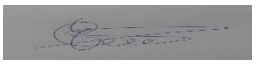
Universitetet i Stavanger

FACULTY OF SCIENCE AND TECHNOLOGY

MASTER'S THESIS

Study programme/specialisation: Environmental Engineering	Spring semester, 2019 Open
Author: **Yaw Boateng**	 …… (signature of author)
Supervisor(s): Krista Michelle Kaster	
Title of master's thesis**: Pathogenic bacteria removal in wastewater using Upflow Anaerobic Sludge Blanket (UASB) Reactor**.	
Credits: 30	
Keywords: *Wastewater, UASB reactor, antibiotic resistance genes, pathogen removal, tetracycline, sulfamethoxazole, erythromycin*	Number of pages: 77 + Front part :8 pages Stavanger, 15/06/2019 date/year

PATHOGENENIC BACTERIA REMOVAL IN WASTEWATER USING UPFLOW ANAEROBIC SLUDGE BLANKET REACTOR (UASB)

MASTER'S THESIS

WATER SCIENCE AND TECHNOLOGY

ENVIRONMENTAL TECHNOLOGY STUDY PROGRAM

DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES

UNIVERSITY OF STAVANGER

2019

ABSTRACT

The search for effective wastewater management strategies continues and never stops due to the importance of ensuring safe environment for all organisms. The Upflow Anaerobic Sludge Blanket (UASB) reactor is cost effective, produces less quantity of sludge and also has smaller size. Such importance makes it an ideal treatment technology which requires a lot of study to know its capabilities and how they can be improved. The main aim of this study was to assess the effectiveness of the UASB reactor in bacterial pathogen and antibiotic resistance genes removal. The pathogens that were considered were total coliforms with focus on *Escherichia coli* and *Enterococcus*. Effects of organic loading rates (OLR) and hydraulic retention time (HRT) were also considered. Qualitative antibiotic resistance genes for sulfamethoxazole *sul* (I, II, III), tetracycline *tet* (A, B, C, D, G, K, L, M, O, *otr* B) and erythromycin *erm* (A, B, C and *msr* A) were studied. There were four sampling periods which were used with no specific intervals between, but the first sample was taking when the reactor was 75 days old. Inlet and outlet samples were analysed. The results showed a high percentage removal of pathogenic microorganisms during period 2 with 100% removal of *E. coli*, 78.5% of other coliforms, 82.5% of *Enterococcus* and 99.2% of total bacteria on plate count. Pathogen removal of period 1 was not effective may be due to microbial communities not stabilized enough though the HRT was one of the longest. The antibiotic resistance gene test also recorded period 2 having the ability to remove tetracycline resistance genes (*tet* E, L, O and *otr* B). PCR was used which was not quantitative and the number of antibiotic resistance genes may have decreased significantly. However, this is impossible to evaluate because theoretically even if there was one gene, it could cause a positive PCR reaction. Furthermore, *tet* D was removed in period 1 and period 3 samples while sulfamethoxazole and erythromycin were not removed in any period. It was found that *tet* A and M were in all periods except period 4 but the UASB reactor was not able to remove them while *tet* B, *tet* K, *sul* II and *erm* A and B were absent in all the samples that were analysed.

Keywords: *wastewater, UASB reactor, antibiotic resistance genes, pathogen removal, tetracycline, sulfamethoxazole, erythromycin*

ACKNOWLEDGEMENTS

My greatest gratitude goes to the Almighty God for His mercies, protection and strength He showed me during all activities of the study. My sincerest gratitude also goes to my advisor, Associate Professor Krista Kaster and all the colleague students I worked with in the laboratory

God richly bless you all.

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ABBREVIATIONS

UASB	Upflow Anaerobic Sludge Blanket
COD	Chemical Oxygen Demand
HRT	Hydraulic Retention Time
OLR	Organic Load Rate
qPCR	Quantitative Polymerase Chain Reaction
BOD	Biological Oxygen Demand
TOC	Total Organic Carbon
USEPA	United States Environmental Protection Agency
WHO	World Health Organisation
UV	Ultraviolet
CFU	Coliform Forming Unit
WWTP	Wastewater Treatment Plant
OTU	Operational Taxonomic Unit
ARG	Antibiotic Resistant Gene
ARB	Antibiotic Resistant Bacteria
CLSM	Confocal Laser Scanning Microscope
RPP	Ribosomal Protection Proteins
PCR	Polymerase Chain Reaction
DNA	Deoxy Ribonucleic Acid
RNA	Ribonucleic Acid
AWWTP	Albireh Wastewater Treatment Plant
MPN	Most Probable Number
ATP	Adenosine Triphosphate
FISH	Fluorescence In-Situ Hybridization
VFA	Volatile Fatty Acids

1.0 INTRODUCTION

Wastewater management has been one of the greatest problems faced over the years and it is a global concern due to the effects of poor wastewater management on the environment and risk to human health. Valipour et al. (2015) added to this by saying that municipal water discharge is a great threat to many ecosystems. According to Belmont et al. (2004) monitoring studies of water bodies reveal that the main source of pollution is sewage discharge and these discharges are done directly or indirectly.

There is therefore the need for new technologies that will help in solving this wastewater discharge problem. Many technologies have been used over years and they are either conventional or nonconventional or they can be aerobic or anaerobic or anoxic. One of such technologies is the Upflow Anaerobic Sludge Blanket (UASB) reactor which uses anaerobic treatment condition. The UASB can achieve high chemical oxygen demand (COD) removal rates and suspended solids in relatively short hydraulic retention times (HRTs), capable of producing less sludge, require small area demand and have moderate construction costs (Samhan *et al*., 2007).

Wastewater contains many pathogenic microorganisms, the most important are those transmitted by the faecal–oral route, which includes bacteria, viruses and parasites (Wen *et al*., 2009). Bacteria is of greatest concern due to its ability to increase in the environment because it does not require a host cell for replication (Ceustermans *et al*., 2007). There is therefore the need for a technology which can reduce these microorganisms to acceptable levels whereby the process is also environmentally friendly and cost effective. This study therefore used such a technology by using the Upflow Anaerobic Sludge Blanket (UASB) bioreactor to assess how it can effectively remove these pathogenic bacteria. Pathogenic bacteria that were of importance and were considered in this study were *Enterococci spp* and total coliforms with focus on *Eschericia coli*. *Eschericia coli* despite been used as indicator organism became further important because of the existence of a pathogenic strain (1057:H7).

One other issue of concern in wastewater treatment when it comes to the removal of bacteria is the development of antibiotic resistance. This has been a serious concern since the wastewater comes to the wastewater treatment plant from all sources including hospitals. Bouki et al. (2013) said different kinds of antibiotics have been used in human therapy, veterinary and animal farming and a huge load of antibiotics are being released into the municipal wastewater which ultimately finds its way into environment. These antibiotic resistance genes when they get to the environment further increases the problem since they can be easily be transferred from one bacterium to the other through horizontal gene transfer. This therefore calls for a solution because when the fight for curing bacterial infections is lost, human life will be at stake. Antibiotics such as tetracycline, sulfamethoxazole and erythromycin were focused on during this study because they represent major groups of antibiotics and are among the abundantly used antibiotic substances in the world as was used by Özkök, (2012).

These reasons therefore call for a search for effective wastewater treatment technologies to help obtain efficient removal of both pathogenic bacteria and antibiotic resistance genes. Though several studies have been performed on these issues, the problem seems to be far from over. The search for effective technologies therefore should never stop and every effort should be made to find solution to this pertinent problem. There is therefore the need for different approaches in solving this issue. One of such approaches is the use of UASB which has an advantage of low cost and having smaller size among others. Hence this study wants to contribute to that objective by assessing its effectiveness.

1.1 Aim and Objectives

The main aim of the study is to assess pathogenic bacteria and antibiotic resistance gene removal in wastewater using an Upflow Anaerobic Sludge Blanket (UASB) reactor.

The specific objectives the study wants to achieve include:

- To assess the effectiveness of UASB in removal of pathogenic bacteria by enumeration.
- To assess the effectiveness of different loading rates on removal of pathogenic bacteria by numbers.

- To assess the effectiveness of UASB in removal of antibiotic resistance genes of pathogenic bacteria of importance.

2.0 LITERATURE REVIEW AND BACKGROUND

2.1 Wastewater treatment

Human activities generate waste in various areas of the environment of which biological waste is inclusive. From the standpoint of sources of generation, wastewater may be defined as a combination of the liquid (or water) carrying wastes removed from residences, institutions, commercial and industrial establishments, together with such groundwater, surface water and storm water as may be present (Cheremisinff, 2002). Monitoring studies of water bodies reveal that the main source of pollution is the discharge of sewage (Belmont *et al.*, 2004).

Wastewater treatment can be simply defined as process of changing wastewater so that its harmful effect on the environment and risks to human life can be reduced or sometimes to make it fit for reuse. As the waste is returned to the environment, it is therefore treated in a wastewater treatment plant where it is subjected to primary treatment, secondary treatment and tertiary treatment. The level of treatment depends on the receiving environment. Treatment helps to reduce the odour, biological oxygen demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC), total phosphorus, total nitrogen, destroy pathogenic organisms of the wastewater and various other parameters. This allows the water to enter the receiving environment without creating any negative effects. After the treatment, sludge which is the solid part is produced whiles the liquid part is either discharged or transformed to another reuse product. The sludge is either returned to be used in the activated sludge system or is further treated and converted to other uses such as compost.

Historically, sewage treatment systems were introduced in cities after Louis Pasteur and other scientists showed that sewage borne bacteria were responsible for many infectious diseases (Henze, 1983). The early attempts in the 1900s at treating sewage usually consisted of acquiring large farms and spreading the sewage over the land, where it decayed under the action of microorganisms (Nira *et al*., 2011). This method was found later not to be effective, so people decided to release sewage into waterbodies using them as sinks. This led to eutrophication where most waterbodies lost their quality and there was the need for a solution. One of the solutions was then to treat these wastes before using these waterbodies as sinks so that there will be no negative effects. But from the early 1970 to about 1990s, wastewater treatment objectives were based

primarily on aesthetic and environmental concerns (Zhou, 2003). Currently, due to advances in scientific research and technology, there have many improvements to wastewater treatment technology more are coming.

