3: PHYLOGENETIC TREE 36
FIGURE 4.4: DENDROGRAM 37
FIGURE 4.5: EFFECTS OF CARBON SOURCE ON PHOSPHORUS REMOVAL 42
FIGURE 4.6: EFFECTS OF VARYING TEMPERATURES ON PHOSPHORUS REMOVAL 42
FIGURE 4.7: EFFECTS OF PH ON PHOSPHORUS REMOVAL 43
FIGURE A.1: CENTRIFUGE OF THE SAMPLE AND PCR RUNNING 57
FIGURE A.2: ISOLATES 57

ACRONYMS

ASM	Activated Sludge Model
BOD	Biological Oxygen Demand
BNR	Biological Nutrient Removal
BPR	Biological Phosphorous Removal
BWWTP	Bugolobi wastewater treatment plant
CSH	Calcium Silicate Hydrate
COD	Chemical Oxygen Demand
DNA	Desoxyribonucleic Acid
DO	Disolved Oxygen
EBPR	Enhanced Biological Phosphorous Removal
GAO	Glycogen Accumulating Organisms
MBR	Membrane Bioreactors
NDP	National Development Plan
ORP	Oxygen Reduction Potential
PAO	Polyphosphate Accumulating Organisms
PCR	Polymerase Chain Reaction
PSF	Phosphate Solubilizing Fungi
pH	Potential of Hydrogen
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PSB	Phosphate-Solubilizing Bacteria
RAS	Return Activated Sludge
RNA	Ribonucleic Acid
SBR	Sequencing Batch Reactor
SDGs	Sustainable Development Goals
UCT	University of Cape Town
VFAs	Volatile Fatty Acids
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plant

ABSTRACT

Enhanced Biological Phosphorous Removal process has worldly been accredited for its efficiency in removing phosphorous from different wastewater treatment plants. This study focused on Enhancing Phosphorous Removal from wastewater using Polyphosphate Accumulation Organisms present in the Bugolobi wastewater treatment plant, Kampala city abattoir and Uganda Breweries limited. Wastewater from the Bugolobi wastewater treatment plant, Kampala Abattoir and Uganda Breweries Limited was assessed to establish a suitable source of the Polyphosphate Accumulating Organisms. The effect of inoculation of Polyphosphate Accumulating Organisms rich wastewater on Phosphorous removal in domestic wastewater was evaluated. Microbial tests isolated the bacteria present in the collected wastewater samples and thereafter Desoxyribonucleic Acid extraction from the bacterial broth was done. The extracted Desoxyribonucleic Acid was stored followed by qualitative and quantitative analysis using a nano drop. Microbial tests identified 16 different species of bacteria in the groups: firmicutes; High gram positive, β-proteobacteria, α-proteobacteria and γ-proteobacteria. Acinetobacter calcoaceticus strain was noted to be the dominate bacteria that accumulated more phosphorous. Different working conditions that enable phosphorus accumulating bacteria to thrive were also determined and amendments of phosphorus accumulating bacteria working condition gave an overall significant difference with acetate as the best carbon source, 25°C as the best optimum temperature and pH 7 for their activity. Of the three wastewater sources, Kampala city abattoir was found to have the most Polyphosphate Accumulating Organisms.. Efficient phosphorous removal in the Enhanced Biological Phosphorous Removal system is highly dependent on Polyphosphate Accumulating Organisms suppressing Glycogen Accumulating Organisms.

KEYWORDS: Wastewater, Wastewater characteristics, Polyphosphate accumulating organisms

CHAPTER ONE: INTRODUCTION

1.1 Background

Phosphorous is a mineral that is naturally found in some foods and is also available as a supplement that helps in the development of teeth, bones and cell membrane and keeps blood pH with in normal range (Uribarri and Calvo 2018).

High phosphorous levels in water bodies have been attributed to human activities such as urban construction, sewage discharge, agricultural practices and residential development (Jenkins and Ives 2013). High phosphorous levels in water bodies lead to low oxygen hence resulting into eutrophication in surface water, stimulates algae growth and other microorganisms like toxic cyanobacteria (blue-green algae) (Ansari and Sarvajeet Singh Gill, 2014). An increase in phosphorus additions of 0.1-5.6 μg/L over a long period cause's algal bloom in parts of water bodies which in turn causes diverse effects on flora and fauna in water bodies (Geissler and Powers 2017).

Wastewater is classified into municipal wastewater, agricultural wastewater, industrial wastewater and hazardous wastewater. Bugolobi wastewater treatment plant has been upgraded to treat wastewater from the Nakivubo Channel that comprises of municipal wastewater originating from parts of sewered areas of Kampala City. The plant also handles storm water and wastewater from the Kampala city abattoir and brewery waste that have high levels of phosphorous.

Abattoir wastewater discharge is a global concern due to the negative effect on public and environmental health. Abattoir wastewater contains large volumes of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN), total phosphorous (TP) and total suspended solids (TSS) (Barbera and Gurnari 2017).

Effluent from the abattoir if not treated contaminates both surface and ground water since it contains animal blood, fat, urine, manure and meat tissue. The organic and nutrient concentration of abattoir wastewater is very high and residuals are partially soluble leading to a high contamination effect on water sources if not treated. Abattoir wastewater can be treated using dissolved air flotation and biological wastewater treatment.

Municipal wastewater is also known as domestic wastewater and is generated from a community of people. Municipal wastewater contains nitrogen, phosphorous and essential elements that

plants need to grow (Tamim Younos and Parece 2016). Municipal wastewater comprises of domestic wastewater, surface runoff and industrial wastewater and constitutes a variety of inorganic substances which may include toxic heavy metals. Municipal wastewater comprises of about 99.9% water, small concentrations of suspended and dissolved organic and inorganic solids as well as sewerage generated from domestic dwellings (Menahem Libhaber and Álvaro Orozco-Jaramillo 2012). Municipal wastewater is contain pathogenic organisms, oxygen demanding wastes, plant nutrients, synthetic organic chemicals, micro plastics, inorganic chemicals, sediments, radioactive substances and oil.

Components of wastewater treatment include bar screens, grit chamber, pre-aeration tank, primary settling tank, aeration tank, secondary settling tank, biological filters and sludge handler (Theodore and R. Ryan Dupont 2019). Wastewater treatment to acceptable limits must be done to ensure that phosphorous levels meet the acceptable standards before disposal into the environment hence the tough laws set on phosphorous effluent limits. Currently there is a growing interest in the need to control phosphorus discharges around the world as reflected in the increasingly stringent regulations and the drive to incorporate phosphorous removal in wastewater treatment (Stewart, Palumbo and Rossi, 2017). These new lower effluent limits can be achieved by looking beyond traditional treatment technologies.

Three options will help remove or stabilise phosphorous in systems namely physical, chemical and microbiological processes (Dölle and Watkins, 2017). Chemical phosphorous removal requires the use of coagulants in removing phosphorous from waste-water. This makes the process expensive since chemicals are expensive to acquire but percentage of metals in the sludge is not environmentally friendly and not good for agricultural use.

Sand filters and membrane technologies are used in the physical method of phosphorous removal hence require high capital investment and a lot of time to recover the capital invested in the technology thus making the process expensive.

The microbiological process is a less expensive process for phosphorous removal as compared to both chemical and physical processes with sludge that is environmentally friendly and highly recommended for agricultural use.

The Biological Phosphorous removal process uses bacteria called Polyphosphate accumulating bacteria to take up phosphorous from waste water (Fukushima *et al.,* 2010) where activated sludge is recycled between anaerobic and aerobic conditions with no chemicals involved.

1.2 Problem Statement

The amount of fresh drinking waters globally is about 3% as compared to 97% of ocean waters (Feinstein 2013). Ocean waters are salty, not good for agriculture or crop production and neither is it good for industrial uses except for cooling purposes (Murray2003). Glaciers and ice caps hold about 68.7% of fresh water but in ice form. Ground water forms 30% of the fresh water while 0.3% of the fresh water is surface water in form of lakes, rivers and swamps (Stephen Henry Schneider et al.2011). Some of the highly ranked countries with fresh water resources include: Brazil (8,233cubic kilometres), Russia (4,503cubic kilometres), United States of America (3,069 cubic kilometres), Canada (2,902 cubic kilometres) cubic kilometres), China (2,840 cubic kilometres), Colombia (2,132 cubic kilometres), European Union (2,057), Indonesia (2,019 cubic kilometres), Peru (1,913 cubic kilometres) and India (1,911 cubic kilometres) (The Economist 2016).

In humans about 55% to 60% of the human body is water hence making water a vital component to human survival. An increase in population globally increases demand for services hence impacting on the need for free water. Human activities like construction, industrialisation, urbanisation, food production and sewerage or wastewater discharge increase chances of contamination of fresh water bodies due to the high phosphorous levels anticipated as a result (Jenkins and Ives 2013).

Bugolobi waste water treatment plant (BWWTP) is located in Kampala and encompasses an ultra-modern sewerage treatment plant that will generate 621 KW of biogas. Bugolobi waste water treatment plant was upgraded from 33,000m^3 to 45,000m^3 and treats both municipal waste water and storm water from the Nakivubo Channel. The current concentration of wastewater influent into the Bugolobi waste water treatment plant is 35 mg/l orthophosphate and 36 mg/l total phosphate (Takouleu, 2021). An increase in Phosphorous loading of the plant is anticipated with storm water treatment and yet the plant was not designed for phosphorous removal. Studies in India have found phosphorous concentrations of total Phosphorous ranging from (0.43 to 1.45) mg/L to (28.47 to 47.89) mg/L in sunny seasons and (0.53 to 1.01) mg/L to (11.78 to 34.88) mg/L in rainy seasons. (Mohammad Saghir Khan et al.2014). Individual contributions differ since Phosphorous is highly constituted in synthetic detergents (Kundu*et al.,* 2015) while the phosphorous concentration in the Nakivubo channel is attributed to wastewater discharge from breweries and abattoirs that is phosphorous rich (Kansiime and Nalubega 2020).

Discharge standards for municipal wastewater range between (5-20) mg/l total phosphorous with ranges of (1-5) mg/L organic waste and yet the Bugolobi wastewater treatment plant effluent is 21mg/l orthophosphate and 22mg/l total phosphate (Hankins, Jancaitis and Olson, 2011).

In order to meet the required disposal standards and reduce eutrophication of the receiving lake, Phosphorous concentrations in the effluent from the Bugolobi Waste Water Treatment Plant have to be reduced. This research therefore was aimed at Enhancing Phosphorous Removal from wastewater using Polyphosphate Accumulating Organisms from different waste streams.

1.3 Objectives

1.3.1 Main Objective

Enhance Biological Phosphorous Removal from wastewater using Polyphosphate Accumulation Organisms from different waste streams (Nakivubo channel) into the Bugolobi wastewater treatment plant.

1.3.2 Specific Objective

1. Quantify Polyphosphate Accumulating Organisms present in different waste streams to establish a suitable source
2. Evaluate the effect of inoculation using wastewater rich in polyphosphate accumulating organisms (PAOs) to remove phosphorous from the Bugolobi wastewater treatment plant.
3. Optimise the working conditions for effective phosphorous removal by Polyphosphate Accumulating Organisms (PAOs).

1.4 Research Questions

1. Do different waste streams have polyphosphate accumulating organisms?
2. Does inoculation of polyphosphate accumulating organisms have an effect on phosphate removal in waste water from Bugolobi?
3. Do optimal working conditions for the effective phosphorous removal by polyphosphate accumulating organisms exist?

1.5 Justification

Bugolobi wastewater treatment plant was upgraded to treat wastewater that includes municipal wastewaters and storm water from the Nakivubo Channel (MN, 2018) that includes wastewater from the Kampala City abattoir and brewery waste. The Kampala City abattoir does not treat its effluent wastewater thus greatly impacting on the phosphorous levels of the Bugolobi WWTP. This increased plant concentrations of total Phosphorous from (0.43 to 1.45) mg/L to (28.47 to

47.89) mg/L in sunny seasons to (0.53 to 1.01) mg/L to (11.78 to 34.88) mg/L in rainy seasons and yet the plant was not designed for phosphorous removal.

Enhanced Biological Phosphorous Removal (EBPR) systems perform better in phosphorous removal than chemical and physical phosphorus removal processes since phosphorous is taken up in activated sludge and it is easy to recover.

A low sludge production with less metal content from the EBPR system makes the EBPR system an environmentally friendly process and the sludge is highly recommended for agricultural use which is a different case with chemical phosphorous removal. About 62% of phosphorous contained in the sludge is released from the stored poly-P in PAOs as compared to the 40% P released from chemical-P sludge (Wu *et al.,* 2016, Hester and Harrison, 2013).

Enhancing Phosphorus removal from wastewater using Polyphosphate Accumulating Organisms (PAO) from different wastewater streams was the main focus in this research.

1.6 Significance/ Importance/ contribution

This research is aimed at meeting the sustainable development goals 6.3 (SDG 6.3) of improving water quality by reducing pollution, eliminating dumping and minimising release of hazardous chemicals and materials, halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally by 2030. The SDG 6.3 can be achieved by scaling down Phosphorous concentrations in the effluent from the Bugolobi Wastewater Treatment plant into Lake Victoria so as to meet the required disposal standards thus reduce eutrophication.

Eutrophication is attributed from the increase in phosphorous loading of the Bugolobi Wastewater Treatment plant as a result of the plant's redesign to handle both wastewater and storm water from the Nakivubo channel. The Bugolobi Wastewater Treatment plant has discharge from breweries and abattoirs that are phosphorous rich (Dietler* *et al.,* 2016a) and yet the plant was not designed for phosphorous removal.

This research contributes to the NDPIII by promoting science, technology, engineering and innovation as well as ICT through the three options that can be used to remove phosphorous from wastewater namely physical, chemical and microbiological processes (Dölle and Watkins, 2017). Microbiological processes are aimed at Enhancing Phosphorous Removal from Wastewater using Polyphosphate Accumulating Organisms (PAO) from different waste streams.

1.7 Scope of the study

1.7.1 General Scope

The research was limited to the influent into the Bugolobi Wastewater Treatment Plant which includes wastewater from sewer locations in Kampala, some feacal sludge and storm water from the Nakivubo channel.

1.7.2 Content Scope

This research concentrated on quantifying PAOs present in different wastewater streams into Bugolobi WWTP, culture the PAOs and derive working conditions favourable for PAOs in the effective removal of phosphorous at the treatment plant.

1.8 Time Scope

This research was carried out for a period of one year with effect from November 2021 to September 2022. The research involved sample collections done in three months, laboratory set ups, data analysis and interpretation done for a period of seven months which was followed by report writing and submission.

1.9 Conceptual Framework

The framework explains how the linkage between Polyphosphate Accumulating Organisms (PAOs) as an independent variable influences wastewater quality of the Bugolobi Wastewater Treatment Plant as a dependent variable. Figure 1.1 shows the conceptual framework.