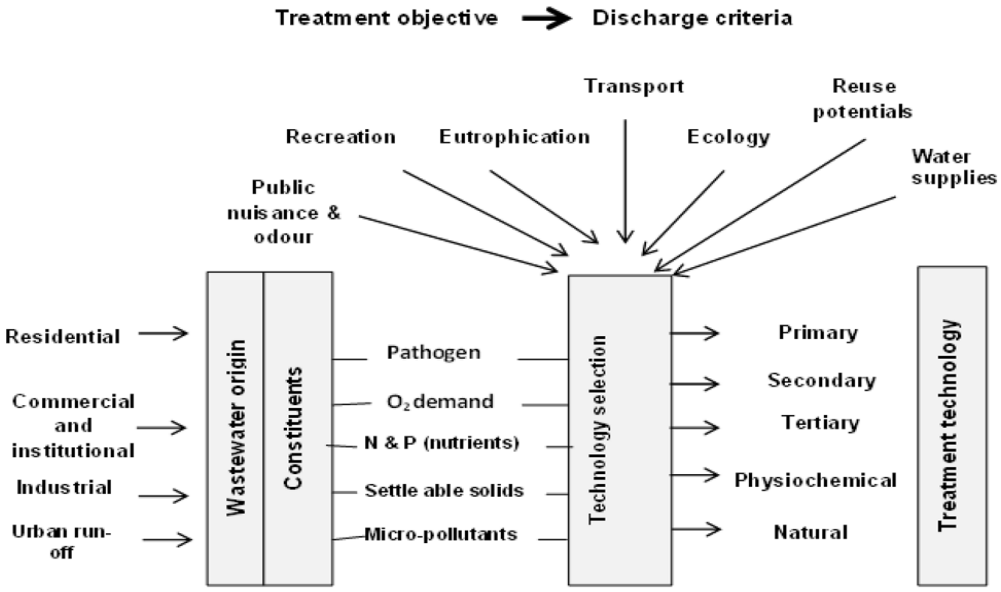

Fig. 1 Treatment Technology Selection Source: Veenstra *et al*., 1997

2.1.1 Wastewater Treatment Technologies

Basically, wastewater treatment technologies can either be conventional or nonconventional with conventional requiring high energy input while the nonconventional are mostly natural systems that require less energy input. The conventional wastewater treatment process consists of combination of physical, chemical and biological processes and operations to remove solids, organic matter and sometimes nutrients from wastewater (Al-Rekabi *et al*., 2007). Biological treatment uses microorganisms for degrading biodegradable organic matter and removing nutrients, for example by using activated sludge process or membrane bioreactor (Riffat, 2012). Physical treatment of wastewater mostly involves screening for removing solid part, sedimentation and filtration whereby physical forces are involved. Lastly, chemical treatment of wastewater concerns itself with the use of chemicals in other to achieve coagulation, flocculation or precipitation.

These conventional processes normally comprise preliminary treatment, primary treatment, secondary treatment and tertiary treatment and are normally performed under aerobic, anaerobic or both conditions depending on what one wants to achieve. The nonconventional treatment processes are ones with free surface and subsurface (horizontal and vertical) flow (Parać, 2015). Examples of nonconventional treatment processes include constructed wetlands, waste stabilisation ponds, etc.

2.2 How Pathogens can be Removed with Wastewater Treatment Technology

To achieve effective pathogen removal, there is the need for a very careful selection of the treatment process since several pathogen groups (viral, bacterial, protozoan and helminthic) must be removed to varying degrees and in developing countries at the lowest possible cost (Jiménez *et al*., 2010). Pathogenic microorganisms' removal from wastewater can be achieved using various removal techniques or technologies. The conventional treatment processes for sewage (primary and secondary processes) remove 95 – 99% of most microorganisms (Koivunen *et al*., 2003). However, their numbers in the sewage effluent usually remain higher than 4 $\log_{10}$ CFU/100 mL (Luczkiewicz *et al*., 2010). Unfortunately, many studies have found that the concentrations of faecal indicators in the treated sewage and biosolids are still more than the standard limits of US EPA and WHO guidelines (Al-Gheethi *et al*., 2018). For these reasons, several other treatment methods are used to reduce the number of pathogens in wastewater before they are either discharged or converted to other uses. Examples of these techniques or technologies include filtration including membrane bioreactors, infiltration, wetlands, stabilization ponds, UASB which will be dealt thoroughly later in this chapter and some disinfection techniques such as heat pasteurization, chlorination, ozonation and UV radiation. Sometimes more than one of these processes are used to increase efficiency of removal of these pathogens. Other times too some of these technologies can be used as alternative standalone treatment methods.

2.2.1 Filtration

It involves the use of materials that can prevent particles of smaller sizes from passing through. Membrane filter is an example. The membrane filtration systems are expensive regarding

construction and maintenance (Neis and Blume 2002). Different filtration systems have been developed which depend on the utilization of raw and low-cost materials such as sands and ceramic (Mohamed *et al*., 2016). Filtration may be the only barrier in some cases; for example, for removing Cryptosporidium oocysts by direct filtration when chlorine is used as the sole disinfectant (LeChevallier and Kwok-Keung, 2004).

***Membrane Bioreactors*: -** The small pore size of the membranes employed for solids separation results in the removal of a wide range of microorganisms (Ottoson, 2005). Studies have reported 5 – 7 log removal of indicator bacteria in membrane bioreactor processes (Gander *et al*., 2000).

2.2.2 Wetlands

Constructed wetlands possess lower operational costs due to the minimum or no external energy input, no large and complex mechanical equipment is needed, no chemicals are required, and sometimes no specialized staff is required to run the facility (Stefanakis, 2014). The removal of pathogenic microorganisms in constructed wetlands is accomplished through a complex of chemical (oxidation, UV radiation, plant biocides, adsorption to organic matter and biofilm), physical (filtration and sedimentation) and biological (predation, biolytic processes, antibiosis, natural die-off) factors, which often act in combination for the removal of pathogenic bacteria (Karathanasis, 2003). Vymazal (2005) presented removal efficiencies and first-order aerial rates for different constructed wetland systems and at the time of the study, it was observed for four different indicator organisms that the removal efficiencies ranged from 65% to 99%. Researchers have reported a bacterial removal rate constant of 0.2 – 0.5 d^{-1} for constructed wetland (Hench *et al*., 2003).

2.2.3 Stabilization Ponds

Waste stabilization ponds are used worldwide for wastewater treatment and are especially suitable for developing countries that have warm climates (Peña and Mara, 2004). Stabilization ponds can achieve removal of 0.03 – 0.05 d^{-1} according to Garcia and Becares (1997). Removal of pathogens occur in a process where they are inactivated because of complex interaction of processes that involve pH which is always high due photosynthetic algae, temperature, ultraviolet radiation

present in the sunlight that reaches the pond surface and photooxidative reactions taking advantage of high dissolved oxygen concentrations (Weaver *et al*., 2016) which was almost similar to what happens in constructed wetlands . Under optimal conditions, removal efficiencies in full-scale systems with several units in series can be as high as 6 $\log_{10}$ for faecal bacteria and 4 $\log_{10}$ for viruses, protozoan (oo)cysts, and helminth ova, though the efficiency of pathogen removal in full-scale systems is highly variable, in practice many systems achieve only 2 to 3 $\log_{10}$ removal (Verbyla *et al*., 2017).

2.2.4 Heat Pasteurization

Heat disinfection requires high temperature and pressure processes. The use of pasteurisation is recognized as an acceptable disinfection process for meeting the inactivation criteria of coliform bacteria (CDPH, 2009). It is a known fact that pathogenic bacteria are inactivated during exposure to heat, especially when the temperature of the treatment is above the optimum temperature of growth (Himathongkham and Riemann 1999). Lucero-Ramirez (2000) revealed that pathogenic bacteria are reduced to less than detectable levels in properly operated heat-drying systems. The retention time and temperature are the most important factors for the removal of pathogenic bacteria (Alcalde *et al*., 2003).

2.2.5 Chlorination

Chlorination is a simple, effective, and relatively cheap method, which can also provide a residual chlorine concentration in the distribution system or in the outflow for additional protection from pathogen growth (Stefanakis, 2015). Tree et al. (2003) said that chlorination has significant effect in the reduction of *E. coli* and *E. faecalis* in sewage-treated effluents. But the occurrence of pathogenic bacteria in treated sewage after chlorination has been observed and the main disadvantage for utilization of chlorine disinfection is the presence of free and combined chlorine residues which is being toxic to aquatic organisms (Al-Gheethi *et al*., 2018).

2.2.6 Ozonation

Ozonation is one of the most effective methods for pathogen treatment in wastewater since it leaves no residues. According to Facile et al. (2000), ozonation destroys the cell wall of the bacteria as well as semi-permeable membrane and the destruction in the cell wall and membrane leads to the

bacterial cell death. Disinfection of treated sewage by ozone is applied because the use of ozone is cheap and low energy is needed (Al-Gheethi *et al*., 2018). The effectiveness of disinfection using ozone depends on the dose, the demand, the quality of the effluent and the transfer efficiency of the ozone system (Paraskeva and Graham 2002).

2.2.7 UV Radiation

Ultraviolet radiation is suitable for inactivation of coliforms and *Salmonella* spp (Keller *et al*., 2003). Thymine dimers can be repaired in a process termed 'photoreactivation' in the presence of light, or 'dark repair' in the absence of light (Jagger, 1967). As a result, the strategy in UV disinfection has been to provide a sufficiently high dosage to ensure that nucleic acid is damaged beyond repair (LeChevallier and Kwok-Keung, 2004). Most of the early work on UV disinfection of *Giardia* (Karanis *et al*., 1992) and *Cryptosporidium* (Campbell *et al*., 1995) relied upon excystation or vital staining to determine viability and found that UV inactivation was not effective for *Giardia* cysts or *Cryptosporidium* oocysts (LeChevallier and Kwok-Keung, 2004).

2.3 Pathogens in Wastewater

Wastewater contains many pathogenic microorganisms, the most important are those transmitted by the faecal-oral route, which include bacteria, viruses and parasites (Wen *et al*., 2009). Parasites include protozoa and helminths. Fungi are also microorganisms sometimes found in wastewater. Traditional indicator bacteria for faecal contamination are coliforms, *Escherichia coli* and *enterococci*. Several studies have shown that the removal of protozoa and viruses differ from that of bacterial indicators (Bonadonna *et al*., 2002). Bacteria is of greatest concern due to their ability to increase in the environment because it does not require a host cell for replication (Ceustermans *et al*., 2007).

Table 2. 1 Densities of pathogens and indicators in sludge

Type	**Organism**	**Density in primary sludges** (/g of dry wt)	**Density in secondary sludges** (/g of dry wt)
Viruses	Various enteric viruses	10^2 - 10^4	3 x 10^2
	Bacteriophages	10^5	-
Bacteria	Total coliforms	10^8 – 10^9	7 x 10^8
	Feacal coliforms	10^7 – 10^8	8 x 10^6
	Enterococci	10^6 – 10^7	2 x 10^2
	Salmonella spp	10^2 – 10^3	9 x 10^2
	Clostridium spp	10^6	-
	Mycobacterium Tuberculosis	10^6	-
Protozoa	*Giardia spp*	10^2 – 10^3	10^2 – 10^3
Helminths	*Ascaris spp*	10^2 – 10^3	10^3
	Trichiuris vulpis	10^2	<10^2
	Toxocara spp	10 – 10^2	3 x 10^2

Source: Straub *et al.,* 1993

The presence of these organisms in household waste differ from those present in sewage especially in terms of numbers and type of microorganism. Presence of pathogens in recycled wastewater remains a major challenge in many countries where water reuse may be the only viable solution to irrigation of food crops (Kalipci, 2011).

2.3.1 Pathogenic Bacteria

There are varieties of bacteria that can be found in wastewater. A large number and diversity of pathogenic (disease-causing) bacteria enter sanitary sewer systems and wastewater treatment plants from domestic wastewater, industrial wastewaters such as slaughterhouses, cat and dog excrement through inflow and infiltration and rats that inhabit the sewer system (Briton, 1994). Not all bacteria in wastewater are pathogenic but the pathogenic bacteria can be found in the sewer system in the wastewater, sediment, biofilm at wastewater treatment plants, sludges, bioaerosols, contaminated surfaces foam, recycle streams, and scum (Gerardi, 2006). Furthermore, many of the

bacterial pathogens are enteric but bacterial pathogens, which cause non-enteric illnesses such as *Legionella spp, Mycobacterium spp,* and *Leptospira* may be detected in wastewaters (Neuman *et al*., 1997).