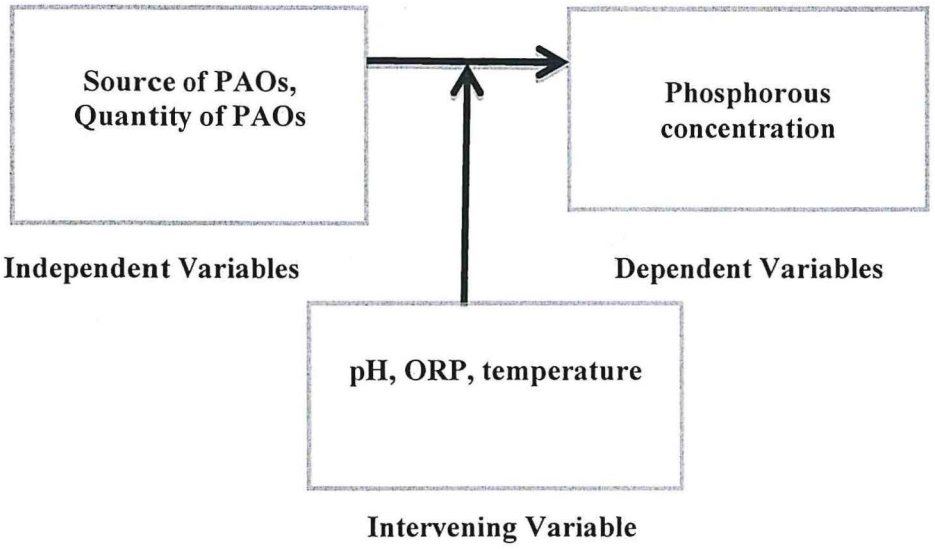


Figure 0.1: Conceptual framework

1.10 Outline of the dissertation

The thesis is organized in a series of chapters which come after an abridgment of the entire research. In total, the dissertation consists of five (05) chapters. Chapter one focuses on the introduction, problem statement, objectives, research questions, significance and justification. Chapter two covers the literature review of the research, Chapter three covers the entire materials and methods, Chapter four contains the results and their analysis (Discussion) and finally chapter five contains the conclusion and recommendations.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Phosphorous (P) is a chemical element having15 as an atomic number and a highly reactive chemical element. Phosphorous exists in two forms namely white phosphorous and red phosphorous with white phosphorous considered as poisonous and causes severe skin burns.

Both rocks and ocean sediments require phosphorous for their formation. This appears in the form of phosphate salt that dissolves into soil water before it is absorbed by plants.

2.2 Concentration of Phosphorous in waste water and storm water

The difference in wastewater characteristics from different areas is influenced by discharges from industries, sewer infiltration and the type of sewer systems (Marcos Von Sperling 2007) Sewer systems include combined and separated systems. The use of phosphate detergents that drain into the channel impacts on the phosphorous levels in the waste water (Porter and Fitz Simons 2009). Different areas have various impacts on waste water thus the difference in characteristics of municipal waste water. Municipal phosphorous ranges are between (4 to 11) mg/l with different places averaging at (6 and 8) mg/l (Schaum 2018)

2.3 Concentration of Phosphorous in waste water receiving bodies

The recommended phosphorous levels for domestic wastewater effluent are 1mg/L (Dotro et al.). Different waste has different limits for example; streams that don't empty into reservoirs have a limit of 0.1mg/L, streams discharging into reservoirs have a limit of not more than 0.05mg/L and a 0.024mg/L limit for reservoirs should not be exceeded. This is according to the United States Environmental Protection Agency 2022.

2.4 Phosphorous implication on public health and environmental pollution

Living organisms require phosphorous as a key nutrient thus humans take in phosphorous through food staffs (e.g. meat and poultry, fish, milk and other dairy products) although too much phosphorous in human bodies causes heart disease, joint pain or fatigue. Humans require phosphorous for cells and tissues growth, waste filtering, energy production, strong bones and teeth, maintain a regular heartbeat, produce Deoxyribonucleic Acid (DNA) and Ribonucleic Acid(RNA) (the body's genetic building block) and to manage how the human body stores and uses energy.

Water bodies acquire phosphorous from agricultural practices known as non-point sources through surface run off and waste water effluent discharges known as point sources (Munn et al.2018). These phosphorous discharges cause environmental pollution thus eutrophication.

Environmental pollution has been regulated through regulatory bodies that enforce laws that regulate phosphorous discharged into the environment by pushing for phosphorous removal at wastewater treatment plants but not targeting agricultural practices and imposing strict limits on wastewater effluent to natural water shade. Phosphorous use must be regulated since it's a limited resource that is highly used for production of fertilisers used for agricultural purposes (Massry 2013).

2.5 The use of PAO in Phosphorous removal

Polyphosphate Accumulating Organisms (PAOs) are micro-organisms that take up phosphorous from wastewater. PAOs in the anaerobic zone absorb and store organic matter using accumulated poly-phosphate reserves (Rossetti et al. 2017). PAOs take up accumulated phosphorous from sedimented sludge in the aerobic zone thus Enhanced Biological Phosphorous Removal (EBPR) (Katerina Stamatelatou and Tsagarakis 2015).

2.5.1 Principal

In normal activated sludge systems, Polyphosphate Accumulating Organisms (PAOs) exist and take up phosphorous as a nutrient for growth (Mannina et al. 2018). The take up of more phosphorous by some of the PAOs present in the enhanced biological phosphorous removal (EBPR) systems than required for cellular growth is known as luxury uptake (Porter and Fitz Simons 2009). There exist different organisms that take up excess phosphorous amounts as energy rich phosphates. The dry weight of the bacteria of about (20-30) % is taken up by phosphorous while the phosphorous content for common heterotrophic bacteria can reach up to 2% (Milo and Phillips 2015). The rate of phosphorous removal in wastewater is proportional to the number of polyphosphate accumulating organisms present in the wastewater. Alteration of anaerobic and aerobic conditions through the use of anaerobic reactors followed by anaerobic reactor stimulates the growth of PAOs (Knowles 2018). The use of fermentative bacteria and sequential batch reactors in the anaerobic zone also creates an environment that supports activity of PAOs. By alternating anaerobic and aerobic conditions, PAOs get an advantage over other bacteria.

2.5.2 Case studies where it has been applied

If chemical process is used for P-removal, the final P concentration is lower than 0.5 mg/l with 90% Phosphorous removal efficiency (Sedlak 2018).

The EBPR process supersedes the chemical removal process in the environmental aspect since no chemicals are used and the process is cost effective (Derin Orhon et al.2009) since it has in recent years been studied in depth. The EBPR system has been recently improved through the addition of membrane bioreactors (MBR), granular sludge reactors and sequencing batch biofilm reactors (SBRs). Studies recently indicate a high level of total N and COD removal (Rimbach and Fuchs 2005). A combination of MBR with EBPR configuration systems by the University of Cape Town (UCT) shows an 88% rate of P-removal with a 0.3mg/L total P as the final total effluent level (Mannina and Springerlink (Online Service) 2017). Without alteration of anaerobic aerobic/anoxic conditions, total P-removal by MBR was only 53%, which further confirms that luxury P uptake by bacteria is unlikely (Spellman 2013).

Biological phosphorous removal is an effective phosphorous removal technique despite certain aspects of this process considered a disadvantage namely complexity that makes it not ideal for small applications and the difficulty in separation of chemically bonded phosphorous hence causing unlikely efficiency in phosphorous recovery for further use and limiting the overall economic benefits of using phosphorous rich sludge.

2.5.3 Operating conditions

2.5.3.1 Carbon Source

EBPR relies upon the accessibility and decision of a substrate/carbon source as promptly biodegradable COD or VFAs for effectively activated sludge while the decision of substrate or carbon source is vital to the EBPR interaction financial matters impacted by the expense and specific utilization of the carbon wellspring of PAOs against GAOs. Microbial rivalry between GAOs with PAOs prompts weakening of phosphorous expulsion particularly when the circumstances favor GAOs over PAOs (Seviour 2010). Acetic acid derivation has effectively been utilized as a carbon source on EBPR frameworks when contrasted with propionate and different substrates and yields a strong stable phosphorous evacuation execution. Studies have as of late recommended propionate as a more ideal substrate than acetic acid derivation for a fruitful EBPR execution (Jr et al. 2013). VFAs are an elective carbon source other than acetic

acid derivation and propionate, (for example, butyrate, lactate, valerate and isovalerate) which can be consumed by PAOs or GAOs yet their impact on PAO-GAO is hazy (Vipin Chandra Kalia and Springerlink (Online Service) 2015). A few examinations show a disintegration of EBPR frameworks because of Glucose being taken care of as a carbon source (Dezotti et al.2018)

2.5.3.2 Temperature

Temperature varieties influence both PAOs and GAOs such that GAOs predominately take up more acetic acid derivation at high temperatures making the EBPR framework idle while PAOs prevail in low temperatures and taking up more acetic acid derivation accordingly an expansion in phosphorous take-up. A SBR reactor working at 20^0C, great phosphorous expulsion execution from an EBPR framework is displayed when contrasted with a 30^0C SBR reactor that showed a low degree of phosphorous delivery and take-up with high anaerobic acetic acid derivation take-up (Kundu et al., 2016).

2.5.3.3 pH

pH is a critical element in the EBPR cycle since the opposition among PAO and GAO is reliant upon the pH levels hence influencing the viability in the phosphorous evacuation process. A high pace of acetic acid derivation take-up by PAOs in the anaerobic and oxygen consuming zones happens at a pH above 7.25 and a low pH favors GAOs to take up the acetic acid derivation (Fujiwara et al., 2009). EBPR frameworks are dependent on PAOs. EBPR system require a pH range between 7.5 to 8.5 for a change in microbial contest from GAOs to PAOs to guarantee high phosphorous evacuation rate in the anaerobic and oxygen consuming zones (Fujiwara et al., 2009). A reduction in VFA take-up results from a pH above 8.0, low acetic acid derivation take-up and low phosphorous up (Motizuki et al., 2004).

2.5.3.4 Dissolved Oxygen

The EBPR framework requires adjustment of anaerobic and vigorous circumstances meaning no oxygen is expected in the anaerobic reactor while oxygen is expected in the high-impact reactor. Presence of disintegrated oxygen in the anaerobic reactor causes contest for VFAs among PAOs and heterotrophic high-impact organic entities which use oxygen as the electron acceptor in this manner coming about into rivalry for VFAs consequently less natural phosphorous evacuation.

PAOs take up VFAs in the anaerobic zone however need oxygen to process capacity items in the high-impact reactor consequently holding phosphorous (Moosavi et al., 2005). Over air circulation of the EBPR cycle adversely influences the natural phosphorous evacuation process since no phosphorous take-up happens because of the consumption of poly-hydroxy-butyrate (PHB) (Peng et al., 2006).

2.5.3.5 Secondary Phosphorous release

Primary phosphorous release happens when phosphorous is released under anaerobic circumstances with concurrent take-up of acetic acid derivation and it's stockpiling as PHB which makes this phosphorous handily eliminated in the vigorous climate (Xu et al., 2011). Energy expected by microorganisms for keeping up with their metabolic exercises are gotten from the released phosphorous from the put away polyphosphate in the nonattendance or deficient presence of VFA under high-impact conditions and which released phosphorous isn't taken out during the auxiliary phosphorous release. The EBPR cycle is adversely impacted by optional phosphorous release because of its sluggish event when contrasted with the quicker pace of the essential phosphorous release hence auxiliary phosphorous should be stayed away from. The anaerobic climate in the auxiliary clarifier because of the deferred slop evacuation or profound ooze cover causes secondary phosphorous release (W. Peng et al., 2007).

2.5.3.6 Solid Retention Time (SRT)

Solid Retention Time (SRT) impacts the opposition among PAOs and GAOs where GAOs effectively contend with PAOs at a long SRT consequently a decline in phosphorous evacuation in the EBPR framework. An expansion in SRT prompts the decrease in phosphorous evacuation accordingly in the decline of biomass yield and abundance sludge release (Zuo and Ji et al., 2019). STR increment is free of phosphorous evacuation productivity in spite of the phosphorous substance in biomass. Phosphorous expulsion isn't reliant upon phosphorous substance in biomass as SRT increments

A SRT of pH 7.5 and 30^0C temperature diminished from 10 to 3 days causing the microbial populace in acetic acid derivation took care of lab scale reactor change from prevailing microbial populace from an enhanced GAO to an improved PAOs culture (INOUE et al., 2020).

2.5.3.7 COD/P ratio

PAOs are reliant upon phosphorous whether the framework is restricted to COD or BOD for an effective EBPR process. The influent COD or BOD to add up to phosphorous proportion is key during the plan and activity of the phosphorous evacuation framework. At a COD:P proportion of 10:20 mg-COD/mg-P PAOs rule while GAOs overwhelm at COD:P proportion >50 mg - COD/mg-P. For a proficient EBPR process, the control of functional circumstances (presence of VFAs) and ideal COD:P proportion must be kept up with in order to use consummation for substrate among PAOs and GAOs. A stoichiometric necessity of COD is required for the expulsion of every P unit (Grady et al, 2011).

2.5.3.8 Cations

Cations in influent waste water in addition to the fact that they tie phosphorous in the activated sludge, they settle the biological phosphorous removal (BPR) process. Since phosphate particles cannot go through cell films all alone, they contain three negative charges that must bond with positively charged particles (magnesium and potassium) to become impartial subsequently empowering the transportation of phosphate atoms (Siwek and Pawelec et al., 2020)

Consumption of magnesium and potassium in the anaerobic zone adversely influences the arrival of phosphates from PAO cell subsequently BPR won't happen. Particle constraints in the EBPR cycle is probably not going to happen in municipal waste water since municipal waste water contains abundance magnesium and potassium. Sewerage treatment plants intended for EBPR might encounter a deficiency of potassium in the influent while enacted slime have overabundance potassium coming about into poor dewatering properties and gushing quality.

2.6 Limitations

The Enhanced Biological Phosphorous Removal process essentially comprises of anaerobic zone followed by aerobic zone rather than an air circulation tank in a conventional activated sludge process. In the EBPR cycle, organic matter take-up and phosphorous delivery happen under anaerobic circumstances while phosphorous take-up happens under aerobic conditions subsequently a low influent phosphorous fixation. Phosphorus gathered in the sludge is taken out by Polyphosphate Accumulating Organisms (PAOs) during sedimentation (R.Ramadori 2017).

2.7 Work on modelling removal

Phosphorous can be taken out as a particulate by sedimentation, filtration or by moving phosphorous into a side-stream utilizing layer treatment.

1.5 to 2.5 % (w/w) phosphorous per volatile solids is contained in typical biomass under specific circumstances and which biomass gathers phosphorous of 6 to 8 %, far in abundance of the dietary necessities. Sedimentation (strong division) is utilized to eliminate collected phosphorus in sludge or biomass (Hart, B.T. and Sly P.G. 2012).

Biomass containing biologically eliminated phosphorus can be utilized as composts. Enhanced Biological Phosphorous Removal (EBPR) process helps in the expulsion of phosphorous from waste water by gathering the phosphorous with biomass. Effectiveness of EBPR frameworks incredibly relies upon the sludge phosphorous substance and strong division productivity.