According to Gerardi (2006), *Camplyobacteria jejuni* and *Leptospira interrogans* represent an elevated risk of disease transmission to wastewater personnel and there are two types of pathogenic bacteria which are "true" pathogens and "opportunistic" pathogens. True pathogens which include *Shigella* are aggressive and are transmitted from person to person and by contact with animals and their wastes while opportunistic pathogens such as *Escherichia coli* are typically found on or in the human body and do not cause disease (except *E. coli* strains such as 0157:H7) unless the body's immune system is weakened by injury, a true pathogen, or physiological disease. The opportunistic pathogens are members of the natural microbial population which have the ability to rapidly increase when there is the availability of nutrients and since wastewaters have high nutrients loads, it gives these opportunistic pathogens the condition to increase in numbers thereby increasing risk of infections from them (Toze, 1997).

Some pathogenic bacteria produce endospores (*Clostridium)* or capsules (*Streptococcus*) which protect them from harsh environmental conditions and disinfection, and this pose a concern to wastewater personnel (Gerardi, 2006). When bacteria can also be removed from wastewater when solids settle in the clarifier, since the suspended and cell-associated pathogenic bacteria are removing the bacteria activated sludge process when they are adsorbed to floc particles.

2.3.2 Viruses

Viruses are generally more resistant to treatment processes, more infectious and require small doses to cause infection than most of the other pathogen types (international nomenclature of diseases, 1983). Viruses also get to wastewater after passing through the oro-faecal route of humans and other animals. Viruses found in wastewater include adenoviruses, enteroviruses, rotavirus, calicivirus, astrovirus and reovirus with the rotavirus been the most infectious (Toze, 1997) with enteroviruses being the most common type of virus found. These enteroviruses course diseases such as upper respiratory tract infections, poliomyelitis, acute gastroenteritis, aseptic

meningitis, pericarditis, myocarditis, conjunctivitis, hepatitis and viral exanthema (international nomenclature of diseases, 1983).

2.3.3 Pathogenic Protozoa

The protozoa are among the most common parasitic pathogens present in environmental samples. They have multistage life cycles, consisting of an active trophozoite stage and a resistant stage (oocyst or cyst) excreted in faeces that is capable of infecting new hosts (Zarlenga, 2004)**.** These protozoa contribute significantly to the staggering caseload of diarrheal disease morbidity encountered in these regions and are also of significant concern in industrialized countries despite improved sanitation (Moss, 2011). *Acanthamoeba* is a genus of free-living amoebae, which are environmental eukaryotic cells distributed worldwide in nature (Martinez, 1997) which supports bacterial growth and survival and saves the bacteria from chlorination (Abd *et al*., 2007). The survival from chlorination therefore increases the risk of humans contracting diseases caused by bacteria and *Acanthamoeba*.

Most waterborne protozoan parasites are causative agent for gastroenteritis, diarrhoea and others related to cellular or tissue infections (Roy *et al*. 2004). According to Mons et al. (2011), *Cryptosporidium* species and *Giardia intestinalis* are major pathogens in the waterborne transmission of infections and they can persist in the environment due to the robustness of the oocysts and cysts. Sewage treatment plants have the potential to be a source of contamination to our watershed if the treatment processes employed do not sufficiently treat the effluents before being discharged into nearby waterbody (Lim *et al*. 2007). Several studies have shown that the removal of protozoa and viruses differ from that of bacterial indicators (Bonadonna *et al*., 2002).

2.3.4 Helminths

Pathogens such as helminths (worms) are responsible for helminthiasis which is a disease of concern with most helminthiasis transmitted by the eggs through a human-water-soil-crop-human pathway (Jiménez *et al*., 2016). Where polluted water is used for agricultural irrigation, helminthiasis is among the main associated diseases that low-income regions face (WHO, 2012).

These eggs are highly infectious, highly persistent in the environment, and very resistant to conventional disinfection/inactivation processes (Strunz, 2014).

2.4 Upflow Anaerobic Sludge Blanket (UASB)

According to Haandel and Lettinga (1994), anaerobic treatment is the preferred method to treat municipal wastewater because of its merits over conventional treatment methods. This includes its ability; to treat high chemical oxygen demand (COD) loads, to withstand fluctuations in the influent, to generate biogas and its effectiveness in treating wastewater in a short period of time (James and Kamaraj, 2002). The UASB process was developed by Lettinga and coworkers in the late 1970s (Metcalf and Eddy, 2003). UASB can achieve high chemical oxygen demand (COD) removal rates and suspended solids in relatively short hydraulic retention times (HRTs), capable of producing less sludge, require small area demand and have moderate construction costs (Samhan *et al*., 2007).

UASB treatment technology has been observed to be very effective for the treatment of high strength effluents from tanneries food processing industries, pulp and paper manufacturing companies and distilleries (Tare and Nema, no year). Carbohydrate rich organic wastewater from starch or canning industry wastewater is easily digestible by microbes and therefore serves as a nutrient-rich starting material for anaerobic hydrogen production, therefore UASB has therefore turned out to be one of the most effective anaerobic wastewater treatment technologies for the treatment of food processing industrial waste (Daud *et al*. 2018). Also, according to Tilley et al. (2014) removal of about 80 to 90% can be obtained with such wastes.

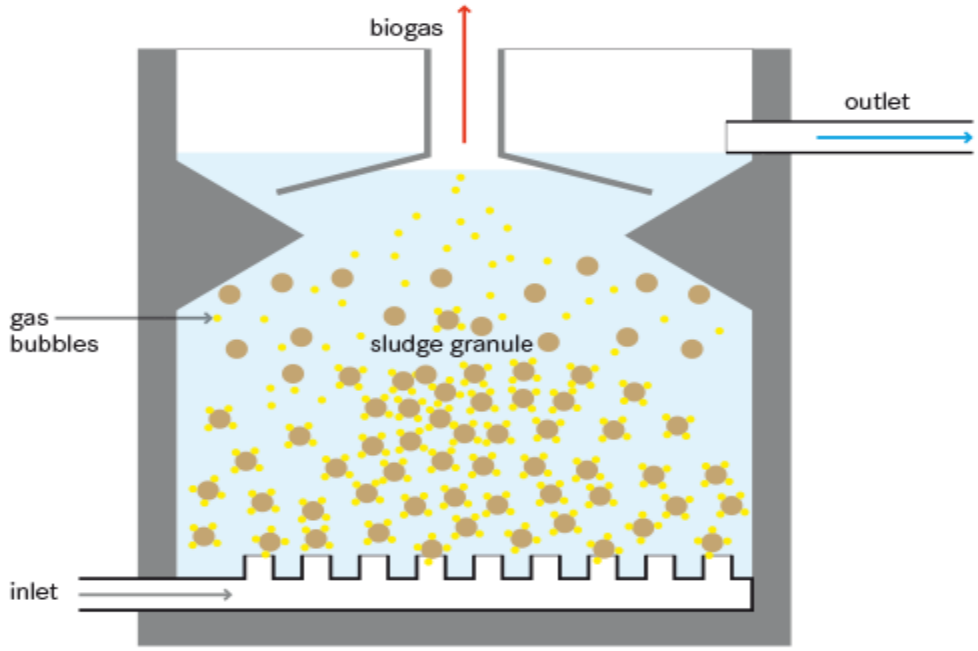

Fig. 2 Schematic diagram of UASB Source: Tilley *et al*., 2014

Tilley et al. (2014) described the procedure of how UASB works by saying that it is a single tank process where wastewater to be treated is introduced from the bottom of the reactor and it flows upward through a blanket of biologically activated sludge, which is generally in the form of granular aggregates as shown in Fig. 2. The suspended sludge blanket then filters and treats the wastewater as the wastewater flows through it. The sludge blanket is comprised of microbial granules of 1 to 3 mm in diameter, i.e., small agglomerations of microorganisms so that because of their weight, resist being washed out in the upflow. The microorganisms in the sludge layer degrade organic compounds and as a result, gases such as methane and carbon dioxide are released. The rising bubbles then mix the sludge without the assistance of any mechanical parts, with the sloped walls deflecting material that reaches the top of the tank downwards and clarified effluent is then extracted from the top of the tank in an area above the sloped walls.

Anaerobic sludge blanket reactors are used in place of primary and secondary wastewater treatment and anaerobic sludge digestion receives wastewater that has only gone through screening and grit removal so their effluents still have relatively high BOD_5 concentrations of 50 – 70 % removal as compared with 85 - 95 % removal for trickling filters or activated sludge. Therefore,

the effluent requires post-treatment with aerobic processes and disinfection, to meet the discharge or reuse requirements proposed by Chernicharo (2007). Tilley et al. (2014) said that if the influent is low-strength or when it contains too many solids, proteins or fats, the reactor may not work properly. Temperature is also a key factor affecting the performance of anaerobic sludge treatment.

2.4.1 Treatment of Municipal Wastewater Using UASB

A study on domestic wastewater treatment without pre-treatment was conducted by Aiyuk et.al. (2010) with UASB reactor and during that study they assessed the performance and stability of the UASB reactor which was a domestic sewage treatment system. They realised that for initial COD of 522 mg/l, the system could remove 80 % of the organic matter and up to 70 % of influent COD was found as suspended solid. They repeatedly discharged sludge from the reactor as was deemed needed. Hampannavar and Shivayogimath in 2010 also used UASB reactor at ambient temperature for anaerobic treatment of sugar industry wastewater and reactor start-up was successful since they achieved granulation within 95 days of operation. It was revealed that the optimum HRT was 6 h and they also observed that methane content in the biogas was between 73% and 82% at steady state conditions. They therefore drew the conclusion that sugar industry wastewater can be treated at maximum loading of 16 g COD/L.d. with low HRT of 6 h at ambient temperature.

A 6 m UASB reactor which was seeded with digested sewage sludge was operated at HRT of 14-17 h and there was a COD reduction reached 85 - 65 % at 20 °C and 70 – 55 % at 13 – 17 °C as was studied by Saghezzo et al. (1998) and they concluded that the UASB reactor concept was a simple, compact and inexpensive technology for sewage treatment, even at relatively low temperatures. This confirmed the results of a study by Fernandes et al. (1985) who used two small 12.4 L capacity UASB reactors to treat settled domestic sewage. There was also a study by Barbosa and Sant'Anna (1989) reported results from 9 months of operation of a 120 L UASB reactor treating raw sewage with 627 mgCOD/l and 357 mgBOD/l, at ambient temperatures (19 – 28 °C). Chemical oxygen demand (COD), BOD and TSS removal increased steadily during the first 4 months of operation. Also, after the start-up phase was over and during the last 5 months of operation, total BOD removal of around 78 % was achieved, while total COD removal reached 74

%. According to Barbosa and Sant'Anna (1989) suspended solid concentration in the effluent did not depend on the variations observed in the influent.
Saghezzo et al. (1998) said full-scale application of the UASB process has been successfully implemented in several countries.