Microorganisms take-up phosphorous as a supplement for cell growth in activated sludge frameworks though over abundance phosphorous take-up results into extravagant take-up. Polyphosphate Accumulating Organisms (PAO) exist in different sorts and the microorganisms are liable for the legitimate working of Enhanced Biological Phosphorous Removal frameworks by taking in abundance measures of phosphorous as energy-rich polyphosphates and subsequently Polyphosphate Accumulating Organisms get a benefit over different microbes in rotating anaerobic and aerobic circumstances. The bacteria dry weight of up to 20-30% can be loaded up with phosphorous and 2% for heterotrophic microbes (Tchobanoglous et al., 2014, Godwin and Cotner, 2015). The Enhanced Biological Phosphorous Removal framework is intended to boost ideal phosphorus expulsion by Polyphosphate Accumulating Organisms.

Change of anaerobic and aerobic conditions using anaerobic reactors followed by aerobic reactors or the utilization of sequential batch reactors animates development of PAOs since anaerobic reactors are adequately feed with volatile fatty acids VFA (for example Acetic acid derivation, propionate and so forth) or fermentative microorganisms from a different tank where maturation happens. Figure 2.1 shows the experimental set up.

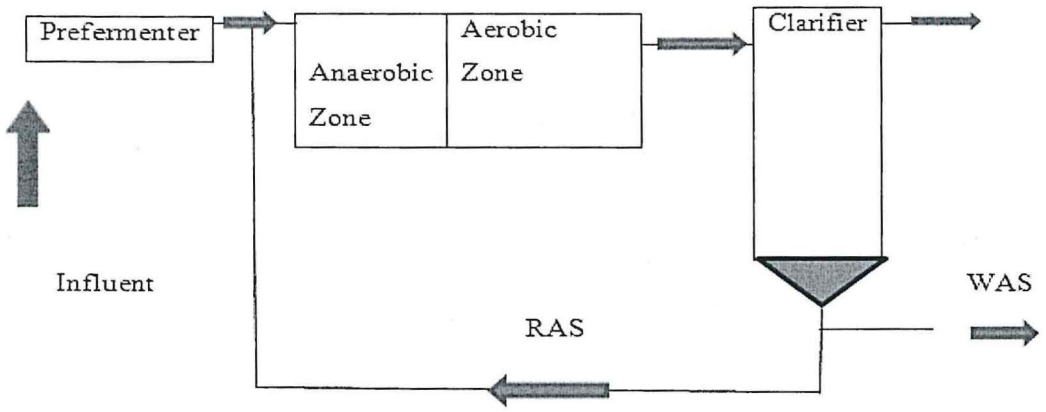


Figure 0.1: Experimental setup

RAS – (Return Activated Sludge)

WAS – (Waste Activated Sludge)

Prefermenter (formation of VFA)

Anaerobic zone (Organic matter uptake and Phosphorous release)

Aerobic zone (Phosphorous uptake)

Clarifier (Sedimentation)

2.7.1 Biochemical Model

In the aerobic zone, approaching wastewater combines as one with activated sludge thus empowering PAOs to utilize VFAs (for example acetic acid derivation, propionate) present in the influent or created by fermentative microbes present in the oxygen consuming tank to form a carbon storage product called poly-hydroxyalkanoates (PHA) in the EBPR framework.

Microbial debasement of natural mixtures in the aerobic zone happens without even a trace of oxygen and nitrate and can likewise be accomplished in a different tank thus fermentation. Stored poly-P and glycogen are utilized by PAOs as an energy source in order to form PHAs while a centralization of 20mgP/L is seen in the anaerobic tank when orthophosphate is released. The recovery of poly-P saves by PAOs works with up-take of adequate orthophosphate consequently guaranteeing a net phosphorous expulsion from the influent. Oxidization of PHAs framed in the anaerobic tank by oxygen gives energy to the development of poly-P holds and renewing glycogen reserves.

2.8 Knowledge gaps

EBPR processes are subject to the qualities of metropolitan wastewater which rely upon different factors like sewer invasion, releases of modern wastewater, utilization of phosphate cleansers and sort of sewer framework (joined or isolated) (Gasperi et al., 2010, Petzet et al., 2013). EBPR frameworks have gotten a wide interest because of lower substance costs, lower sludge creation, an expanded potential for phosphorus recuperation with around 90% of the approaching phosphorus contained in the slop (Petzet et al., 2013; Bi, Guo and Chen, 2013) in spite of the prevalence of Synthetic phosphorous evacuation frameworks.

EBPR has become critical accordingly thus the need to find and propose procedures and control measures that incline toward the improvement of PAO and suppress the development of GAO in EBPR actuated ooze frameworks.

CHAPTER THREE: METHODOLOGY

This research was aimed at Enhancing Phosphorous Removal from Waste Water using Polyphosphate Accumulating Organisms from different waste streams.

3.1 Description of the study area

The Bugolobi wastewater treatment plant (BWWTP) has a capacity of 45,000m^3 and was the case study of this research. The BWWTP has been upgraded to treat wastewater from the Nakivubo Channel, Kampala city abattoir and brewery wastewater. Nakivubo channel comprises of municipal wastewater originating from parts of the sewered areas of Kampala city and storm water. The BWWTP is located at Latitude 0.318483^{0}and Longitude 32.606890^0 as presented in figure 3.1 below.



Figure 0.1: Bugolobi Waste Water Treatment Plant

3.2 Study Design

This study involved sample collections placed in a cool box. The samples were transported and then placed in a refrigerator at 4°C so as to carryout microbiological and molecular characterization of Phosphate Accumulating Organisms. The samples were collected from Bugolobi WWTP, Kampala abattoir and Uganda Breweries Limited.

Microbial tests were used to isolate the bacteria present in the collected wastewater samples and there after DNA extraction from the bacterial broth was done using a Quick-DNA™ Fungal/Bacterial Miniprep kit using manufacturer instructions (Zymo Research Corporation, USA, Ca). The extracted DNA was stored at -20^0C followed by qualitative and quantitative analysis using a nano drop. Different working condition that enable phosphorus accumulating bacteria to thrive were then determined which include pH, temperature and carbon source.

3.3 Research Approach

3.3.1 Quantification of Polyphosphate Accumulating Organisms present in different waste streams

3.3.1.1 Sample Collection

During this study to identify a suitable source of PAOs, various samples were collected during morning hours between 7:00am to 11:00am in the dry season. Six samples of wastewater were collected from Bugolobi Wastewater Treatment plant in sterile sample 0.5Ltr bottles at different points (i.e. Inlet, Aeration zone, Sedimentation zone, Filters, Effluent and cesspool emptier). One sample of 0.5Ltrs effluent water was picked from the abattoir while four different samples of 0.5Ltrs were collected from Uganda Breweries Limited at different points of the Wastewater Treatment Plant (i.e. Influent, aerobic zone, anaerobic zone and the effluent). The one sample collected from the abattoir was because the abattoir has no treatment unit for the waste water it produces thus this one sample was collected at the effluent point of the abattoir draining to the Nakivubo channel. The samples collected from both the BWWTP and UBL were picked up from the different points at each treatment plants.

The collected samples were placed in a cool box and transported to the laboratory (Department of Plant Sciences, Microbiology and Biotechnology, College of Natural Sciences of Makerere University) where they were immediately placed in a refrigerator and kept at 4°C in preparation

for the microbiological and molecular processes to be carried out. Figure 3.2 shows the different areas where samples were collected.

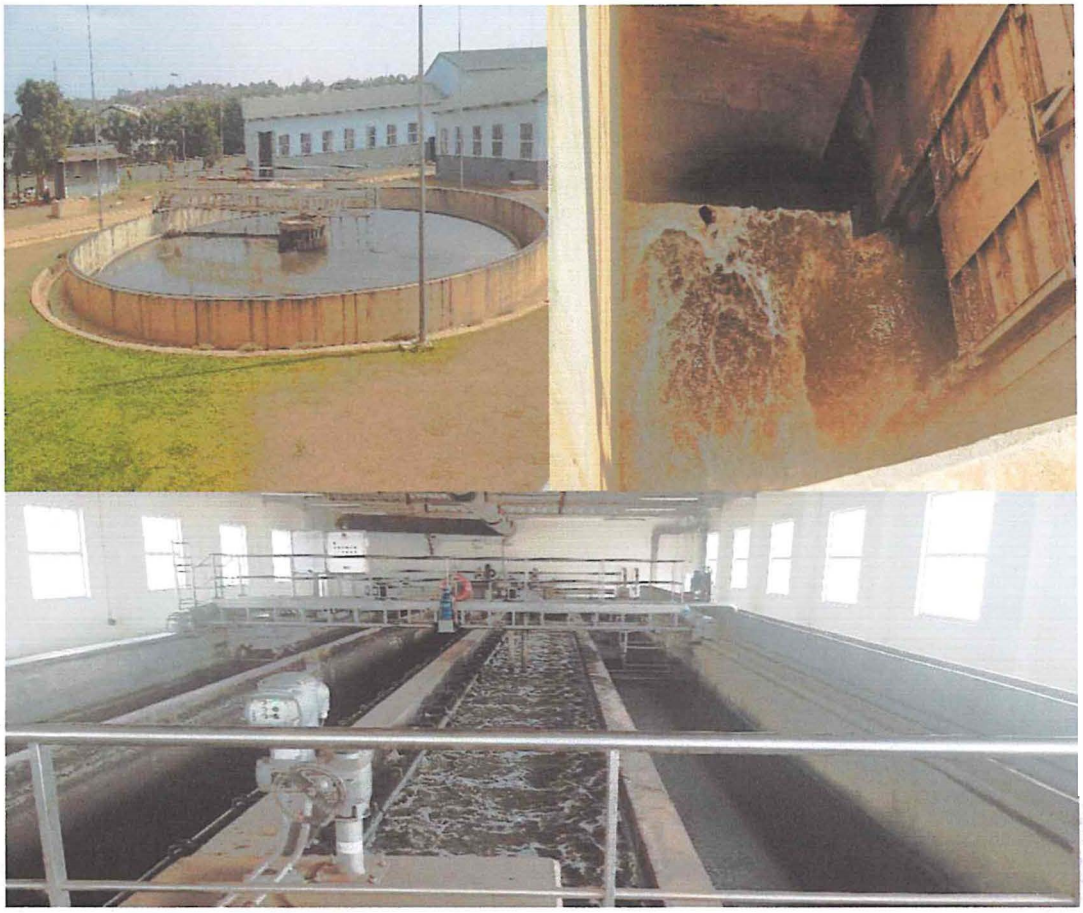
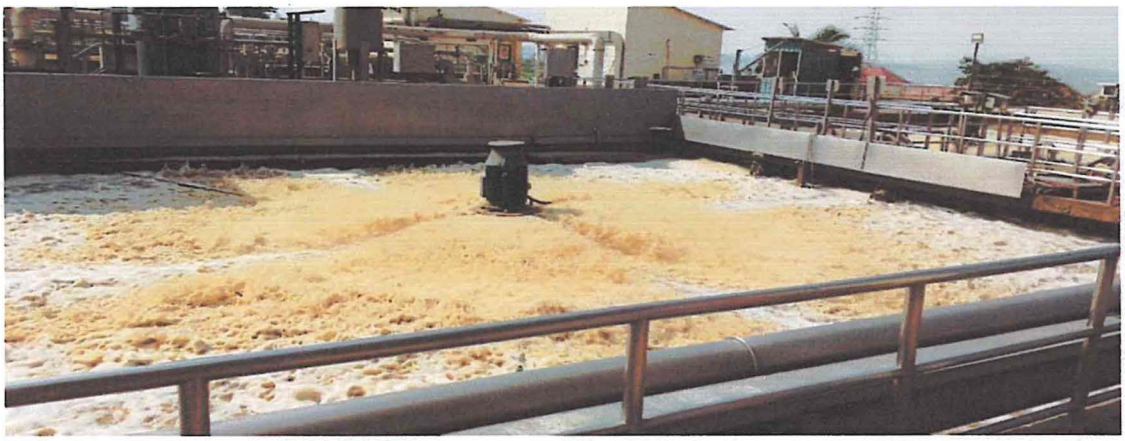


Figure 0.2: Sample collection points from Bugolobi Waste Water Treatment Plant



Figure 0.3: Sample storage in a refrigerator





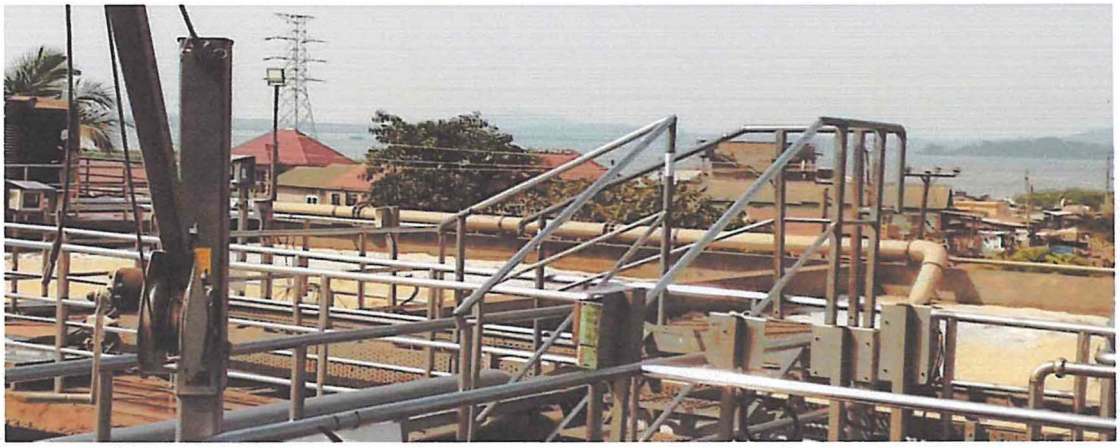


Figure 0.4: Sample collection points from Uganda Breweries Limited

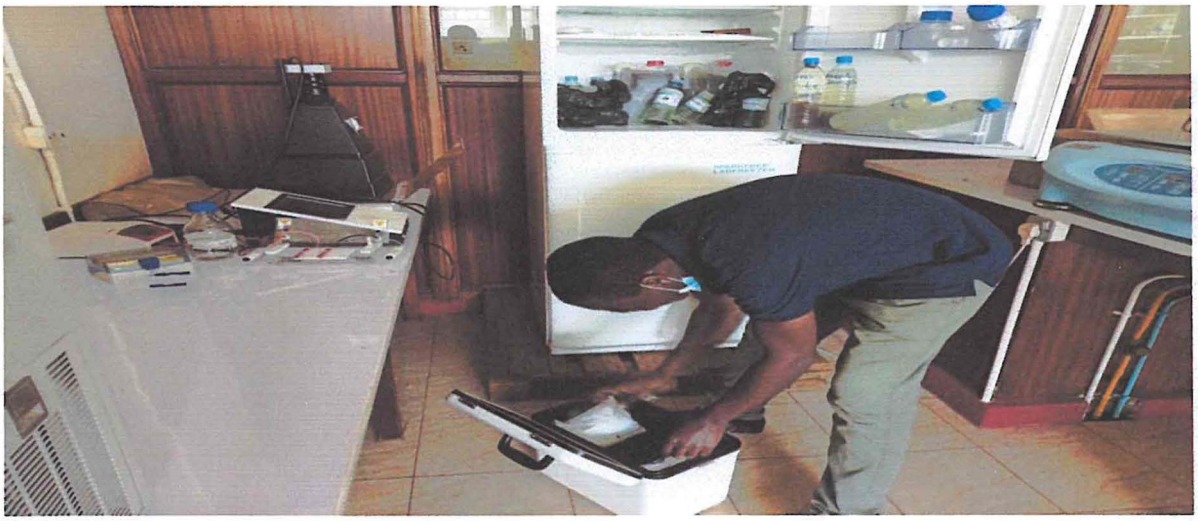


Figure 0.5: Refrigeration of samples

3.3.1.2 Sample preparation

The eppendorf 5430R refrigerated centrifuge w/digital display was used and operates at a versatile high speed micro centrifuge offering up to a maximum speed of 17,500rpm, with an acceleration of (30,130 x g) and a temperature range of (-11°C to 40°C) between 12 different compatible rotors.

All samples were centrifuged at 8000rpm followed by filtration by Whatman No.1 filter paper (or filtration alone for stream samples). The pellet was discarded while the supernatant was placed in a falcon tube and then kept at 4°C until the time for microbial analysis.



Figure 0.6: Centrifuge of samples

3.3.1.3 Analysis for phosphate Accumulating Bacteria

3.3.1.3.1 Microbiology Procedures

Microbiological analysis for PAOs was done according to Ajeeta Anand and Hideki Aoyagi (Anand and Aoyagi*et al.,* 2019) and the procedure used to prepare the reagents and analysis for the micro-organism was as follows;

Preparation of a Selective media

Two selective Medias were prepared in the laboratory of pH 7.2 and the other media was of pH3.5 so as to identify PAOs.

The selective media of pH 7.2 was prepared using Sodium acetate, Magnesium sulphate heptahydrate, Potassium dihydrogen phosphate, Sodium Nitrate, di-sodium hydrogen phosphate, Ammonium sulphate, enriched with toluidine blue as shown in Table 3-1.

Table 3-1: Composition of Selective media with pH=7.2

Composition	Concentration(g/l)
Sodiumacetate	5
Magnesium sulphate heptahydrate	0.5
Potassium dihydrogen phosphate	0.332
Sodium Nitrate	0.8
di-sodium hydrogen phosphate	1.418
Ammonium sulphate	0.17
Toluidine blue	0.22

Oxoid CM0003 Nutrient Agar was added to the media of pH 7.2 at 1% and autoclaved for 20 minutes at a temperature of 121^0C. The media was cooled to a temperature of 60^0C and 1% toluidine blue was added in weight per volume which then turned the media from colourless to blue. 10mls of media was then poured on the labelled plates and left to cool.

Samples were spread on the media using a sterilized glass spreader and then incubated for 5days at 30^0C. Colonies formed within 3days of incubation but left for up to 5days for more microbes to be formed. Phosphate accumulating bacteria formed blue colonies in the media with toluidine blue since the dye stains phosphate granules in the bacteria while none phosphate accumulating bacteria appeared colourless on this media. Since bacteria does not thrive in acidic conditions, an acidic media of pH 3.5 was formed with no toluidine blue to identify phosphate accumulating bacteria since the accumulated phosphate neutralizes the acidity of the media.

Table 3-2: Composition of selective acidic media with pH=3.5

Composition	Concentration(g/l)
Sodium acetate	5
Magnesium sulphate heptahydrate	0.5
Potassium dihydrogen phosphate	0.332
Sodium Nitrate	0.8
Di-sodium hydrogen phosphate	1.418
Ammonium sulphate	0.17

An inoculating loop was used to transfer non similar coloured blue colonies in each sample onto the acidic media and incubated at 30^0C for 3days. Once bacteria was placed on the acidic media that appeared colourless, the blue dye absorbed by the bacteria was released onto the acidic media leaving the bacteria to appear colourless and the dye was only visible within 24hours thus leaving the bacteria colourless.

The bacteria were quantified by counting the non-similar blue colonies on each plate and then colonies from the acidic media were transferred to the nutrient agar media to study colony morphology and for gram and methylene blue staining.

28g of nutrient agar were placed in a litre of water, mixed and boiled to dissolve the agar which was followed by sterilizing the solution by autoclaving at 121^0C for 15 minutes. The media was cooled to about 60^0C and 10ml of the media was poured on each plate and left to solidify. Solidification of the media took 20 minutes before bacteria was transferred to it using a sterilized loop from the acidic media to the nutrient agar media and then incubated for 24hours at 30^0C to observe the colonies before carrying out gram staining.

Gram Staining

A fixed smear was prepared from the collected samples from the abattoir, Bugolobi wastewater treatment plant and Uganda Breweries Limited to achieve a mono layer of cells before the sample was flooded with crystal violet solution and left to stand for thirty (30) seconds.

The stain was gently washed off with running tap water for 5 seconds and thereafter excess water was shaken off.

The smear was then flooded with Gram's iodine solution for one minute and the stain was gently washed off with running tap water for five (5) seconds and the excess water was shaken off.

Decolorizing with decolorizing solution was done by slowly dropping the decolorizing solution above the smear on the slightly tilted slide until no more purple colour eluded off the smear.

The stain was gently rinsed off with running water for five (5) seconds and thereafter excess water was shaken off.

The slide was then covered with counterstain (safranin) for 30 seconds and the stain was gently rinsed off with running water for 5 seconds and there after excess water was shaken off. Slides were viewed at 10000X

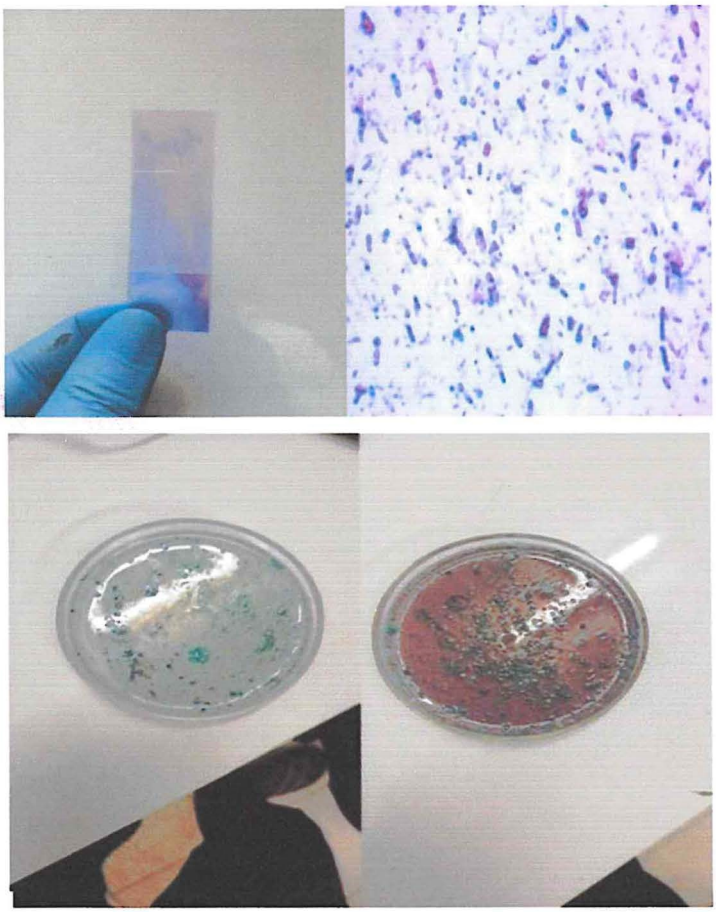


Figure 0.7: Gram Staining

3.3.1.3.2 Molecular Characterization

DNA was extracted from the bacterial broth using Quick-DNA™ Fungal/Bacterial Miniprep kit using manufacturer instructions (Zymo Research Corporation, USA, Ca.) and the extracted DNA was stored at -20^0C before it was qualitatively and quantitatively analyzed using nano drop. PCR wasthen conducted on samples obtained.

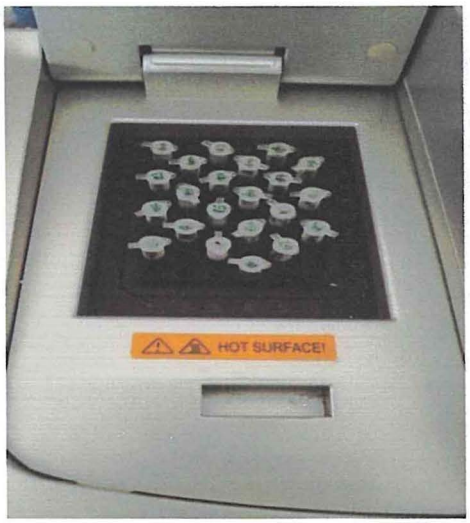


Figure 0.8: PCR

Table 3-3: Reaction components

Reagent	Working stock	PCR concentration used	Volume
2XMasterMixwithstandardbuffer (New England Biolabs,USA)	2X	1X	12.5 µl
Forwardprimer(27F)	20 µM	0.2 µM	0.25 µl
Reverseprimer(1492R)	20 µM	0.2 µM	0.25 µl
Template	250ng/µl	10ng/µl	1 µl
Nucleasefreewater	-	-	11 µl
Total			25

Table 3-4: PCR Thermo cycling program (35 cycles)

Step	Segment	Temperature(˚C)	Time (Min:Sec)
1	Initial Denaturation	94	03:20
2c	Denaturation	94	00:25
3c	Annealing	48.5	00:30
4c	Extension	72	00:50
5	Final extension	72	03:20
6	Hold	4	∞

GEL Electrophoresis

1.5% basic agarose was used and TBE (recipe – Tris, borate, EDTA) buffer was used at 100volts and a current of 300A. Samples were loaded into wells (indentations) at one end of a gel and an electric current was applied. The gene ruler used was a BH 100bp DNA Ladder RTU (100-1,500 bps)

Sequencing

The PCR products of waste water from Bugolobi Waste Water Treatment plant, Kampala City Abattoir and Uganda Breweries Limited were flown to Macrogen Europe B.V, Meibergdreef 57,1105BA, Amsterdam, the Netherlands using FedEx account number473205645.

Samples were sequenced using Sanger sequencing methods and the resulting sequences were analysed using bioinformatics tools.

BLAST, Clastaw omega and phylodraw

Sequences were subjected to BLAST against existing nucleotide sequences in National Centre for Biotechnology Information (NCBI) database toidentify present species/Operational Taxonomical Units (OTU), a cladogram was obtained by phylodraw.

As reflected in the phylogenetic tree, acinetobacter calcoaceticus (strain ICA01 16S ribosomal RNA) partial sequence is the bacteria that accumulated more phosphorous.

3.3.2 Effect on inoculation of Polyphosphate Accumulating Organisms (PAOs)

Evaluating the effect of inoculation using wastewater rich in polyphosphate accumulating organisms (PAOs) to remove phosphorous from the Bugolobi wastewater treatment plant (BWWTP) involved the following:

3.3.2.1 Experimental design

Basing on the amount of Phosphate accumulating bacteria found in the waste water samples from the different waste streams (i.e. the Kampala city abattoir waste, Bugolobi waste water treatment plant and Uganda brewery ltd waste), it was discovered that waste water from the Kampala city abattoir had more Phosphate accumulating bacteria than the waste water from the Bugolobi Waste water treatment plant and Uganda Breweries Limited since the Kampala city abattoir does not treat it's waste water yet it drains into the Bugolobi waste water treatment plant through the

Nakivubo channel thus impacting on the quantity of Phosphate Accumulating Bacterial in the Bugolobi Waste Water Treatment plant.

As a result, 80ltrs of waste water was collected from the abattoir effluent that drains into the Nakivubo channel to the Bugolobi WWTP. The temperature of the wastewater was measured to be 24^0C and kept constant. Phosphorous levels were measured to be 6.1mg/l, noted before two experimental set ups ran in order to evaluate the effect on inoculation of PAO rich waste water on Phosphorous removal in wastewater from the Bugolobi Wastewater Treatment Plant. The setups ran by gravity for two days after inoculating 3ml of Phosphate Accumulating bacteria solution in each tank with a constant flow rate of $20.8m^3/h$.

The setups relied on the ability of wastewater microorganisms to release phosphorus in a more concentrated form in the anaerobic zone 'stripper-tank' while the Phosphorous depleted wastewater moved to the aeration zone for phosphorus accumulation. The alteration of aerobic and anaerobic processes results into resource recovery and high treatment efficiency since biological processes alone do not produce effluent that complies with the current effluent discharge limits when treating high organic strength wastewaters.

The phosphorous levels drastically dropped from 6.1mg/L to 2.2mg/L by end of the 48hours.



Figure 0.9: Collection of wastewater from the Kampala City Abattoir and a reactor set up

3.3.3 Optimising the working conditions for effective phosphorous removal by Polyphosphate Accumulating Organisms (PAOs)

An experimental set-up was run parallel to the control setup but with different parameter tests runs. The set up was subjected to various conditions of pH, temperature and carbon source. The pH alterations involved pH4 pH7 and pH9, temperatures of 25^0C, 35^0C and 45^0C with acetate and glucose as the carbon sources. The alternations of the different conditions helped in finding the favourable conditions for PAOs to perform better with an intension of stabilising the EBPR system. EBPR systems may experience operational upsets and performance deterioration (Manuel 2017). The conditions where altered so as to establish the optimal working conditions for effective phosphorous removal by Polyphosphate Accumulating Organisms (PAOs).

Oxidation-reduction potential (ORP)

Oxidation-reduction potential was measured to determine the ability of the waste water to self-cleanse since wastewater may comprise of waste products like contaminants, dead plants and animals. A Hanna pH meter-ORP meter was used to obtain the ORP in the wastewater sample as reflected in the table A-2 before running the wastewater in the set-up and after collecting it at the clarifier. Table A-2 is in the appendix.

pH variation

20g of Sodium thioacetate was weighed and placed in each tank. The tanks were filled with waste water and the pH adjusted to pH4, pH7 and pH9respectively using glacial acetic acid and sodium hydroxide for acidity and alkalinity respectively.

Temperature variation

The setup was subjected to different temperatures i.e. (25^0C, 35^0C and 45^0C) so as to ascertain the most favourable temperature for PAOs to take up more phosphorous from wastewater. A water bath was used in temperature regulation.

Carbon source variation

Two different setups were used with a temperature of 25^0Cand pH 7. One set-up was fed with 20g of glucose as the carbon source in each tank while the other setup was fed with 20g of Sodium thioacetate as the acetate source. The setups were left to run for two days after inoculating 3ml of the phosphate accumulating bacteria solution in each tank. The water that collected at the clarifier was taken for testing.