2.5 Antibiotics in Wastewater

Antibiotics can simply be defined as antimicrobial agents used to fight microbes. Antibiotics are found in wastewater and wastewater treatment plants due to the enormous use of antibiotics to help in the treatment of bacterial infections. Antibiotics have been discharged into wastewater treatment plants for decades from households, livestock industry, hospitals and pharmaceutical industries which has resulted in multiple classes of antibiotics including tetracyclines, sulphonamides, fluoroquinolones, macrolides, β-lactams and others been widely detected in different wastewater treatment plants' influents and effluents worldwide (Zhang and Li, 2011).

According to Fateme and Mariya (2019), antibiotics such as tetracycline, sulfamethoxazole, ciprofloxacin, norfloxacin, trimethoprim and ofloxacin are found in high concentrations in the sludge of different wastewater treatment plants. Clarke and Smith (2011), found in their study the presence of antibiotics such as norfloxacin, ofloxacin, ciprofloxacin, and doxycycline in biosolids which were measured in the sludge of a Swedish wastewater treatment plant. Martin et al. (2015) conducted a study on sludge from four sludge stabilization treatments including anaerobic digestion, aerobic digestion, composting and the lagoon which were monitored to detect the occurrence of 22 pharmaceutically active compounds and revealed that concentrations of studied compounds were 179 μg/kg of sludge dry matter in primary sludge, 310 μg/kg of sludge dry matter in secondary sludge and 142 μg/kg of sludge dry matter in mixed sludge. There has mostly been incomplete removal of antibiotics during wastewater treatment and the antibiotics get released into the environment (Kümmerer, 2009).

Zhang (2016) said that concentrations for the same antibiotic from influents in different sites may vary significantly, sometimes by 1~2 orders of magnitude due to multiple reasons, including antibiotics consumption pattern, seasonal fluctuations including hourly and the size of catchment area of the wastewater treatment plant. After the antibiotics leave the wastewater treatment plant,

they pose further threat to the environment. They either become part of sludge or get discharged into receiving water bodies. Those that become part of the sludge gets into the soil when the sludge is processed and used as organic fertilizer. The antibiotics that get into the effluent that is discharged into receiving water bodies also get diluted but might be accumulated in this waterbody with time since there will be more additions. Due to their persistent occurrence in low concentrations, the toxic effects on the environment are more likely to be chronic rather than acute (Ferrari *et al*., 2004). The major classes of antibiotics can be found in Table 2.2.

Table 2. 2 Major classes with groups and subgroups of antibiotics

Class	Group	Subgroup	Example
ß-lactams	Penicillins	Benzyl-penicillins	Phenoxypenicillin
		Soxazolylpenicillins	Oxacillin
		Aminopenicillins	Amoxicillin
		Carboxypenicillins	Carbenicillin
		Acylaminopenicillins	Piperacillin
	Cephalosporins	Cefazolin group	Cefazolin
		Cefuroxim group	Cefuroxim
		Cefotaxim group	Cefotaxim
		Cefalexin group	Cefprozil
	Carbpenems	-	Meropenem
Sulfonamides			Sulfamethoxazole
Tetracyclines		-	Doxycycline
Macrolides			Erythromycin A
Aminoglycosides		-	Gentamicin 1c
Glycopeptides			Vancomycin
Quinolones			Ciprofloxacin

Source: Kümmerer, 2009; Özkök, 2012

Many antibiotics can be found in wastewater, but this study concentrated on three antibiotics based on the fact that it represents major groups of antibiotics and are among the abundantly used antibiotic substances in the world as was used by Özkök, (2012) on her study on inhibitory impact of selected antibiotics on biodegradation characteristic and microbial population under aerobic

conditions. As a result of that sulfamethoxazole represented sulfonamides group, tetracycline for tetracyclines and erythromycin for representing macrolides.

2.5.1 Sulfamethoxazole Antibiotic

Sulfamethoxazole has been documented by many researchers as a contaminant in wastewater streams, surface water and groundwater and besides that it has also been detected in sludge as well as in fish (Hallgren and Wallberg, 2016). Wastewater treatment plants in the urban zones are considered as one of the main areas of potential development and spreading of antibiotics such as sulfamethoxazole into the environment (Manaia *et al*., 2016). Sulfonamides constitute one of the most consumed antimicrobial families and sulfamethoxazole is one of the most widely used synthetic sulfonamide antibiotics worldwide (Kumar and Xagoraraki, 2010). It prevents the formation of dihydrofolic acid which is a compound that bacteria must be able to produce in order to survive and it is a low adsorptive polar antibiotic, which makes its fate in aqueous environments be of high concern (Nguyen, 2018). According to Müller et al. (2013), for sulfamethoxazole to reduce in concentration in effluents is mainly due to microbial activity. Rossmann et al. (2014) in their study observed sulfamethoxazole in the influents and effluents of a German wastewater water treatment plant at concentrations up to 2 μg/L. Another study in Seine, Charmoise and Prédecelle (France) was undertaken by Dinh et al. (2011) and they detected sulfamethoxazole concentrations of 1.4 μg/ L. The highest concentration of sulfamethoxazole was about 6000 ng/L in wastewater (Batt *et al*., 2006; Zhang, 2016).

2.5.2 Tetracycline Antibiotic

The first member of the family of tetracyclines was discovered in 1945 by Benjamin Duggar, and received the name of aureomycin (chlortetracycline), which was produced from the natural fermentation of the bacteria *Streptomyces aureofaciens* that is naturally present in the soil (Borghi and Palma, 2014). Tetracycline is one of the antibiotics that has been extensively used in human and veterinary medicine for several decades but though its usage in human treatment has decreased in recent years, its consumption in agricultural and animal husbandry settings is still common (Tehrani and Gilbride, 2018).

According to Hasan et al. (1985), tetracycline acts as an inhibitor of protein synthesis by avoiding the binding of aminoacyl-tRNA to the A site of the bacterial ribosome. Tetracycline as an antibiotic has been identified as being susceptible to light and it can therefore be degraded by photocatalytic processes as was proposed by Kümmerer (2009). Osińska et al. (2017) demonstrated higher percentage of amoxicillin and tetracycline resistance in *Escherichia coli* isolated from treated effluent than in *E. coli* isolated in the inflow of the same wastewater treatment plant (WWTP) during their study. Vergeynst et al. (2015) in a Belgian case study, found 1.4 μg/L of tetracycline as maximum concentration in influent and effluent of wastewater treatment plant. Opriş et al. (2013) similarly found tetracycline in wastewater treatment plant influents at maximum concentration of 146 μg/L. Rossmann et al. (2014) reported the occurrence of tetracyclines in influents and effluents of a German wastewater treatment plant with concentrations ranging from 1 to 2 μg/L. However, according to Carvalho and Santos (2016), tetracyclines were not detected by most other researchers in wastewater treatment plant of most European countries.

2.5.3 Erythromycin

Erythromycin is an antibiotic which can be used to treat respiratory tract infections, skin infections, infections of chlamydia, syphilis, eye infections such as conjunctivitis and pelvic inflammatory diseases. It was first discovered in 1952 by McGuire and co-workers as metabolite product from a strain of *Streptomyces erythraeus*, which was later assigned to the genus *Saccharopolyspora* and it is a microorganism that is found in the soil (Schafhauser *et al*. 2018).

According to Carvalho and Santos (2016), hospital effluents signify one of the main sources of macrolides in wastewater treatment plants and therefore in the natural environment and they further signify critical class of compounds due to their consumption in hospitals and their stability once excreted outside the body as urine or faeces. Macrolides, having a lactone ring that is substituted with hydroxyl (or neutral or amino sugars), alkyl, and ketone groups, inhibit bacterial protein synthesis and usually are used as penicillin substitutes (Gobel *et al*., 2005; Zhang and Li, 2011). Wastewater treatment plant effluents from Spain and Switzerland as was reported by Suarez et al. in 2010 revealed high erythromycin concentrations of 0.08 – 2.5 μg/L. A closed bottle test at initial concentration of 2.46 mg/L as was reported by Alexy et al. (2004) revealed that

erythromycin could not be readily biodegraded. Erythromycin-H_2O is commonly found in wastewater treatment plants than Erythromycin (Zhang and Li, 2011).

2.6 Antibiotic Resistant Genes in Wastewater

According to Zhang (2016), activated sludge has been widely used as a biological wastewater treatment process for over 100 years and plays an important role in control of conventional pollutants, including suspended solid, biological oxygen demand BOD/ chemical oxygen demand COD, nutrients (Nitrogen/Phosphorus), etc. with high bacterial diversity in the activated sludge. Activated sludge may contain more than 3000 OTUs (operational taxonomic units) in wastewater treatment plant as was proposed by Zhang et al. (2012) with the same sequencing depth of about 17000 16S rRNA gene sequences per sample, using 97 % similarity as the cut-off for a species level OUT as they further proposed.

Conventional biological treatment process therefore can provide a positive environment for the maintenance and dissemination of antibiotic-resistant bacteria and the antibiotic resistance genes (ARG) (Tehrani and Gilbride, 2018). Zhang (2016) further proposed that activated sludge may be an important hotspot for the dissemination of ARGs into environment and consequent exposure to human beings and livestock cannot be neglected. As in the activated sludge process, the average generation time of bacteria is about six to nine days which means that there could be more than 600 generations within 10 years of operation in which to develop resistance which is a slow generation time. Antibiotic resistant genes and antibiotic resistant bacteria have been isolated by various researchers from a variety of different environments including soils, wastewater treatment plants, water bodies etc. as shown in Fig. 3.

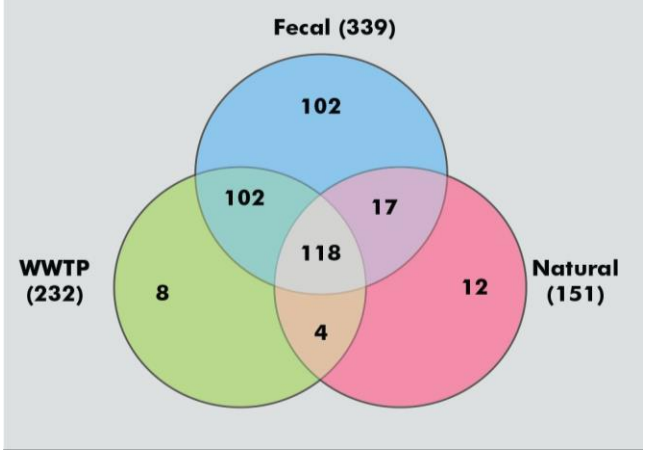

Fig. 3 Antibiotic resistant genes in different environments Source: Li *et al.*, 2015; Zhang, 2016

Antibiotic resistance genes can persist in the environment even when there is no antibiotic pressure (Fateme and Mariya, 2019). In a study conducted by Du et al. in 2015 which was about antibiotic resistance genes (*tet* X, *tet* W, *tet* G, *sul* I and *intI* 1) from a municipal wastewater treatment plant using effluent and inlet samples from membrane bioreactor revealed that anaerobic and anoxic treatments methods were more effective than aerobic due to microorganisms having lower bioactivity under anaerobic condition.

The occurrence and distribution of ARGs in five wastewater treatment plants was studied by Munir et al. (2011), with *sul* I, *tet* W and *tet* O as the antibiotic resistant genes of focus with their associated bacteria and it was observed that antibiotic resistance genes and antibiotic resistant bacteria removal ranged 2.37 - log to 4.56 - log in that activated sludge.