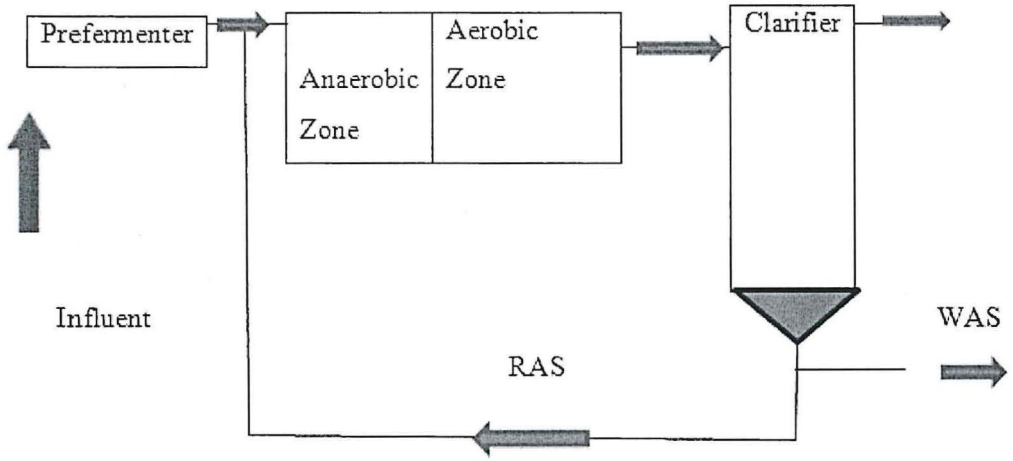


Figure 0.10: Experimental setup

RAS – (Return Activated Sludge)

WAS – (Waste Activated Sludge)

Prefermenter (formation of VFA)

Anaerobic zone (Organic matter uptake and Phosphorous release)

Aerobic zone (Phosphorous uptake)

Clarifier (Sedimentation)

3.3.3.1 Inoculum with PAO

80ltrs of waste water samples was collected from the city abattoir in four twenty litre (20ltr) jericans and the Phosphorous content was measured at the time of collection before the wastewater was left to ferment in the laboratory for two weeks.

3.4 Data analysis and presentation

3.4.1 Statistical analysis

Microbiology data was analysed using descriptive analysis while molecular biology data was analysed using NCBI database. R statistical package (version 3.4.3) developed by the R development core team was also used to analyse data. The Data was checked for normality and homogeneity of variances and analysed by one way analysis of variance (ANOVA) to test for significance of the different conditions on phosphorous removal.

3.4.2 Analysis of data

A list of microbes was generated, samples sequenced using Sanger sequencing methods and the resulting sequences were analysed using bioinformatics tools. Sequences were then subjected to BLAST against existing nucleotide sequences in the National Centre for Biotechnology Information (NCBI) database to identify present species/Operational Taxonomical Units (OTU) and a cladogram was obtained by phylodraw.

3.5 Quality assurance

Prior to use, all transfer equipment and glassware were thoroughly cleaned and autoclaved to avoid external contaminations. Nuclease free water was used in all preparations and all analysis was done in triplicates.

All experimental set-ups were subjected to similar conditions of temperature, light intensity and water supply.

3.6 Ethical consideration

A clearance letter from Kyambogo University and the research approval committee was obtained to carry out research and permission to carry out research from Bugolobi waste water treatment plant and other authorities from the research community was obtained. The research was conducted following standard procedures and all the sources of information review papers, manuscripts, textbooks and reports by national and international organisations were cited in this dissertation.

CHAPTER FOUR: RESULTS AND DISCUSSION

This chapter presents results and discussions from different experimental setups as regards enhancing phosphorous removal from wastewater using Polyphosphate Accumulation Organisms from different waste streams.

4.1 Polyphosphate Accumulating Organisms present in different waste streams

Different colonies formed on the media and phosphate accumulating bacteria formed blue colonies on the media with toluidine blue since the dye stained phosphate granules in the bacteria while none phosphate accumulating bacteria appeared colourless on this media.

Isolates were quantified as shown in table 4-1 by counting non-similar blue colonies from each plate and colonies from the acidic media were transferred to the nutrient agar media to study colony morphology and for gram and methylene blue staining.

Phosphate accumulating bacteria was identified in all the wastewater samples tested. Bugolobi Wastewater treatment plant had more isolates twelve (12) in number followed by isolates in the wastewater from the Kampala city abattoir that where eleven (11) in number. Wastewater from the Kampala city abattoir was used in the experimental set-up since Kampala city abattoir waste directly drains into the Bugolobi wastewater treatment plant without treatment thus positively influencing the quantity of PAOs present in the Bugolobi wastewater treatment plant. Bugolobi wastewater treatment plant also receives wastewater from the Nakivubo channel which comprises of municipal wastewater, storm water, industrial wastewater and abattoir wastewater.

Phosphorous concentration in the Nakivubo channel is attributed to wastewater discharge from breweries and abattoirs that is phosphorous rich (Kansiime and Nalubega 2020). Phosphate accumulating bacteria in the wastewater was identified as colourless after incubation at 30^0C for 3days since the dye was only visible within 24 hours on the acidic media of pH 3.5.

Table 4-1: Isolation of organisms, gram and methylene blue staining (chemical and morphological characterisation)

Sample	Isolates (Diversity)	Description of mixed culture
(A) Aerated grit (BWWTP)	5	Gram negative bacilli, smooth margins, blue and colorless, serrated margins, mostly blue and few colorless colonies
(B) Cesspool (BWWTP)	4	Scanty shiny and creamy and gram-positive streptococci.
(C) Filters (BWWTP)	3	Gram negative short rods, few blue and many colorless colonies, gram positive streptococci and slender rods.
(D) Inlet (BWWTP)	12	Many blue and few colorless colonies, gram positive streptococci and slender rods, Gram negative short rods.
(E) Primary Sedimentation (BWWTP)	8	Moderate blue colonies, gram positive streptococci, gram negative bacilli and creamy wide colonies.
(F) Final effluent (BWWTP)	2	Most colonies were colorless, only few identical blue colonies, gram positive streptococci.
(G)Abattoir effluent	11	Many rough colonies with undefined boundaries, gram positive streptococci and gram negative rods.
(H) Brewery Influent	2	Dark blue and pale blue colonies of gram negative bacilli.
(I) Brewery Anaerobic zone	5	Some rough blue colonies and small smooth pale blue colonies, gram negative bacilli
(J) Brewery Effluent	1	Single blue creamy colonies of gram negative rods.
(K) Brewery Aerobic zone	8	Dark blue colonies with smooth boundaries and small colonies. Gram positive streptococcus, gram negative slender rods

NOTE:

Aerated grit sample from Bugolobi wastewater treatment plant – (**A**)

Cesspool emptier sample from Bugolobi wastewater treatment plant – (**B**)

Filters sample from Bugolobi wastewater treatment plant – (**C**)

Inlet sample from Bugolobi wastewater treatment plant – (**D**)

Primary Sedimentation sample from Bugolobi wastewater treatment plant – (**E**)

Final effluent sample from Bugolobi wastewater treatment plant – (**F**)

Abattoir effluent to Bugolobi wastewater treatment plant – (**G**)

Brewery Influent – (**H**)

Brewery Anaerobic zone – (**I**)

Brewery Effluent – (**J**)

Brewery Aerobic zone – (**K**)

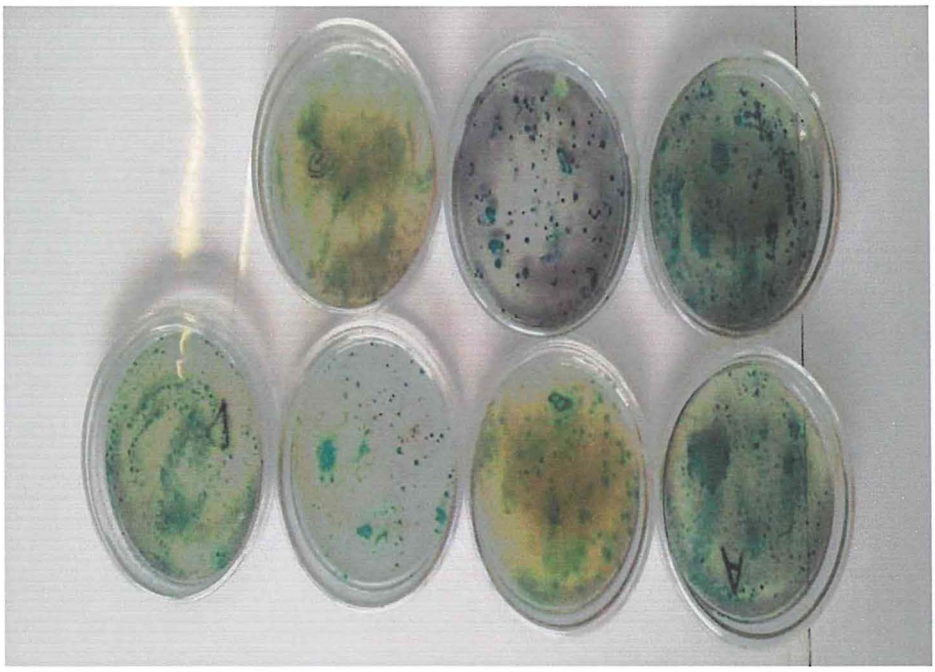


Figure 0.1: Gram Staining

Results on Molecular characterisation of phosphate degrading bacteria

Gel electrophoresis results

Gel electrophoresis of the PCR amplicons obtained from 27F and 1492R primer pairs from the 16S Ribosomal RNA gene with 100 base pair DNA ladder. The size of the first band up on the

ladder corresponds to 1500 base pairs which is the estimated size of the PCR amplicons from the used 16S primers.

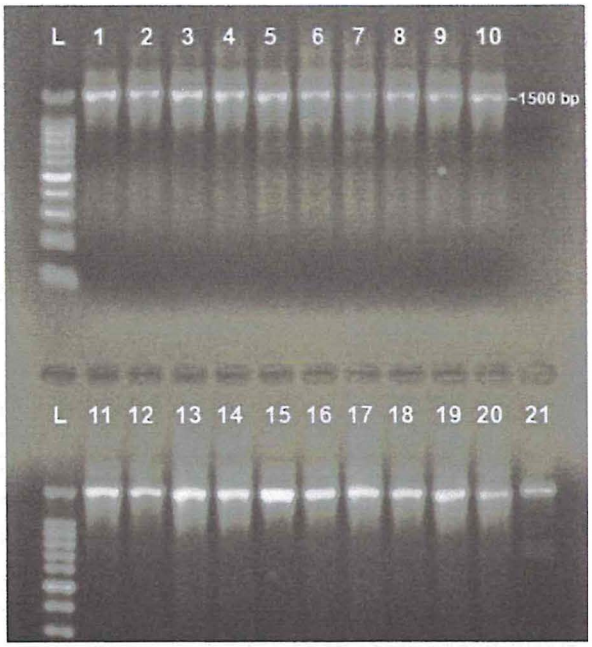


Figure 0.2: Gel electrophoresis of the PCR amplicons

Molecular Phylogenetics of PAOs

The evolution of PAOs is shown on the phylogenetic tree constructed from NCBI using the accession numbers of the sequences obtained from the Sanger sequencing. The method used for tree construction was the "First Minimum Evolution Method" (Appendix I)

The bacterial species closest to the roots are the ancestral species (firmicutes) and the species goes on evolving through high GC Gram+, Beta-proteobacteria, Alpha-proteobacteria up to the last evolving species of the gamma-proteobacteria which are typical of the Phosphate Accumulating Organisms (Seviour et a.l., 2000)

This phylum evolved approximately 1.5 billion years ago during the Paleoproterozoic era. This evolution is also believed to have been caused by rise in atmospheric oxygen concentrations. Gamma-proteobacteria, are fundamental in marine and coastal ecosystems because they are the major groups involved in the nutrient cycling and despite their fame as pathogens they find application in a huge number of fields such as bioremediation and biosynthesis.