One other problem of concern is the fact that antibiotic resistance genes can be transferred between pathogenic microorganisms, non-pathogenic microorganisms and distantly related microorganisms by horizontal gene transfer (Pruden *et al.*, 2006). According to Karkman et al. 2017 wastewater treatment process creates conditions that sometimes favour horizontal gene transfer with high bacterial densities, stress caused by pollutants such as heavy metals and antibiotics and biofilms formed during the purification process. The focus of this study is on qualitative assessment of antibiotic resistance genes for some selected antibiotics (sulfamethoxazole, tetracycline and erythromycin) which my reason for using them had been stated earlier.

Sulfonamide resistance gene is generally coded by the mutations in the highly conserved regions of DHPS gene (*sul*) (Sköld, 2000; Özkök, 2012). According to Antunes et al. (2007) different sulfonamide resistant mechanisms have been detected due to mutations on the *sul* gene and which spread through mobile genetic elements. In the environment, there are four different bacteria sulfonamide resistance genes which have been defined (*sul* I, *sul* II, *sul* III, *sul* A). *Sul* I and *sul* II were detected in stool samples taken from cattle farms (Srinivasan *et al*. 2005; Özkök, 2012). They are commonly present in Gram-negative bacteria with 57% similarity between them (*Sul* I and *sul* II) and in 2003 *Sul* III gene presence in *E. coli* strain was isolated from pigs in Switzerland according to (Perreten and Boerlin 2003). In this current study, *sul* I, *sul* II, *sul* III were analysed for their presence using the UASB influent and effluent samples.

Roberts in 2005 mentioned that over the last 50 years, more than 38 tetracycline resistance determinants have been identified in a variety of bacterial genera. The main resistance mechanisms provided by these determinants were efflux pump proteins and ribosomal protection proteins (RPPs) (Roberts, 1996). The efflux determinant resistant genes that can be found in activated sludge are *tet* A, *tet* B *tet* C, *tet* D, *tet* E and *otr* B whiles the ribosomal protection proteins that can be found in activated sludge are *tet* M, *tet* O, *tet* Q, *tet* S and *otr* A (Zhang *et al*., 2009). Grossman in 2016 attributed the resistance to tetracyclines to one or more of the following processes such as the acquisition of mobile genetic elements that carry tetracycline-specific resistance genes, mutations within the ribosomal binding site, and chromosomal mutations leading to increased expression of intrinsic resistance mechanisms. Zhang et al. (2009) studied antibiotic resistance genes including *tet* M, *tet* O, *tet* S, *tet* Q, *tet* W, and *mec* A have been identified in microbial communities of hospital wastewaters because of the wide consumption of antibiotics in the hospital (Zhang *et al*., 2009). During this study, samples taken from UASB system qualitatively analysed tetracycline were for the presence of *tet* A, *tet* B, *tet* C, *tet* D, *tet* E, *tet* G, *tet* K *tet* L, *tet* M, *tet* O and *otr* B genes, which covered both efflux protein and ribosomal protection genes as was done in Özkök (2012).

Erythromycin inhibit protein synthesis by binding to the 50S ribosomal subunit (Gaynor and Mankin, 2003). Several different macrolide resistance genes have been identified in gram-positive

bacteria as well as in gram-negative bacteria (Roberts, 2004). Whereas *erm* A gene can be found in 7 genera and *erm* C in 16 *erm* B gene can frequently be found in gram-positive and gram–negative aerobic and anaerobic bacteria in many different ecosystems with a wider host range of 33 genera due to its association with mobile genetic elements (Roberts, 2008). It has been said that *erm* A and *erm* C are responsible genes for macrolide resistance in *Staphylococcus* species (Aktaş *et al*., 2007). According to some researchers, *msr* A was not previously found in activated sludge biomass though the gene codes ATP dependent efflux mechanism and causes resistance against the antibiotic erythromycin both in gram-positive and gram-negative bacteria (Martineau *et al.,* 2000; Roberts 2008; Özkök 2012). In the current study, inlet and outlet samples of UASB were analysed for the presence of *erm* A, *erm* B, *erm* C and *msr* A from erm class of erythromycin genes were examined except *mph* A.

2.7 Applications of UASB in Pathogen Removal in Wastewater

The primary mechanism that is responsible for the removal of pathogens from wastewater treated in anaerobic sludge blanket reactors is retention in the sludge and their attachment to microbial extracellular polymeric substances in sludge blanket granules. Unfortunately, this has not been well-studied (Oakley *et al*., 2017). The retention of pathogens in the sludge may occur due to physical filtration as wastewater passes through the dense layer in the sludge blanket, or due to microbiological factors like pathogens been retained in the sludge blanket by the same mechanisms as granule formation (Chernicharo, 2007). According to Oakley et al. (2017) physical-chemical factors such as temperature, reaction times, NH_3 toxicity and volatile fatty acids toxicity can affect pathogen removal efficiency in UASB.

Samhan et al. (2007) conducted a study on removal of pathogenic microorganisms in pilot-scale UASB septic tanks and Albireh urban wastewater treatment plant (AWWTP) in Palestine. The UASB septic tanks were located at AWWTP and were fed continuously with raw municipal wastewater from the aerated grit chamber of AWWTP. The two-pilot scale UASB septic tanks (R1 and R2) were operated at two different hydraulic retention times (HRT) of 2 d for R1 and 4 d for R2. Both raw wastewater and treated effluent were tested for microbial pathogens (indicator bacteria, protozoa and trophozoites) using microscopic and their specific culture media. The removal efficiencies were 15.5 % for R1 and 15 % for R2 for faecal coliform. There were also

removal efficiencies of 6.9 % for R1 and 11 % for R2 for faecal *streptococcus*. Though *Salmonellae* was detected in 30 % of analysed influent samples, it was not detected in any sample from the effluent of both treatment systems and the treated effluent of R1 and R2 were cysts or trophozoites free.

Another study was conducted by Pant and Mittal (2007) on the microbial profile of a UASB reactor-based sewage treatment plant and possible risk due to the pathogenicity of the treated wastewater which was located at a suburb of Delhi in India. During the study, the frequency of occurrence of *Salmonella*, *Shigella* and *Vibrio* was 100% at all the stages of sewage treatment but it was realised that the recovery of *Vibrio* was the highest among all the pathogens with the order of removal of all the pathogens being the same at the different stages of the treatment. They further said that ratio of counts of faecal coliforms and faecal streptococci at different stages of treatment exposed the origin of contamination which was said to be from human source. The average density of faecal coliforms in the treated sewage was 4.6 x 10^5 MPN/100 ml, which exceeds their prescribed limit of 1,000 MPN/100 ml.

Sylvestre et al. (2014) performed a study on the performance of two treatment systems with the aim of reducing indicators of biological contamination in swine production wastewater with system I consisting of two UASB reactors, with volumes 510 and 209 L. System II also consisted of a UASB reactor, anaerobic filter, trickling filter and a decanter with volumes of 300, 190, 250, and 150 L, respectively. The average removal efficiencies of that were obtained for total coliforms and thermotolerant coliforms in system I were 92.92% to 99.50% and 94.29% to 99.56%, respectively. In system II, it increased between 99.45% to 99.91% and 99.52% to 99.93%, respectively. Average removal rates of helminth ova in system I were 96.44% to 99.11%, reaching 100% as in system II. In the reactor sludge, the counts of total and thermotolerant coliforms ranged between 10^5 and 10^9 MPN $(100\ mL)^{-1}$, while helminth ova ranged from 0.86 to 9.27 ova g^{-1} TS.

El-Khateeb et al. (2009) in their study also reported highly efficient reduction of faecal bacteria in a UASB reactor in Egypt which showed removals of more than 1 $\log_{10}$ for total and thermotolerant coliforms, faecal streptococci, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, salmonella and staphylococci.

2.8 Techniques used in Detection Pathogens in Wastewater

There are two basic techniques that are used to detect pathogens in wastewater. They normally include culture dependent and culture independent methods.

2.8.1 Culture Dependent Technique

This method is mostly used for the detection and quantification of bacteria and sometimes viruses (Toze, 1997). It mostly involves the use of technique called Most Probable Number (MPN). Most Probable Number (MPN) determines the average number of bacteria in a sample through probability tests (Sutton, 2010). It consists of inoculating a series of tubes with appropriate decimal dilutions of the sample (APHA, 2005). The sample is subsequently subjected to a confirmation test. Though the method has been in use for several decades, its main setback is that it only provides estimates rather than real values (Rompré *et al*., 2002).

2.8.2 Culture Independent Techniques

Flow Cytometry (FC): - According to Xue et al. (2016), it is an alternative fast and reliable method to monitor bacterial abundance and viability of planktonic cells or cells in suspension. The technique uses fluorescent dyes to stain the water samples before they are detected quantitatively and based on the fluorescence intensity and the scattering generated (Anna *et al*., 2015). The main disadvantage of using this technique is its susceptibility to errors due to the formation of cell clusters and also the attachment of cells to inorganic compounds (Van der Kooij *et al*., 2014). It is also expensive to acquire, requires skilled professionals. Sometimes it is difficult to detect pathogenic microbes at low concentration as was discussed by Xue et al. (2016).

Enzymatic Assays: - Enzymatic Assay is a rapid assay that has been used in the study of the beta-D-glucuronidase (GLUase) activity of *E. coli* (Naga *et al*., 2016). The technique involves the use of the substrate 4-methylumbelliferyl-b-D-glucuronide measured as the rate of production of fluorescent methylumbelliferone (MU) and hydrolysis of the substrate which is estimated by fluorometry and this technique is preferred because of its fastness in speed and high reliability (George *et al*.,2004).

Fluorescence In-situ Hybridization (FISH): - This technique effectively extends epifluorescence microscopy to allow for the fast detection and enumeration of specific microorganisms (Jonach *et al.*, 2014). This technique uses fluorescent labelled oligonucleotides probes which bind specifically to microbial DNA in the sample, allowing the visualization of the cells using an epifluorescence or confocal laser scanning microscope (CLSM) (Kongsted *et al*., 2013). The process is cost involved.

Polymerase Chain Reaction (PCR): - It involves the amplification of the fragments of DNA (Chandra *et al*., 2016). PCR has helped to increase precision in microbial studies and is achieved through the extraction of nucleic acids (DNA/RNA), the amplification of a target gene or genes via PCR and the post-PCR analysis (Bożena *et al*., 2015). MultiplexqPCR and quantitative real time (qPCR) are the most useful PCR-based techniques in detecting faecal bacterial pathogens in wastewater (Diana *et al*., 2015). Though it is costly, but it is very accurate. PCR method was used for the detection of antibiotic resistance genes during this study and qualitative method was used.

3.0 MATERIALS AND METHODS

3.1 UASB Reactor Setup

The UASB reactor had a volume of 3 L and was made from polyethylene and constructed by Ytre Vanntank (ID 350 x 8). The reactor has an external cooling jacket which was set to a temperature of 16 ℃ and the temperature was maintained using a thermo-heating circulator. The reactor was fed continuously with an inlet sample from primary domestic effluent which had a volume of 25 L and the feeding was done in batches. The feed or inlet samples were kept in a refrigerator at a temperature of 8 ^{o}C constantly during the process. With the help of an adjustable-flow peristaltic pump (ISMATEC ISM4408), the feed was pumped from the container in the refrigerator into the UASB reactor. The organic loading rates were changed during each period from period 1 to period 4 in the order 4.30 g/L.d, 5.60 g/L.d, 6.40 g/L.d and 5.14 g/L.d. The liquid part of the effluent was allowed to drain into a sink unless outlet samples were being collected. Produced biogas was also transported upward due to the pressure from the influent upflow liquid and this was used by others for their study. Fig. 4 which can be found below, shows the experimental UASB reactor setup.

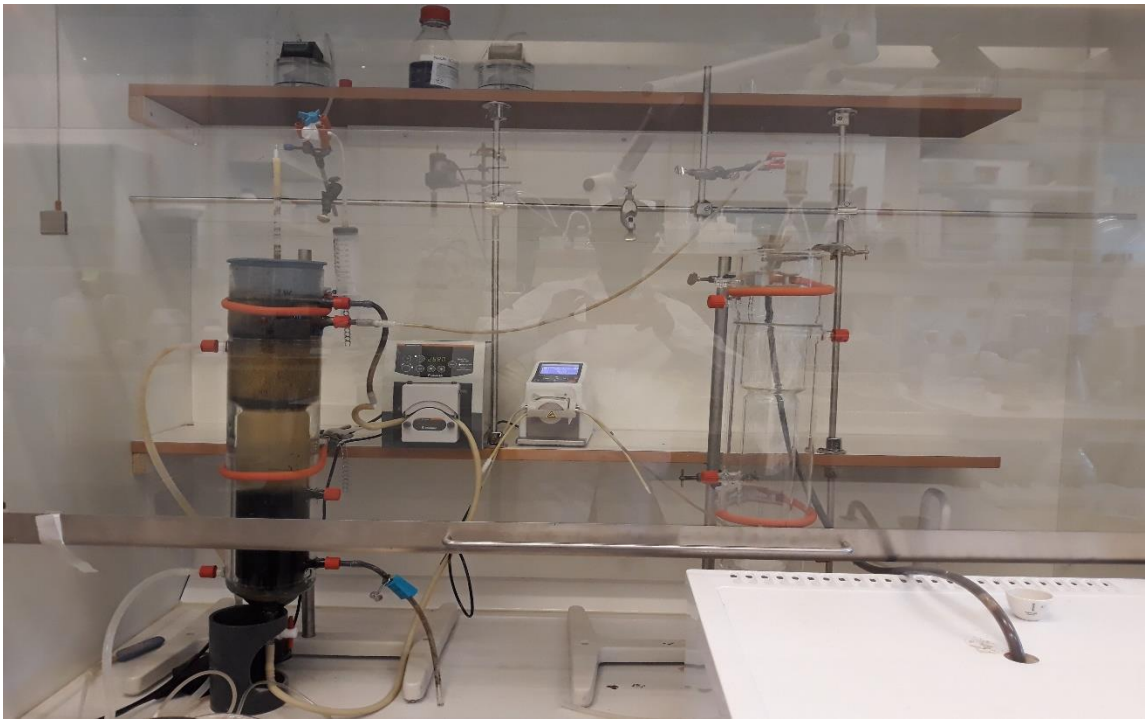

Fig 4. UASB Reactor setup during experiment

3.2 Enumeration of Pathogenic Bacteria

Samples were collected from the inlet and outlet of the UASB reactor which were then subjected to enumeration process in other to obtain bacteria numbers from both stages of the pathogen removal process**.**

3.2.1 Collection of Samples

Samples were collected from the feed (inlet) and the outlet of the reactor. There were four (4) periods samples were collected but were not of a specific time interval and all within a period of three (3) months. Samples were put in sterile bottles that were autoclaved for this specific purpose. A volume of 1 L of samples were obtained for the bacteria enumeration procedure whiles a volume of 100 mL was used for the antibiotic resistance gene test. The samples for the bacteria enumeration test was analysed directly after taking it to the microbiology laboratory to keep the numbers that were initially present as possible as it can be. The other for the antibiotic resistance gene test was kept frozen in a freezer at minus 20 ℃ until use.

3.3.2 Media Used

Chromogenic coliform selective agar - Chromogenic agar is the selective media for detection of total coliforms which also helps in identification of *E. coli*. This agar which was used was manufactured by Oxoid^{TM}. During the media preparation, 30 g of the media powder was dissolved in 1L of distilled water which was then allowed to boil till all it was dissolved completely. The pH was then measured, and the expected range was 6.8 ± 0.2 at 25 ^{0}C. The media was then poured into the media plates and after cooling were stored in fridge 4 ^{o}C.

Slanetz-Bartley agar – It is a media used for selective enumeration of *Enterococci*. The agar manufacturer was Sigma-Aldrich^{TM}. The media was prepared by dissolving 42 g of the media powder in 1 L of distilled water which was then heated to dissolve completely. The final pH which was to be expected was 7.2 ± 0.2 at 25 ^{o}C. The agar was then kept in a fridge at 4 ^{o}C after cooling solidifying.

Bile Esculin agar - It is a selective media for *Enterococcus* and was used as confirmatory test for growth in Slanetz-Bartley agar. It was also manufactured by Sigma-Aldrich^{TM}. Preparation of the

media was done by dissolving 56.65 g of media powder in 1 L of distilled water. It was then stirred to dissolve and was autoclaved at 121 ^{o}C for 15 min. The final pH expected was 7.1 ± 0.2 at 25^{o}C. The media was allowed to cool and then was poured in the media plates. It was then kept in fridge at 4 ^{o}C.

Plate count agar – This media was used for the enumeration of total bacteria in the wastewater sample. The manufacturer was Oxoid^{TM}. The media was prepared by dissolving 24 g in 1 L of distilled water. It was then heated and stirring to dissolve completely. The media was then sterilized by autoclaving at 121 ^{o}C for 15 min. The final pH that was expected was 7.2 ± 0.2 at 25 ^{o}C. Since the media was to be used for pour-plate count, it put into smaller glass bottles and kept in fridge at 4 ^{o}C. It was heated to melt when it was time to be used.

3.2.3 Culturing of Bacteria

In order to enumerate the number of bacteria present in the inlet and outlet sample serial dilutions in peptone water (8.5g NaCl, 1 g peptone powder, 1 L of distilled water, pH 7- 8) were used. Different dilutions used as depending on the type of media as seen in Table 3.1, 100 µL. All the dilutions were made in a sterile cabinet in order to avoid contamination.

Table 3. 1 Dilutions Used During the Study

Sample	**Media**		
	Chromogenic Agar (Coliform)	Plate Count Agar	Enterococcus Selective Agar
Inlet	$10^{-3} – 10^{-6}$	$10^{-4} – 10^{-8}$	$10^{-3} – 10^{-6}$
Outlet	$10^{-3} – 10^{-6}$	$10^{-3} – 10^{-8}$	$10^{-3} – 10^{-6}$

After the dilutions were made, 100 µL of diluted samples were pipetted and spread onto the chromogenic agar and the Enterococcus agar plates. Since the media was kept at 4 ^{o}C in a fridge,

it was allowed to get to room temperature before streaking was done to avoid thermal shock if possible. The streaking was done with a metal rods and caution was taken to prevent cross contamination. In doing this the metal rods were kept in alcohol and heated red hot after every single use. But care was also taken to allow it to cool down enough to prevent it from killing some of the bacteria.

For the plate count media, pour plate method was used so after pouring 1mL of diluted sample it was then swirled gently to mix the sample and agar. Caution was taken so the media was allowed cool down to approximately 50 ℃ before pouring.

After inoculating the bacteria on the media, they were then sent to an incubator based on their required temperatures. Chromogenic agar for coliform enumeration was kept at 36 ^{o}C for 24 h, Enterococcus selective media was kept at 36 ^{o}C for 48 h and plate count agar was kept at 22 ^{o}C for 62 hours. For growth on Enterococci media, the organisms were subjected to confirmatory test using Bile Esculin Azide agar, which was cultured at 44 ^{o}C for 24 h.

3.3 Qualitative Determination of Antibiotic Resistance genes

The antibiotic resistance genes determinations were performed qualitatively using DNA extraction, and Polymerase Chain Reaction (PCR). The bands that were obtained after gel-electrophoresis were then analysed for the presence or not for the antibiotic resistance genes.

3.3.1 DNA Extraction

Filtration was done before was extracted with filtration apparatus. The filter apparatus was sterilized including the filter paper and cutting instruments. 100 ml of sample was filter onto a 0.22 µm nitrocellulose filter (Millepore) it. The DNA extraction was performed with Power soil DNA kit manufactured by MO BIO Laboratories, Inc. UltraClean™ which has now been bought by Qiagen. The DNA extraction was performed as described by the manufacturer. The components of the DNA extraction reagents were not revealed on the kit. The filter paper was then cut into pieces, placed in a PowerBead tube and vortexed to mix well. 60 µl of solution C1 was then added to the sample. The sample was then inverted severally and vortexed for 5sec during each of

the inversions. The PowerBead tubes containing the sample were then placed in the fast prep and vortexed at 6 m/s for 60sec. The DNA extraction was then as describe by the manufacture with the exception of the final step where 50 µL of solution C6 was added. The DNA was then stored at minus 20 ℃ until use.

3.3.2 Polymerase Chain Reaction (PCR)

The protocols for the PCR of the antibiotic resistance genes were described by Özkök (2012). In all, PCR reactions a total volume of 25 µL was used.

3.3.2.1 Resistance to Sulfamethoxazole

The PCR mixtures were composed of 2.5 µL 10X PCR Buffer solution, 1 µL of 2.5 mM dNTP mixture, 2 µL of $MgCl_2$ (25 mM) solution, 1 µL of each 25 µM *sul* forward and reverse primers, 0.2 µL 5U/µL Taq DNA Polymerase and 1 µL of extracted DNA (inlet or outlet or positive control). Sterile water was then added to top it up to reach the final volume of 25 µL. The thermal cycler conditions that were used were 9 min pre-denaturation at 95 ^{o}C, 40 cycles of 15 sec denaturation at 95^{o}C, 30 sec with annealing temperatures of for *sul* I (55.9^{o}C), *sul* II (60.8 ^{o}C) and *sul* III (60.0 ^{o}C) as can be seen in Table 3.2, 1 min elongation at 72 ^{o}C, and elongation at 72 ^{o}C for 5 min..

Table 3. 2 Primers, Sequences and Annealing Temperature for Sulfamethoxazole Resistance

Gene	Primers	Sequence	Annealing Temp. ^{o}C	Amplicon Size	Reference
sul I	*sul* I-FW	cgcaccggaaacatcgctgcac	55.9	163	
	sul I-RV	tgaagttccgccgcaaggctcg			
sul II	*sul* II-FW	tccggtggaggccggtatctgg	60.8	191	(Pei *et al*., 2006)
	sul II-RV	cgggaatgccatctgccttgag			
sul III	*sul* III-FW	tccgttcagcgaattggtgcag	60.0	128	
	sul III-RV	ttcgttcacgccttacaccagc			

Source: Özkök, 2012

3.3.2.2 Resistance to Tetracycline

For the tetracycline resistance genes, *tet* genes such as *tet* A, B, C, D, E, G, K, L and *otr* B and ribosomal protection proteins such as *tet* M and O as described by according to Özkök in 2012, they had been previously detected in wastewater and activated sludge. The PCR mixture for *tet* genes consisted of 2.5 µL 10X PCR Buffer solution, 1 µL of 2.5 mM dNTP mixture, 2 µL of $MgCl_2$ (25 mM) solution, 1 µL of each 25 µM *tet* forward and reverse primers, 0.2 µL 5U/µL Taq DNA Polymerase and 1 µL genomic DNA. A final volume of 25 µl waster reached using sterile molecular grade water. The thermal cycler conditions can be found in Table 3.3.