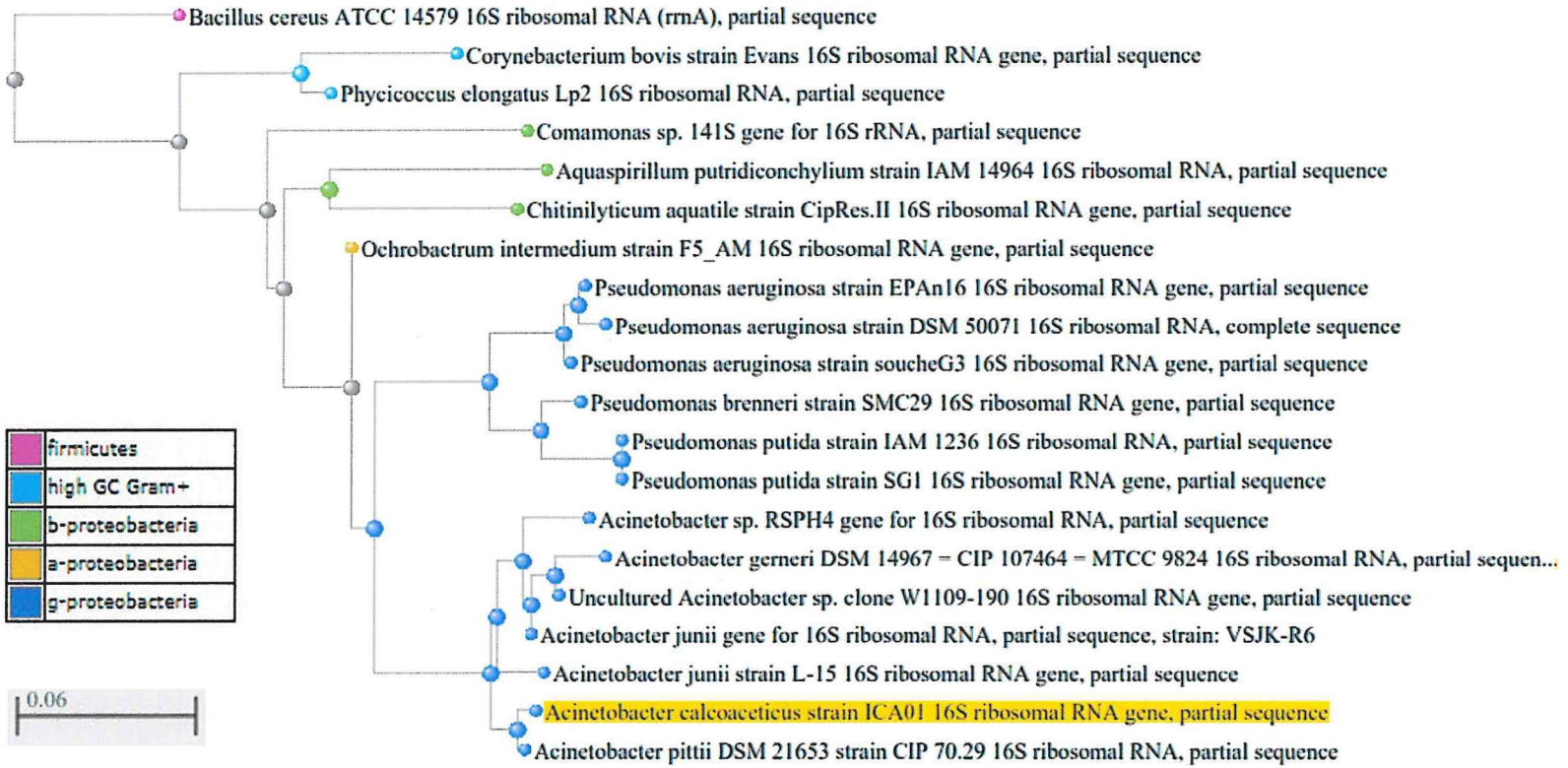


Figure 0.3: Phylogenetic tree

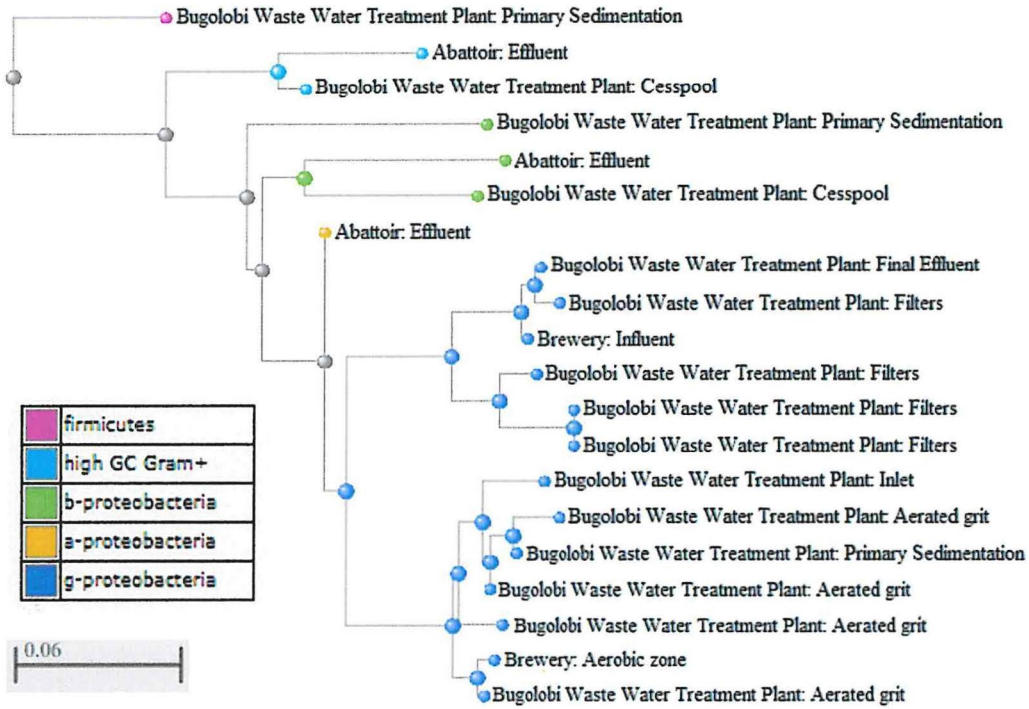


Figure 0.4: Dendrogram

4.2 Effect on inoculation of Polyphosphate Accumulating Organisms (PAOs)

To determine the effect on inoculation of polyphosphate accumulating organisms' rich wastewater on phosphorous removal in wastewater from the Bugolobi Wastewater treatment plant, it was noted that untreated effluent from the abattoir directly drains into the Nakivubo channel. The Nakivubo channel was noted to collect storm water, brewery wastewater and surface runoff. Municipal wastewater comprises of water together with relatively small concentrations of suspended and dissolved organic and inorganic solids. Phosphorous concentration in the Nakivubo channel is attributed to wastewater discharge from breweries and abattoirs that are phosphorous rich (Kansiime and Nalubega2020).

An abattoir operates on three modules namely a production model, service model and meat market. The production model includes a slaughter floor, lairage, chiller, tripe room, meat cutting and processing while a service model includes a water supply, effluent disposal, solid waste and blood disposal, hide and skin processing, electric light and power.

The high volumes of wastewater from the abattoir are attributed to the large amounts of slaughtering process and cleaning of the facility. The abattoir processes harvest a distinctively extreme organic waste comprising of condemned meat, bones, horns, hairs blood, oil, minerals and organic solids, salts, aborted foetuses and chemicals added during handling operations. Abattoir wastewater has pH that ranges from (4.9 to 8.1), turbidity that ranges from (200 to 300), colour that ranges of (175 to 400) mg/L Pt scale) and K that ranges from (0.01 to 100) mg/L. These volumes of abattoir wastewater must be subjected to treatment before disposal into the environment because of its content levels of organics and nutrients.

Abattoir wastewater drains into the Nakivubo channel that also drains into the Bugolobi Wastewater Treatment plant (BWWTP) thus abattoir waste was used in the experimental set-up since abattoir effluent was noted to influence the quantity of Phosphate accumulation bacteria at the Bugolobi Waste Water Treatment Plant. The results suggested that a reasonable amount of oxygen (13.4mV) was present in the waste-water sample before running the set-up with a drastic reduction in oxygen levels (-113.4mV) at the end of the run.

Table 4-2: Effect of inoculation on critical parameters

Parameter	Before Inoculation(Mean±SEM)	After Inoculation(Mean±SEM)
ORP (mV)	13.4±0.00	-113.4±0.00
pH	7.2±0.20	6.5±0.30
Phosphorous (mg/L)	6.1±0.30	5.4±0.10
Temperature (°C)	24.6±0.20	27.3±0.00
Values are Means±SEM (n=3), [SEM, standard error of the mean]		

Oxidation-reduction potential (ORP)

A high value of ORP (13.4mV) before inoculation indicates the presence of a lot of oxygen in the waste-water which greatly contributes to high efficiency rate of bacteria phosphorous accumulation in the aerobic chamber since bacteria works efficiently with a high rate of ORP. At the end of the reaction all the oxygen reserves were depleted to deficit levels and thus the negative value of the ORP (-113.4mV). A reduction in ORP after inoculation with PAOs shows that the increased number of PAOs (bacteria) uses up oxygen in the waste-water as is it breaks down phosphorous. The ranges used in this study were <-200 mV (reduction of sulphur compound, acetate fermentation and methane formation; -199 to -51 mV (acid formation); -50 to +49 mV (nitrate/ nitrite reduction) and > +50 mV (aerobic degradation).

The low value of ORP shows that PAOs used up the oxygen as they broke down phosphorous in the waste-water which also explains the alterations of aerobic and anaerobic conditions during the bacterial phosphorous removal process. The alteration of aerobic and anaerobic processes results into resource recovery and high treatment efficiency since biological processes alone do not produce effluent that complies with the current effluent discharge limits when treating high organic strength wastewaters.

Anaerobic treatment uses less complex equipment requirements and it is effective in high strength wastewater such as abattoir wastewater. Anaerobically treated wastewater effluent does not enable complete stabilization of organic matter. Anaerobic effluent requires post treatment using aerobic treatment so as to meet the required disposal standards since solubilized organic matter in the anaerobic effluent is more suited for treatment using aerobic processed hence the anaerobic and aerobic alterations. Aerobic processes operate at higher rates than the conventional anaerobic treatment process since oxygen requirements and treatment time is directly proportional to an increase in wastewater strength.

Alteration of anaerobic and aerobic conditions alterations enable resource recovery and high treatment efficiency since effluent as a result of biological processes alone does not meet the standards.

pH

Waste-water pH before treatment ranges between pH 6 to pH 8 (Cordova and F. Wijaya 2007) depending on the source of waste-water with the variations in pH resulting from organic loading in the waste-water. The bacteria in the aerobic zone use oxygen and gives off carbon dioxide thus lowering the pH. This in turn reacts with the water to produce a weak acid (carbonic acid). The resultant weak acid might be responsible for lowering of the pH from 7.2 to 6.5.

Phosphate accumulating bacteria needs to be maintained in a specific narrow range of pH for maximum removal of phosphorus. Typical pH ranges of 6.5 – 8.5 are optimal for the different PAOs therefore the change from 7.2 to 6.5 probably had no implication on phosphorus removal.

Temperature

During the respiratory process, breakdown of the available carbon source occurs resulting in the evolution of energy in the form of heat thus the change in temperatures before and after inoculation

4.3 Optimal working conditions for effective phosphorous removal by Polyphosphate Accumulating Organisms (PAOs).

Three different parameters were tested for at different ranges so as to ascertain the favorable working conditions for PAOs to blossom along with a negative control where nothing was tampered with, not even inoculation of the bacteria. These included pH4, pH7 and pH9, Temperature (T25°C, T35°C, and T45°C) and Carbon source (Glucose and acetate).

Table 4-3: Phosphorus levels across different enhancements, original and a negative control

Treatment	Phosphorous (Mean±SEM)
Temperature of 25°C	1.97±0.15a
Temperature of 35°C	4.83±0.12d
Temperature of 45°C	5.47±0.15e
pH of 4	2.77±0.06b
pH of 7	1.77±0.25a
pH of 9	2.70±0.10b
Glucose	5.17±0.15de
Acetate	4.27±0.06c
Negative control	5.97±0.15f
Initial sample	6.17±0.12f

Values are Means±SEM (n=3), means with different letters of the alphabet within a particular row indicate a significant difference between the values, Turkey's test (P<0.05) for phosphorous in the amendment. [SEM, standard error of the mean]

Carbon Source

The water that collected at the clarifier was tested and the results showed that the setup fed with acetate greatly reduced the phosphorous levels from 6.1mg/L to 4.267mg/L as compared to the set up fed with glucose.

PAOs rapidly absorbed acetate and utilize it to synthesis PHA hence utilizing stored poly P as an energy source under anaerobic circumstances with the orthophosphate produced by poly P breakdown discharged into the bulk liquid. The organisms with stored PHA utilized them as a carbon and energy sources to develop and ingest phosphate to manufacture poly P in the absence of any organic molecules in the aerobic zone. Importantly, both acetate and glucose appeared to contribute to anaerobic PHA synthesis and the PHA was then used aerobically for glycogen production (Maurer *et al.*,n.d.; Mino *et al.*,n.d.; Seviour*et al.*, 2003; Smolders et al., 1994; van Loosdrecht*et al.*, 1997).

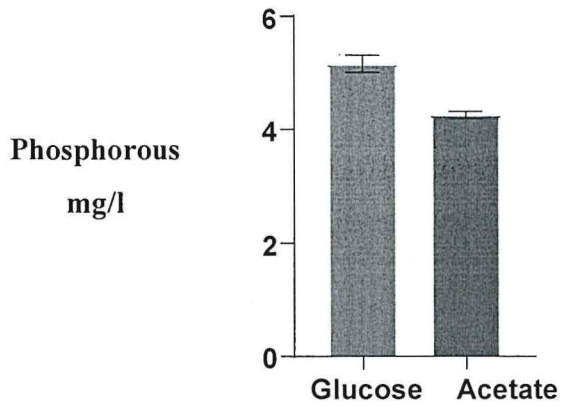


Figure 0.5: Effects of carbon source on Phosphorus removal

Temperature

Temperature variations of 25°C, 35°C and 45°C were considered and it was noted that at a temperature of 25°C, the highest rate of phosphorous removal was noted. An increase in temperature to 45°C registered the lowest rate of P-removal in the setup since PAOs are more competitive than GAOs at lower temperatures. Temperature also affects the transformations of glycogen and polyhydroxyalkanoates and the activities of exopolyphosphatase and polyphosphate kinase activities. These results agree with most documented studies, though there are some few contradicting results that were documented. (Brdjanovic, Logemann, et al., n.d.; Brdjanovic, Mark, *et al.,* n.d.; Chen et al., 2014; Erdal*et al.*,n.d.; López-Vázquez*et al.,* 2008; Oehmen*et al.,* n.d.). It influences the metabolic activities of the microbial population, gas-transfer rates and the settling characteristics of the biological solids (Mulkerrins*et al.,* 2004)

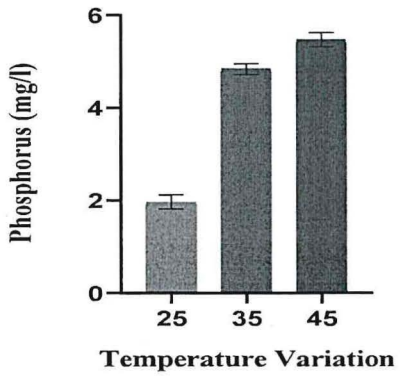


Figure 0.6: Effects of varying Temperatures on Phosphorus removal

pH

The experiment used acetate wastewater as pH was monitored throughout the anaerobic and aerobic stages since the metabolism of PAOs in acetate wastewater is dependent on pH. Maximum acetate absorption occurred at a pH 7 whereby most heterotrophs could not handle pH levels higher than 9.5 or lower than 4.0. The ratio of anaerobic P released to acetate absorption was not constant as it was affected by the mixed liquor pH which influenced the energy demand for acetate transport. Nitrification, denitrification, P-release, and P-uptake are all processes that have a certain pH. (Kim *et al.*, 2016; Zhang *et al.*, 2005)

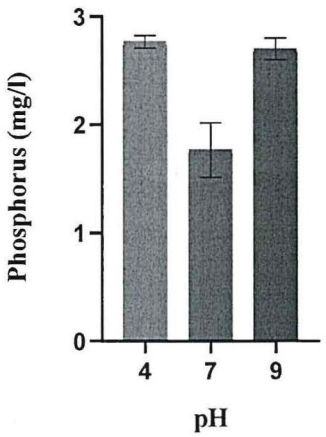


Figure 0.7: Effects of pH on Phosphorus removal

When the set up was subjected to pH 4 at a temperature of 35^0C, the phosphorous levels reduced from 6.1mg/l to 5.1mg/l

A minimal phosphorous reduction noted when the setup was subjected to pH 9 and 45^0C with acetate as the carbon source from 6.1mg/l to 5.3mg/l.

A reduction in phosphorous levels from 6.1mg/l to 2.9mg/l was noted when the experimental set up was subjected to pH7 at a temperature of 25^0C and fed with acetate at the same time.

CHAPTER 5: CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The EBPR system has not been commonly used in Uganda due to the operational knowledge gap despite studies showing a high rate of efficiency in the control of nutrient discharge to aquatic environment. Wastewater treatment studies that have been widely carried out on the EBPR systems prove cost effectiveness of the system since no chemicals are required and demonstrate excellent consistent performance as regards phosphorous removal under rigorous discharge conditions.

Too much phosphorus can cause increased growth of algae and large aquatic plants, which can result in decreased levels of dissolved oxygen– a process called eutrophication. High levels of phosphorus can also lead to algae blooms that produce algal toxins which are harmful to human and animal health.

Microbial studies of this dissertation showed that different waste water streams had different numbers of bacteria isolated with the Bugolobi Inlet having the highest number of microbes (12) and Brewery effluent with the least number of microbes (1). Among the 20 samples that were sent in for genotyping, 16 different species of bacteria were obtained lying in the groups: firmicutes; High GC gram positive, β-proteo bacteria, α-proteo bacteria and γ-proteo bacteria.

Optimal working conditions for effective phosphorous removal by polyphosphate accumulating organismsgave an overall significant difference at $P<0.05$(ANOVA) with acetate as the best carbon source, [$P=0.001$, ANOVA], 25°C as the best optimum temperature and pH 7 for their activity, [$P=0.001$, ANOVA]

The system is also not free from occasional upsets resulting from permit violations and inconsistent operational environmental conditions thus affecting the stability and reliability of the system.

5.2 Recommendation

Efficient phosphorous removal in the EBPR system is highly dependent on PAOs suppressing GAOs. Approaches that favour the development of PAOs but suppress the growth of GAOs in the EBPR active sludge systems have to be found and suggested.

There is need to obtain a more detailed insight on how the EBPR process works since the EBPR system experiences disturbances and prolonged periods of insufficient phosphorous removal at full scale despite the seemingly desirable conditions at which the EBPR process works.

REFERENCES

Adam, C. (2008) Sustainable and Safe Re-use of Municipal Sewage Sludge for Nutrient Recovery – SUSAN [URL] http://www.susan.bam.de/

Adam, C., B. Peplinski, M. Michaelis, G. Kley& F. G. Simon (2009) Thermochemical treatment of sewage sludge ashes for phosphorus recovery. Waste Management, 29(3), 1122-1128.