Table 3. 3 Thermal Cycler conditions for Tetracycline Resistance genes

Gene	Thermal Cycler Conditions
tet A	Pre-denaturation: 9min at 95^{o}C, 40 cycles: 45sec at 95^{o}C, 45sec at 55^{o}C, 90sec at 72^{o}C. Final incubation: 7min at 72 ^{o}C.
tet B & *tet* C	Pre-denaturation: 2min at 95^{o}C, 30 cycles: 30sec at 95^{o}C, 30sec at 57^{o}C, 50sec at 72^{o}C.
tet D	Pre-denaturation: 9min at 95^{o}C, 30 cycles: 45sec at 95^{o}C, 45sec at 57^{o}C, 90sec at 72^{o}C. Final incubation: 7min at 72 ^{o}C.
tet E	Pre-denaturation: 9min at 95^{o}C, 35 cycles: 30sec at 95^{o}C, 30sec at 55^{o}C, 50sec at 72^{o}C. Final incubation: 7min at 72 ^{o}C.
tet G, *tet* K, *tet* L & *tet* M	Pre-denaturation: 9min at 95^{o}C, 30 cycles: 30sec at 95^{o}C, 30sec at 57^{o}C, 50sec at 72^{o}C.
tet O & otr B	Pre-denaturation: 9min at 95^{o}C, 35 cycles: 30sec at 95^{o}C, 30sec at 55^{o}C, 50sec at 72^{o}C. Final incubation: 7min at 72 ^{o}C.

Source: Özkök, 2012

3.3.2.3 Resistance to Erythromycin

The determination of resistance to erythromycin was performed according to a method reported by Martineau et al. (2000) which was found in Özkök (2012). In that method, the presence of *erm* A, *erm* B, *erm* C and *msr* A genes were determined by multiplex PCR which besides the use of primers to amplify the specific resistance gene, also adds an internal control mixture which amplifies the 16S rRNA gene that results in a 241bp PCR product, showing that the PCR system

worked effectively. The PCR mixture of *erm* and *msr* genes in each tube contained 2.5 µL of 10X PCR buffer solution, 2 µL of 2.5 mM dNTP mixture, 2 µL of $MgCl_2$ (25 mM) solution, 1 µL of each 25 µM genes specific forward and reverse primers, 0.4 µl of 5U/µl Taq DNA Polymerase and 1 µL genomic DNA of which positive controls were included. The solution mixture also contained 16S rRNA universal primers which had a dilution factor of 2:10 except the negative control. This differed from the 1:10 that was used in Özkök (2012) since there was no positive results until the dilution factor was changed. The mixture was then topped up with sterile water to reach the 25 µL final volume. The Thermal Cycler conditions were similar for all *erm* and *msr* A genes. The conditions used were 9 min at 95 ^{o}C pre-denaturation, 30 cycles of 30sec at 95 ^{o}C 52 denaturation, 30 sec at 55 ^{o}C annealing and 30 sec at 72 ^{o}C elongation.

3.3.3 Agarose Gel-electrophoresis

Positive PCR reaction were determined using a 1% agarose gel run at 100 V for 60 min. The DNA fragments were then visualised using a gel doc (BioRad).

4.0 RESULTS

4.1 Physicochemical Parameters of UASB Reactor on Sampling Day

During the sampling period the reactor worked under the parameters that can be seen in Table 4.1. From the table, it can be seen that the highest HRTs were recorded in periods 1 and 3 with values of 4.80 h with the least been period 3 (2.4 h). The highest organic loading rate (OLR) was recorded in period 3 (6.40 g/L.d) with least been recorded in period 1 (4.30 g/L.d). The UASB reactor was run at a constant temperature of 16^{o}C for all the sample taking periods. The highest flow rate was at period 2 (20.16 L/d) with the least being 12.96 L/d for period 1 but there was slight difference of 1.15 L/d, between values recorded for period 2 and 4. The UASB reactor was working within the pH range of 7 – 8.

Table 4. 1 Sampling Period Parameters

Sampling Period	**Parameter**			
	Hydraulic Retention Time (h)	Organic Loading Rate (g/L.d)	Temp. (^{o}C)	Flow Rate (L/d)
Period 1	4.80	4.30	16	12.96
Period 2	2.40	5.60	16	20.16
Period 3	4.80	6.40	16	14.40
Period 4	3.79	5.14	16	19.01

4.2 Performance of UASB in Pathogen Removal

The performance of UASB in pathogen removal was analysed for all of samplings as shown in Tables 4.2 - 4.5. with their pathogen removal percentages for various media that was used for culturing.

The highest pathogen removal percentage efficiency was recorded for plate count (33.3%) with the least being Coliform (14.3%) but in all the samples in period 1, none of them had a removal efficiency above 50% as has been represented in Table 4.2.

Table 4.2 Pathogen Removal in Period 1

Treatment type	**Total Coliform in CFU/mL**		**Enterococcus in CFU/mL**	**Plate count in CFU/mL**
	Coliform	*E. coli*		
Inlet	2.1×10^5 (0.3)	4.0×10^3 (2.0)	8.3×10^3 (2.1)	3.3×10^6 (1.4)
Outlet	1.8×10^5 (0.1)	3.0×10^3 (2.0)	6.3×10^3 (1.5)	2.2×10^6 (0.1)
Percentage Removal	14.3	25.0	24.1	33.3

Values in brackets are standard deviations

During period 2, the highest pathogen removal was recorded in *E. coli* with a 100% removal efficiency while the least was observed in coliforms. There was a general high removal efficiency during period 2 with none of the pathogens recording less than 75%.

Table 4. 3 Pathogen Removal in Period 2

Treatment type	Total Coliform in CFU/mL		Enterococcus in CFU/mL	Plate count in CFU/mL
	Coliform	*E. coli*		
Inlet	7.9×10^4 (2.1)	2.3×10^3 (1.2)	4.0×10^3 (3.0)	1.3×10^6 (0.4)
Outlet	1.7×10^4 (0.2)	0	0.7×10^3 (1.5)	0.01×10^6 (0.004)
Percentage Removal	78.5	100	82.5	99.2

Values in brackets are standard deviations

In period 3, plate count recorded the highest removal efficiency of 94.7% while *E. coli* recorded the least with a percentage removal of 60.06% as shown in Table 4.4.

Table 4.4 Pathogen Removal in Period 3

Treatment type	Total Coliform in CFU/mL		Enterococcus in CFU/mL	Plate count in CFU/mL
	Coliform	*E. coli*		
Inlet	1.8×10^5 (0.5)	3.3×10^3 (1.2)	4.7×10^3 (2.1)	5.7×10^5 (1.8)
Outlet	0.5×10^5 (0.1)	1.3×10^3 (1.5)	1.3×10^3 (0.6)	0.3×10^5 (0.2)
Percentage Removal	72.2	60.6	72.3	94.7

Values in brackets are standard deviations

The highest percentage removal was recorded in plate count 93.5% while the lowest was recorded in coliforms 82.1% with only 0.4% difference between coliforms and Enterococcus. However, there was no *E. coli* recorded in both inlet and outlet samples as seen in Table 4.5.

Table 4. 5 Pathogen Removal in Period 4

Treatment type	Total Coliform in CFU/mL		Enterococcus in CFU/mL	Plate count in CFU/mL
	Coliform	*E. coli*		
Inlet	6.7×10^4 (1.2)	0	4.0×10^3 (1.0)	7.8×10^5 (2.8)
Outlet	1.2×10^4 (0.4)	0	0.7×10^3 (1.2)	0.5×10^5 (0.3)
Percentage Removal	82.1	-	82.5	93.5

Values in brackets are standard deviations

4.2 Qualitative Antibiotic Resistance Gene Results

The antibiotic resistance gene test was performed qualitatively on both inlet and outlet samples for sulfamethoxazole (*sul*), tetracycline (*tet*) and erythromycin (*erm and msr A*) during all the four periods. Positive control samples were available for *sul* I, *tet* A, *tet* C, *tet* D, *otr* B, *erm* A, *erm* B, *erm* C and *msr* A.

Antibiotic resistance genes for sulfamethoxazole for period 1 can be found in Table 4.6. The only antibiotic resistance genes that were present were found in both inlet and outlet for *sul* I.

Table 4. 3 Antibiotic Resistance Genes for Sulfamethoxazole in Period 1

Primer	Inlet	Outlet
sul I	+	+
sul II	-	-
sul III	-	-

For tetracycline, the resistance genes were found in both inlet and outlet samples for *tet* A, *tet* C, *tet* G and *tet* M. However, they were found in the inlet of *tet* D but not the outlet as has been represented in Table 4.7.

Table 4. 7 Antibiotic Resistance Genes for Tetracycline in Period 1

Primer	Inlet	Outlet
tet A	+	+
tet B	-	-
tet C	+	+
tet D	+	-
tet E	-	-
tet G	+	+
tet K	-	-
tet L	-	-
tet M	+	+
tet O	-	-
otr B	-	-

Erythromycin reported antibiotic resistance genes in only *erm* C during the period 1 with resistance in both inlet and outlet samples as has been represented in Table 4.8.

Table 4. 8 Antibiotic Resistance Genes for Erythromycin in Period 1

Primer	Inlet	Outlet
erm A	-	-
erm B	-	-
erm C	+	+
msr A	-	-

The only resistance to sulfamethoxazole in period 2 was recorded in the inlet and outlet of *sul* III as has been represented in Table 4.9.

Table 4. 9 Antibiotic Resistance Genes for Sulfamethoxazole in Period 2

Primer	Inlet	Outlet
sul I	-	-
sul II	-	-
sul III	+	+

In period 2, tetracycline resistance genes were found in both inlet and outlet samples for *tet* A and *tet* M. But the resistance gens were found in the inlet of *tet* E, *tet* L, *tet* O and *otr* B but not their outlet samples as has been represented in Table 4.10.

Table 4. 10 Antibiotic Resistance Genes for Tetracycline in Period 2

Primer	Inlet	Outlet
tet A	+	+
tet B	-	-
tet C	-	-
tet D	-	-
tet E	+	-
tet G	-	-
tet K	-	-
tet L	+	-
tet M	+	+
tet O	+	-
otr B	+	-

Erythromycin also reported antibiotic resistance genes in only *msr* A during the period 2 with resistance in both inlet and outlet samples as has been represented in Table 4.11.

Table 4. 11 Antibiotic Resistance Genes for Erythromycin in Period 2

Primer	Inlet	Outlet
erm A	-	-
erm B	-	-
erm C	-	-
msr A	+	+

There was resistance to sulfamethoxazole in period 3 which was recorded in the inlet and outlet of *sul* III. However, there was resistance in the outlet of *sul* I and not the inlet as has been represented in Table 4.12.