Anand, Ajeeta, and Hideki Aoyagi. "A High Throughout Isolation Method for Phosphate-Accumulating Organisms." *Scientific Reports*, vol. 9, no. 1, Dec. 2019, 10.1038/s41598-019-53429-2. Accessed 21 Apr. 2022. (Anand and Aoyagi. *et al.*, 2019)

Ansari, Abid A, and Sarvajeet Singh Gill.*Eutrophication: Causes, Consequences and Control.* Dordrecht, Springer, 2014.Barakat, M.A. and Schmidt, E. (2010).Polymer-enhanced ultrafiltration process for heavy metals removal from industrial wastewater.Desalination, 256(1–3), pp.90–93.

Barbera, Marcella, and Giovanni Gurnari. *Wastewater Treatment and Reuse in the Food Industry*. Cham, Springer International Publishing, 2017.

Bi, D., Guo, X. and Chen, D. (2013). Phosphorus release mechanisms during digestion of EBPR sludge under anaerobic, anoxic and aerobic conditions. Water Science and Technology, 67(9), pp.1953–1959.

Chen, Huanjun, *et al.*, "Nitrogen and Phosphorus Removal by GAOs and PAOs Using Nitrate and Limited Oxygen as Electron Acceptors Simultaneously and the Impact of External Carbon Source in the Anoxic Phase."Journal of Environmental Chemical Engineering, vol. 9, no. 6, Dec. 2021, p. 106520, 10.1016/j.jece.2021.106520. (Chen et al.2021)

Choi, J., Lee, B., Kim, K. and Baek, D. (2016).Improved Coagulant for High Efficiency Phosphorus Removal in Secondary Effluent of Waste Water Treatment Plant.Journal of the Korean Society of Water and Wastewater, 30(6), pp.683–690.

Cordova, Hendra, and Andry F. Wijaya."Self-Tuning Pid Neural Network Controller to Control Nonlinear Ph Neutralization in Waste Water Treatment."*IPTEK the Journal for Technology and Science*, vol. 18, no. 3, 30 Aug. 2007, 10.12962/j20882033.v18i3.168.

Cornel, P. & C. Schaum (2009) Phosphorus recovery from wastewater: needs, technologies and costs. Water Science & Technology, 59(6), 1069–1076.

Deniz, T. and Bratby, J.R. (2018). Road Map to Achieving Effluent Total Phosphorus Limit of 0.043 Mg/L. Proceedings of the Water Environment Federation, 2018(5), pp.811–815.

Derin Orhon, et al. *Industrial Wastewater Treatment by Activated Sludge*. IWA Publishing, 2 Feb. 2009.

DezottiMárcia, et al. *Advanced Biological Processes for Wastewater Treatment : Emerging, Consolidated Technologies and Introduction to Molecular Techniques*. Cham, Switzerland, Springer, 2018.

Dotro, Gabriela, et al. *Treatment Wetlands*. London, Iwa Publishing, 2017.

Dölle, K. and Watkins, C. (2017).Algae to Remove Phosphorous in a Trickling Filter.Journal of Advances in Biology & Biotechnology, 11(2), pp.1-5.

Feinstein, Stephen. *Conserving and Protecting Water*. Enslow Publishers, Inc., 1 Apr. 2013.

Freni, Gabriele, et al. "The Influence of Rainfall Time Resolution for Urban Water Quality Modelling." *Water Science and Technology*, vol. 61, no. 9, 1 May 2010, pp. 2381–2390, https://doi.org/10.2166/wst.2010.162. Accessed 27 Oct. 2020.

Freni, G., Mannina, G. and Viviani, G. (2010).Emission standards versus immission standards for assessing the impact of urban drainage on ephemeral receiving water bodies.Water Science and Technology, 61(6), pp.1617-1629.

Fujiwara, Kazuya, et al. "Impact of PH on Anaerobic Substrate Uptake by PAOs and GAOs in an EBPR Activated Sludge Process Analyzed by MAR-FISH." Journal of Water and Environment Technology, vol. 7, no. 4, 2009, pp. 215–223, 10.2965/jwet.2009.215.(Fujiwara et al., "Impact of PH on Anaerobic Substrate Uptake by PAOs and GAOs in an EBPR Activated Sludge Process Analyzed by MAR-FISH")

Fukushima, T., Onuki, M., Satoh, H. and Mino, T. (2010). Effect of pH reduction on polyphosphate- and glycogen-accumulating organisms in enhanced biological phosphorus removal processes. Water Science and Technology, 62(6), pp.1432-1439.

Gasperi, J., Gromaire, M.C., Kafi, M., Moilleron, R. and Chebbo, G. (2010). Contributions of wastewater, runoff and sewer deposit erosion to wet weather pollutant loads in combined sewer systems. Water Research, 44(20), pp.5875–5886.

Geissler, Catherine, and Hilary J Powers.*Human Nutrition.*13th ed., Oxford Oxford University Press, 2017.

Godwin, C.M. and Cotner, J.B. (2015). Aquatic heterotrophic bacteria have highly flexible phosphorus content and biomass stoichiometry. The ISME Journal, 9(10), pp.2324–2327.

Hankins, W., Jancaitis, J. and Olson, K. (2011). Hudson, Massachusetts Utilizes Dissolved Air Flotation Tertiary Treatment to Achieve <0.1 mg/L Total Phosphorous. Proceedings of the Water Environment Federation, 2011(15), pp.1911-1921.

Henze, M, M. C. M. van Loosdrecht, G. A. Ekama& D. Brdjanovic (2008) Biological wastewater treatment: principles, modelling and design. IWA publishing.

INOUE, DAISUKE, et al. "Polyhydroxybutyrate Accumulation Ability and Relevant Microbial Populations in Acetate-Fed Mixed Microbial Cultures Enriched from Activated Sludge under Different Temperature Conditions." Japanese Journal of Water Treatment Biology, vol. 53, no. 2, 2017, pp. 57–67, 10.2521/jswtb.53.57.Accessed 15 Mar. 2020. (INOUE et al.2020)

Jr, Franklin, et al. *Middleton's Allergy : Principles and Practice (Expert Consult - Online).* London, Elsevier Health Sciences, 2013.

Jenkins, S H, and K J Ives.*Phosphorus in Fresh Water and the Marine Environment.* Elsevier, 22 Oct. 2013.

Kansiime, Frank, and MaimunaNalubega.*Wastewater Treatment by a Natural Wetland: The Nakivubo Swamp, Uganda.* CRC Press, 2020.

Katerina Stamatelatou, and Konstantinos P Tsagarakis. *Sewage Treatment Plants : Economic Evaluation of Innovative Technologies for Energy Efficiency.* London, Iwa Publishing, 2015.

Knowles, Christopher. *Diversity of Bacterial Respiratory Systems.* CRC Press, 18 Jan. 2018.

Kundu, S., Coumar, M. V., Rajendiran, S., Rao, A., &Rao, A. S. (2015). Phosphates from detergents and eutrophication of surface water ecosystem in India.*Current Science*, *108*(7), 1320–1325. http://www.jstor.org/stable/24905495

Le Corre, K., E. Valsami-Jones, P. Hobbs & S. Parsons (2009) Phosphorus recovery from wastewater by struvite crystallization: A review. Critical Reviews in Environmental Science and Technology, 39(6), 433-477.

Littler, M., Littler, D., Lapointe, B. and Barile, P. (2006). Toxic Cyanobacteria (blue-green algae) associated with groundwater conduits in the Bahamas. Coral Reefs, 25(2), pp.186-186.

Liu, Y., S. Kumar, J.-H.Kwag& C. Ra (2013) Magnesium ammonium phosphate formation, recovery and its application as valuable resources: a review. Journal of Chemical Technology & Biotechnology, 88(2), 181-189.

Makinia, J., et al. "Internal vs. External (Alternative) Carbon Sources for Denitrification and EBPR Accomplished by a Full-Scale Process Biomass." Proceedings of the Water Environment Federation, vol. 2009, no. 4, 1 Jan. 2009, pp. 16–30, 10.2175/193864709793901130. (Makinia*et al.*, 2009)

Mannina, Giorgio, et al. *Advances in Wastewater Treatment*. London, Iwa Publishing, 2018.

Mannina, Giorgio, and Springerlink (Online Service. *Frontiers in Wastewater Treatment and Modelling : FICWTM 2017*. Cham, Springer International Publishing, 2017.

Manuel, Carlos. *The Competition between Polyphosphate-Accumulating Organisms and Glycogen-Accumulating Organisms*. CRC Press, 13 July 2017.

Marcos Von Sperling. *Wastewater Characteristics, Treatment and Disposal*. London ; New York, Iwa Pub, 2007.

Massry, Shaul. *Phosphate Metabolism*. Springer Science & Business Media, 21 Nov. 2013.

---. *Phosphate Metabolism*. Springer Science & Business Media, 21 Nov. 2013.

Menahem Libhaber, and Álvaro Orozco-Jaramillo. *Sustainable Treatment and Reuse of Municipal Wastewater : For Decision Makers and Practicing Engineers*. London ; New York, Iwa Publishing Alliance House, 2012.

Merrylin, J., Kumar, S.A., Kaliappan, S., Yeom, I.-T. andBanu, J.R. (2013). Biological pretreatment of non-flocculated sludge augments the biogas production in the anaerobic digestion of the pretreated waste activated sludge. Environmental Technology, 34(13–14), pp.2113–2123.

Meyer, Richard J., and Stacie A. Brown.*Challenges of the Unseen World: A Laboratory Course in Microbiology*. Washington, DC, ASM Press, 2018.

Milo, Ron, and Rob Phillips. *Cell Biology by the Numbers*. Independence, Taylor & Francis Group, 2015.

Murray, Maynard. *Sea Energy Agriculture*. Austin, Tex., Acres U.S.A, 2003.

Munn, Mark D, et al. *Understanding the Influence of Nutrients on Stream Ecosystems in Agricultural Landscapes*. Reston, Virginia, U.S. Department Of The Interior, U.S. Geological Survey, 2018.

Mohammad Saghir Khan, et al. *Phosphate Solubilizing Microorganisms Principles and Application of Microphos Technology*.Cham Springer International Publishing, 2014.

Mokhtar, G. (2012). Microfiltration, Nano-filtration and Reverse Osmosis for the Removal of Toxins (LPS Endotoxins) from Wastewater.Journal of Membrane Science & Technology, 02(03).

Moosavi, G.H., et al. "Simultaneous Organics and Nutrients Removal from Municipal Wastewater in an Up-Flow Anaerobic/Aerobic Fixed Bed Reactor." Journal of Applied Sciences, vol. 5, no. 3, 15 Feb. 2005, pp. 503–507, 10.3923/jas.2005.503.507. (Moosaviet al.2005)

Motizuki, M. "Low PH Facilitates Uptake of Proteins by Cells through a Non-Endocytic Pathway." Journal of Biochemistry, vol. 135, no. 6, 1 June 2004, pp. 713–719, 10.1093/jb/mvh087. (Motizuki*et al.*, 2004)

Nielsen, S. (2006). Size-dependent growth rates in eukaryotic and prokaryotic algae exemplified by green algae and cyanobacteria: comparisons between unicells and colonial growth forms. Journal of Plankton Research, 28(5), pp.489-498.

Özacar, M. & İ. A. Şengil (2003) Enhancing phosphate removal from wastewater by using polyelectrolytes and clay injection. Journal of Hazardous Materials, 100(1–3), 131-146.

Parsons, S. A. & J. A. Smith (2008) Phosphorus Removal and Recovery from Municipal Wastewaters. Elements, 4(2), 109-112.

Peng, Weihua, et al. "Sludge Blanket Formation and Its Effects on Secondary Clarifier Performance." Journal of Environmental Engineering, vol. 133, no. 7, July 2007, pp. 758–764, 10.1061/(asce)0733-9372(2007)133:7(758). (W. Peng*et al.,* 2007)

Peng, Yong-zhen, et al. "Anoxic Biological Phosphorus Uptake and the Effect of Excessive Aeration on Biological Phosphorus Removal in the A2O Process."Desalination, vol. 189, no. 1-3, Mar. 2006, pp. 155–164, 10.1016/j.desal.2005.06.023.Accessed 11 Apr. 2020. (Y. Penget al.2006)

Petzet, S. & P. Cornel (2013) Phosphorus Recovery from Wastewater. In R. E. Hester & R. M. Harrison (eds.), Waste as a Resource. Royal Society of Chemistry.

Petzet, S., B. Peplinski& P. Cornel (2012b) On wet chemical phosphorus recovery from sewage sludge ash by acidic or alkaline leaching and an optimized combination of both. Water Research, 46(12), 3769-3780.

Porter, Ruth, and David W FitzSimons. *Phosphorus in the Enviroment*. John Wiley & Sons, 16 Sept. 2009.

Richardson, A. E. & R. J. Simpson (2011) Soil Microorganisms Mediating Phosphorus Availability Update on Microbial Phosphorus. Plant Physiology, 156(3), 989-996.

Rimbach, Gerald, and Jürgen Fuchs. *Nutrigenomics*. CRC Press, 23 May 2005.

Rossetti, Simona, et al. *Activated Sludge Separation Problems : Theory, Control Measures, Practical Experiences*. London, Iwa Publishing, 2017.

Schaum, Christian. *Phosphorus : Polluter and Resource of the Future - Removal and*

Recovery from Wastewater. London, Iwa Publishing, Gb, 2018.

Sedlak, RichardI. *Phosphorus and Nitrogen Removal from Municipal Wastewater*.Routledge, 27 Apr. 2018.

Seviour, J.R., Maszenan, M.A., Soddell, A.J., Tandoi, V., Patel, C.K.B., Kong, Y & Schumann, P. (2000). Minireview: Microbiology of the 'G-bacteria' in activated sludge. Environmental microbiology 2, 581 – 593

Seviour, R J. *The Microbiology of Activated Sludge*. London, Iwa, 2010.

Spellman, Frank R. *Handbook of Water and Wastewater Treatment Plant Operations, Third Edition*. CRC Press, 21 Oct. 2013.

Stephen Henry Schneider, et al. *Encyclopedia of Climate and Weather*. New York, Oxford University Press, Cop, 2011.

Stewart, M., Palumbo, R. and Rossi, C. (2017) Performance Testing and Long Term Operations Demonstrate Successful Application of Discfilter System for Stringent Effluent Phosphorus Limits. Proceedings of the Water Environment Federation, 2017(3), pp.210-218

Tamim Younos, and Tammy E Parece. *Sustainable Water Management in Urban Environments*. Cham, Springer International Publishing, 2016.

Takouleu, J.M. (2021). Uganda: The Bugolobi-Nakivubo wastewater treatment plant is finally operational. Retrieved from https://www.afrik21.africa/en/uganda-the-bugolobi-nakivubo-wastewater-treatment-plant-is-finally-operational/ on 25-June-2022

Tchobanoglous, G., M. Abu-Orf, G. Bowden & W. Pfrang (2014) Wastewater Engineering: Treatment and Resource Recovery. McGraw-Hill Education, New York.

Theodore, Louis, and R. Ryan Dupont. *Water Resource Management Issues*. CRC Press, 26 Nov. 2019.

The Economist. *World in Figures*. Springer, 1 May 2016.

"Urban Storm-Water Quality Management: Centralized versus Source Control." *Journal of Water Resources Planning and Management*, vol. 136, no. 2, Mar. 2010, pp. 268–

278, https://doi.org/10.1061/(asce)0733-9496(2010)136:2(268). Accessed 26 Apr. 2022.

Uribarri, Jaime, and Mona S. Calvo. *Dietary Phosphorus : Health, Nutrition, and Regulatory Aspects*. Boca Raton, FL, CRC Press, Taylor & Francis Group, 2018.

Vipin Chandra Kalia, and Springerlink (Online Service. *Microbial Factories : Biodiversity, Biopolymers, Bioactive Molecules: Volume 2*. New Delhi, Springer India, 2015.

WEF (2011) Nutrient removal.WEF, Alexandria, Va.

Xu, Xiangyang, et al. "Enhanced Denitrifying Phosphorous Removal in a Novel Anaerobic/Aerobic/Anoxic (AOA) Process with the Diversion of Internal Carbon Source." Bioresource Technology, vol. 102, no. 22, Nov. 2011, pp. 10340–10345, 10.1016/j.biortech.2011.08.108. (Xu*et al.,* 2011)

Zuo, N, and F Y Ji."The Influence of SRT on Phosphorus Removal and Sludge Characteristics in the HA–A/A–MCO Sludge Reduction Process."Laser Physics Letters, vol. 10, no. 2, 17 Dec. 2012, p. 025601, 10.1088/1612-2011/10/2/025601.Accessed 25 Oct. 2019. (Zuo and Ji*et al.,* 2019)

Zuo, N. and Ji, F.Y. (2012).The influence of SRT on phosphorus removal and sludge characteristics in the HA–A/A–MCO sludge reduction process.Laser Physics Letters, 10(2), p.025601.

APPENDICES

APPENDEX I: Sequential Analysis

Table A-1: Sequence Analysis

Sample ID	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len
Go	Corynebacteriumbovis strain Evans 16S ribosomal RNA gene, partial sequence	Corynebacteriumbovis	2658	2658	99%	0.00E+00	99.79%	1445
Ao	Acinetobactergerneri DSM 14967 = CIP 107464 = MTCC 9824 16S ribosomal RNA, partial sequence	Acinetobactergerneri	2287	2489	89%	0.00E+00	99.29%	1505
Co	Pseudomonas putida strain SG1 16S ribosomal RNA gene, partial sequence	Pseudomonas putida	2327	2327	92%	0.00E+00	99.69%	1525
D6	Acinetobacter sp. RSPH4 gene for 16S ribosomal RNA, partial sequence	Acinetobacter sp. RSPH4	2366	2366	93%	0.00E+00	98.32%	1460
A2	Acinetobacterjunii gene for 16S ribosomal RNA, partial sequence, strain: VSJK-R6	Acinetobacterjunii	2470	2470	97%	0.00E+00	99.07%	1265
A5	Acinetobacterjunii strain L-15 16S ribosomal RNA gene, partial sequence	Acinetobacterjunii	2366	2366	95%	0.00E+00	90.97%	466
Eo	Uncultured Acinetobacter sp. clone W1109-190 16S ribosomal RNA gene, partial sequence	Uncultured Acinetobacter sp.	2289	2289	90%	0.00E+00	99.34%	1236
H	Pseudomonas aeruginosa strain soucheG3 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2252	2252	86%	0.00E+00	98.93%	1196
C8	Pseudomonas brenneri strain SMC29 16S ribosomal RNA gene, partial sequence	Pseudomonas brenneri	2271	2271	87%	0.00E+00	98.56%	929
K	Acinetobactercalcoaceticus strain ICA01 16S ribosomal RNA gene, partial sequence	Acinetobactercalcoaceticus	2534	2534	98%	0.00E+00	99.26%	1590
F6	Pseudomonas aeruginosa strain EPAn16 16S ribosomal RNA gene, partial sequence	Pseudomonas aeruginosa	2263	2263	87%	0.00E+00	97.11%	1391
E3	Comamonas sp. 141S gene for 16S rRNA, partial sequence	Comamonas sp. 141S	2265	2265	87%	0.00E+00	97.61%	1520
G5	Aquaspirillumputridiconchylium strain IAM 14964 16S ribosomal RNA, partial sequence	Aquaspirillumputridiconchylium	2171	2171	82%	0.00E+00	88.59%	1477

B2	Chitinilyticumaquatile strain CipRes.II 16S ribosomal RNA gene, partial sequence	Chitinilyticumaquatile	2207	2207	85%	0.00E+00	92.05%	1393
G4	Ochrobactrumintermedium strain F5_AM 16S ribosomal RNA gene, partial sequence	Brucellaintermedia	2549	2549	98%	0.00E+00	99.51%	1437
A9	Acinetobacterpittii DSM 21653 strain CIP 70.29 16S ribosomal RNA, partial sequence	Acinetobacterpitti	2651	2651	99%	0.00E+00	99.68%	1442
C3	Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA, partial sequence	Pseudomonas aeruginosa	2325	2325	92%	0.00E+00	99.57%	1521
E4	Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	Bacillus cereus	2271	2271	87%	0.00E+00	98.35%	1012
B5	Phycicoccuselongatus Lp2 16S ribosomal RNA, partial sequence	Phycicoccuselongatus Lp2	2555	2555	98%	0.00E+00	99.45%	1425
C1	Pseudomonas putida strain IAM 1236 16S ribosomal RNA, partial sequence	Pseudomonas putida	2201	2201	85%	0.00E+00	92%	1353

APPENDIX II: Inoculation results of critical parameters

Table A-2: Effect of inoculation on critical parameters

Parameter	Before Inoculation			After Inoculation		
ORP (mV)	13.4	13.4	13.4	-113.4	-113.4	-113.4
pH	7.2	7.4	7	6.5	6.8	6.2
Phosphorous (mg/L)	6.1mg/L	6.4	5.8	5.4	5.5	5.3
Temperature (°C)	24.6	24.4	24.8	27.3	27.3	27.3

Table A-3: Effect of varying conditions on phosphorus removal

	Total Phosphorus, mg/l	Sample ID	Result			Average
1	Original	Prefermenter	6.1	6.3	6.1	6.167
2	Negative control	No enhancement	5.8	6	6.1	5.967
3	Temperature	T25°C	2.0	1.8	2.1	1.967
		T35°C	4.9	4.9	4.7	4.833
		T45°C	5.6	5.3	5.5	5.467
4	pH	pH 4	2.8	2.7	2.8	2.767
		pH 7	1.8	1.5	2	1.767
		pH 9	2.8	2.6	2.7	3
5	Carbon source	Glucose	5.2	5.3	5	5
		Acetate	4.3	4.2	4.3	4.267



Figure A.1: Centrifuge of the sample and PCR running



Figure A.2: Isolates

APPENDIX III: Letter of acceptance for sample collection at NWSC Bugolobi

NATIONAL WATER & SEWERAGE CORPORATION

HEAD OFFICE

TELEGRAMS WATERS KAMPALA
Telephone : 256 - 313 - 315000/100
Twitter : @nwscug
Facebook : www.facebook.com/waterug
Email : info@nwsc.co.ug

P.O. Box 7053
Plot 3 Nakasero
KAMPALA

Our Ref: BSS/R&D/22-03

Date: March 22, 2021

The Sen. Manager, Sewerage Services, KW

Re: PERMISSION TO ACCESS AND COLLECT RAW SEWAGE FROM THE NAKIVUBO WW TREATMENT PLANT - BUGOLOBI

This is to introduce to you Mr. Andrew Ssempala Bbumba, a student of Kyambogo University, pursuing a MSc. in Sanitation Engineering, who has been granted permission to collect raw sewage from the Nakivubo WW Treatment Plant. The student requires the samples for laboratory analysis at the University as part of his Masters research project. The samples are therefore meant for academic purposes only.

In this regard, you are kindly requested to accord him access to the treatment plant to enable him successfully accomplish his research project. This admission is valid up to end of April 2022.

Anticipating your usual cooperation.

Christopher Kanyesigye
Manager, Research and Development

APPENDIX IV: Introductory Letter to Makerere University

MAKERERE UNIVERSITY
P.O. Box 7062 Kampala, Uganda
http://www.sci.mak.ac.ug
http://www.science.ac.ug
Tel: +256-414-540765 URL:
Fax: +256-414-532780
Email: pmb@cns.mak.ac.ug

COLLEGE OF NATURAL SCIENCES
DEPARTMENT OF PLANT SCIENCES, MICROBIOLOGY AND BIOTECHNOLOGY

November 1, 2021,

The in-Charge Research Laboratory
Dear Sir,

RE: INTRODUCTION OF MR SSEMPALA ANDREW BBUMBA – 17/U/14705/GMEW/PE

I take great pleasure in introducing to you the above-mentioned gentleman. He is a student pursuing a master's degree in water and sanitation engineering at Kyambogo University.
He seeks to have his research project work done in the Research laboratory in the fields of microbiology and molecular biology anticipated to run for five months starting on 8th November 2021. He is expected to buy his reagents and reaction mixes for his work unless decided otherwise by the office of the chief technician.
I therefore kindly request you to guide him and provide him with all the necessary help needed to accomplish his laboratory work.

Yours sincerely,

Abubakar Sadik Mustafa
Ass. Head of Labs & stores
P. O. Box 7062 Kampala - UGANDA
Tel: +256-779-764915

APPENDIX V: Letter of shipment of PCR product samples for sequencing analysis

MAKERERE UNIVERSITY
P.O. Box 7062 Kampala, Uganda
URL: http://www.sci.mak.ac.ug
http://www.science.ac.ug
Tel: +256-414-540765
Fax: +256-414-532780
Email: pmb@cns.mak.ac.ug

COLLEGE OF NATURAL SCIENCES
DEPARTMENT OF PLANT SCIENCES, MICROBIOLOGY AND BIOTECHNOLOGY

10th January, 2022

To Whom It May Concern

Freight, Custom clearance and delivery of products worldwide C/O FEDex

RE: SHIPMENT OF PCR PRODUCT SAMPLES FOR SEQUENCING ANALYSIS BY MACROGEN EUROPE, AMSTERDAM, THE NETHERLANDS

I am a master's student at Kyambogo University currently conducting research titled "Enhancing phosphorus removal from waste water using polyphosphate accumulating organism from different water waste streams"

In this research, I am charged with a number of objectives one of which involves molecular Identification (sequencing) of microbes from the different waste water streams.

I have managed to collect the samples from the different parts of sample collection, extracted the nucleic acids, performed the polymerase chain reactions and now the products need to be sent to Macrogen Europe, Amsterdam, the Netherlands for sequencing since this company has the expertise and competence in sequencing services

This is therefore to request that you allow the shipment of these samples that **ARE NOT DANGEROUS, CORROSIVE, OXIDATING, TOXIC, INFECTIOUS or RADIOACTIVE AND DO NOT IMPLY RISK IN AIR TRANSPORTATION** according to the actual rules (IATA and ICAO). The samples have no commercial value and are intended for research purposes only.

Your consideration of our request is highly appreciated.

Yours sincerely

Ssempala Andrew .B.

Tel: +256 782383936

Email: andrewsempala@ymail.com

APPENDIX VI: Letter of Authorisation from Macrogen Europe

Letter of Authorization (LOA)

Humanizing Genomics macrogen EUROPE

January 7th, 2021

To: Federal Express Corporation Uganda

This letter serves as confirmation that **Macrogen Europe B.V.** a division of **Macrogen Inc.** has authorized the following shipper to arrange for any number of FedEx shipments to be picked up and billed to the designated payor account.

Effective Date:

This LOA shall continue to be in effect until termination by either FedEx or Payor giving not less than 14 days written notice of its intention to terminate. Any such termination will be without prejudice to any liabilities previously accrued and arising under this LOA and settlement shall be in accordance with this LOA.

Shipper's Company Name:
Address: Kampala / Bukoto /Uganda
Phone Number: +256782383936
Email: andrewsempala@ymail.com
Name: Ssempala Andrew

Recipient's Company Name: *Macrogen Europe B.V.*
Address: Meibergdreef 57, 1105BA, Amsterdam, the Netherlands
Phone Number: +31 (0)20 333 7563
Email: info@macrogen-europe.com
Name: *Maria Teresa Borrero Borrego*

Attention: In connection with above -mentioned shipper, we hereby confirm our agreement to pay all charges including both Freight & Duty/Tax and authorize FedEx to charge **Macrogen Europe B.V.** using FedEx Account Number **473205645.**

We acknowledge that FedEx may at its option decline to bill to our FedEx account if it is deemed by FedEx as an account not in good credit standing.

Sincerely yours,

Sales Account Manager

Valid through: 31 December 2022. Only for shipments to Macrogen Europe B.V.

APPENDIX VII: Clearance letter from Kyambogo University

KYAMBOGO UNIVERSITY

Department of Civil and Building Engineering

P. O. BOX 1, KYAMBOGO – P. O. BOX 7181 KAMPALA, UGANDA
Website: www.kyu.ac.ug, Email: civil@kyu.ac.ug
TEL: +256-41-4287340, FAX: +256-41-4289056/4222643

February 8, 2020

SM. WRM
Could you have a brief with the student and guide regarding his admission
3/3/2020

Manager,
International Resource Center,
National Water & Sewerage Cooperation – Bugolobi,
Kampala, Uganda.

Dear Eng./Dr./Sir/Madam,

RE: INTRODUCTION LETTER FOR SSEMPALA ANDREW BBUMBA

Mr. Andrew Ssempala Andrew Bbumba is a student of Kyambogo University undertaking Master of Science in Water and Sanitation Engineering from the Department of Civil and Building Engineering. He is conducting a research study on "Assessment of the effectiveness of biological nutrient removal from waste water". The researcher is being supervised and co-supervised by Dr. Charles Onyutha and Eng. Dr. Anne Nakagiri, respectively.

In his research, Andrew Ssempala will require support on a number of occasions for the various specific objectives of his study (see next page). Both primary and secondary data are required. Importantly, the student will need support regarding experimental sites and setup, laboratory testing of samples, etc. This letter is therefore to introduce the researcher and seek your support where necessary. The necessary support rendered to ensure timely completion of this research will be duly acknowledged in the dissemination of the results.

I shall be grateful for any assistance rendered to Mr. Andrew Ssempala to allow him conduct his research study timely.

MRKD
The research is fish
and a shower at Mekerere
will be done at
Lab. The study is
hoped to be
completed as planned
into an above chapter,
remotely. I am pleased to
can provide
help any

Yours Sincerely,

THE HEAD OF DEPARTMENT
CIVIL AND BUILDING ENGINEERING
KYAMBOGO UNIVERSITY

Dr. Lawrence Muhwezi
Head of Department of Civil and Building Engineering

Cc. Dean, School of Graduate Studies, Kyambogo University
Dr. Charles Onyutha - Department of Civil and Building Engineering, Kyambogo University
Eng. Dr. Anne Nakagiri - Department of Civil and Building Engineering, Kyambogo University

Cc. Uganda Breweries
CC. Nile Breweries Limited.

(SSEMPALA ANDREW)