Table 4. 4 Antibiotic Resistance Genes for Sulfamethoxazole in Period 3

Primer	Inlet	Outlet
sul I	-	+
sul II	-	-
sul III	+	+

Tetracycline resistance genes were found in both inlet and outlet samples in period 3 for *tet* A, *tet* G and *tet* M. Also, the resistance genes were found only in the inlet of *tet* D. Furthermore, they were found only in outlet of *tet* E and not the inlet as has been represented in Table 4.13.

Table 4.5 Antibiotic Resistance Genes for Tetracycline in Period 3

Primer	Inlet	Outlet
tet A	+	+
tet B	-	-
tet C	-	-
tet D	+	-
tet E	-	+
tet G	+	+
tet K	-	-
tet L	-	-
tet M	+	+
tet O	-	-
otr B	-	-

Results obtained from period 3 for erythromycin shoed antibiotic resistance genes in only *msr* A with resistance in both inlet and outlet samples as has been represented in Table 4.14.

Table 4. 14 Antibiotic Resistance Genes for Erythromycin in Period 3

Primer	Inlet	Outlet
erm A	-	-
erm B	-	-
erm C	-	-
msr A	+	+

There was no resistance to sulfamethoxazole in period 4 as was recorded in the inlet and outlet samples as has been represented in Table 4.15.

Table 4. 6 Antibiotic Resistance Genes for Sulfamethoxazole in Period 4

Primer	Inlet	Outlet
sul I	-	-
sul II	-	-
sul III	-	-

Tetracycline resistance genes were found in both inlet and outlet samples in period 4 for *tet* E, *tet* O and *otr* B as has been represented in Table 4.16.

Table 4. 7 Antibiotic Resistance Genes for Tetracycline in Period 4

Primer	Inlet	Outlet
tet A	-	-
tet B	-	-
tet C	-	-
tet D	-	-
tet E	+	+
tet G	-	-
tet K	-	-
tet L	-	-
tet M	-	-
tet O	+	+
otr B	+	+

Results obtained from period 4 for erythromycin indicated no antibiotic resistance genes in both inlet and outlet samples as has been represented in Table 4.17.

Table 4. 8 Antibiotic Resistance Genes for Erythromycin in Period 4

Primer	Inlet	Outlet
erm A	-	-
erm B	-	-
erm C	-	-
msr A	-	-

5.0 DISCUSSION

5.1 Effectiveness of UASB in Pathogen Removal

High pathogenic bacteria removal efficiencies are important due to the release of the wastewater after treatment into the environment. High removal efficiency of pathogenic microorganisms saves receiving waterbodies from contamination and also prevents the risk associated with pathogenic bacteria to human health.

During period 1, the overall pathogen removal efficiency of the UASB was low compared to the other periods though the hydraulic retention time (HRT) was among the two highest that were recorded and also had a low flow rate. The organic loading rate was also the lowest that was recorded but from Musa et al. (2018) on their study on slaughterhouse waste using UASB reactor where the reactor worked for 127 days, it was found that increase in organic loading rate corresponded with a decrease in removal efficiency. This therefore means there was a deviation because the UASB reactor was 75 days old when the period 1 samples were taken and comparing the removal efficiencies obtained to period 2 where the organic loading rates were increased from 4.30 g/L.d to 5.60 g/L.d at110 days , the removal efficiency increased. The results obtained on plate count also attested to that fact since less than 40% of the total bacteria percentage removal was recorded. This could be due to the fact that the reactor was still in its early stages of operation and might not have developed fully to work effectively as was proposed by Musa et al. (2018).

In period 2, the organic loading rate was increased from 4.3 g/L.d in period 1 to 5.6 g/L.d while the hydraulic retention time was reduced to the lowest. The overall removal efficiency was high with *E. coli* recording 100% removal while the lowest was 78.5% for coliforms. This could have been as result of the fact that the conditions that were present were conducive for removal of the pathogenic microorganisms by the UASB reactor. The results obtained during plate count confirmed the efficiency of the reactor in period 2 since 99.2% of total bacteria percentage removal was observed. According to Torkian et al. in 2012, the reduction in removal efficiency could be related to high organic loading rate and low hydraulic retention time. However, comparing the removal efficiencies obtained in period 2, this result is an exception because the organic load was rather high. This was also an exception to Musa et al. (2018) statement that increasing organic

loading rate at early reactor working stages decreases removal efficiency. The flow rate at the period 2 was also the highest among all the periods and this corresponded with the low hydraulic retention time that was used.

The highest organic loading rate which was used in this study was in period 3 with a value of 6.40 g/L.d. A hydraulic retention time of 4.80 h was also used which was a decrease in organic loading rate and increase in hydraulic retention time when compared to period 2. The removal efficiencies for coliform, *E. coli*, *Enterococcus* and total bacteria count during the period 3 (72.2%, 60.6%, 72.3% and 94.7% respectively) were lower than period 2 (78.5%, 100%, 82.5%, 99.2% respectively) but higher than period 1 (14.3%, 25%, 24.1%, 33.3% respectively). However, from the removal efficiencies, period 2 performed better than period 3.

During period 4, the organic loading rate was reduced to 5.14 g/L.d with the hydraulic retention time was also reduced to 3.79 h. This resulted in high removal efficiencies and it increased when compared to period 3. However, total bacteria count on plate count media was slightly reduced from period 3 to period 4. There was also no *E. coli* in the inlet samples. This could have been as a result of the fact that there was high precipitation that day and that might have caused dilution in wastewater inlet samples.

The highest percentage removal of coliforms was recorded in period 4 (82.1%) while that of *E. coli* was recorded in period 2 (100%). This agrees with Sylvestre et al. (2014) whose study on total coliform removal using UASB reactor in swine production waste obtained removal efficiencies between 92.9 % to 99.5%. Highest removal efficiencies of *Enterococcus* were recorded in period 2 and period 4 while the highest total bacteria removal on plate count was observed in period 2 (99.2%). From these results that were observed in period 2 during this study it was found that the parameters that were used in period 2 were more effective when using the UASB reactor. Future studies should therefore consider using different temperatures with these parameters to assess the best removal efficiencies that can be obtained.

5.2 Qualitative Detection of Antibiotic Resistance in Various Periods

The qualitative detection of *sul* (I, II, III), *tet* (A, B, C, D, G, K, L, M, O, *otr* B) and *erm* (A, B, C) and *msr* A antibiotic resistance genes were performed. According Nordgard et al. (2017), the mechanism of resistance to tetracycline happens through efflux, ribosomal protection and enzymatic inactivation; Resistance to sulfamethoxazole happens by development of resistant forms of DHPS enzymes and mutations in *dhp* gene; and the mechanism of resistance to erythromycin happens through target modification, mutations in 23S rRNA, efflux pumps and enzymatic inactivation.

In general, there were antibiotic resistance genes recorded in various periods during the study. There was also removal of some of the antibiotic resistance genes, but PCR was used which was not quantitative and the number of antibiotic resistance genes may have decreased significantly. However, this is impossible to evaluate because theoretically even if there was one gene, it could cause a positive PCR reaction.

In period 1 the resistance to sulfamethoxazole was found in both the inlet and outlet of samples for *sul* I. This was similar to a study conducted by Nordgard et al. in 2017 where resistance gene of *sul* I in resistant bacteria were isolated in wastewater treatment plants in Tromso. For tetracycline resistance genes, resistance genes such as *tet* A (was also present in the study by Nordgard et al. in 2017), C, G and M were present in both the inlet and outlet of the wastewater treatment plant. However, *tet* D was present in only the inlet sample but was absent in the outlet and this could be as a result of the fact that the UASB reactor was able to remove *tet* D from the wastewater during period 1. Resistance to erythromycin was also found in *erm* C while the other erythromycin resistance genes were not detected. Though the bacteria removal efficiency during period 1 was generally low, the UASB was able to remove *tet* D antibiotic resistance genes under those working parameters.

Resistance to sulfamethoxazole was determined by the presence of *sul* III in the inlet and outlet samples during period 2. Generally, the UASB reactor performed effectively during period 2 on the removal of tetracycline resistance genes as it was observed from the outlet samples. As it was recorded in period 1, similar happened since *tet* A and *tet* M were present in both the inlet and

outlet samples of period 2. However, the UASB reactor was able to remove *tet* E, L, O and *otr* B resistance genes that were present in the inlet samples from the outlet samples but there was the presence of *msr* A resistance gene in both the inlet and outlet. The removal of these tetracycline resistance genes could have been as a result of the higher removal efficiency of bacteria that was present during period 2. This also reveals that the organic loading rate (5.6 g/L.d) and low hydraulic retention time (2.4 h) according to this study that were effective for high bacteria removal efficiency as well as removal of some tetracycline resistance genes.

During period 3, the qualitative antibiotic resistance gene results that was obtained indicated that for sulfamethoxazole, the antibiotic resistance for *sul* I was absent from the inlet but was found in the outlet and this could have been as result of the fact that there was an error in the PCR reaction but there was not enough time for the test to be repeated. There was also the presence of *sul* III resistance genes in both the inlet and outlet samples. For tetracycline, the antibiotic resistance genes *tet* A, B and M were present in both the inlet and outlet samples while *tet* D was present only in the inlet samples since it was removed from the outlet samples by the UASB. However, *tet* E had an issue similar to *sul* I where it was absent in the inlet samples but was present in the outlet samples. Erythromycin resistance had results similar to period 2 where there were *msr* A resistance genes present in both the inlet and the outlet samples due to ineffective removal.

In the final period, sulfamethoxazole and erythromycin resistance genes were absent in both the inlet and the outlet samples. This could be as a result of the precipitation that day which caused the samples to be diluted because there was reduction in bacteria numbers in samples. Despite this, tetracycline resistance genes such as *tet* E, O and *otr* B were present in both the inlet and outlet samples of the UASB reactor.

5.3 Comparing Sulfamethoxazole Resistance Genes in Various Periods

Sulfamethoxazole resistance genes that showed up during the study were *sul* I and *sul* III with *sul* II resistance genes absent in all inlet and outlet samples at all sampling periods. Also, *sul* III was more prominently seen than *sul* I. This was because *sul* III was present in both inlet and outlet samples of period 2 and 3 while *sul* I was present in the inlet and outlet samples of period 1 but only in the outlet samples of period 3 which could probably due to and error in the PCR reaction.

The positive controls that were available for sulfamethoxazole resistance genes were only *sul* I which therefore was used to confirm the results that were obtained. Results obtained for *sul* II and III differed from that of Özkök (2012) since *sul* II was present in the activated sludge that was analysed but *sul* III was absent.

5.4 Comparing Tetracycline Resistance Genes in various periods

Tetracycline resistance genes were present in all the samples that were analysed. This indicates the extent of usage of this antibiotic in our health and veterinary services which had made them prominent in various parts of our environment. According to Chen et al. (2016), in recent years at least 39 different genes encoding resistance for tetracycline and due to the numerosity of these genes it was not surprising they were present in the wastewater used in the UASB reactor. From the study it was found that *tet* A and *tet* M were present in all inlet and outlet samples which symbolises that the UASB reactor was not effective in removing them from the wastewater. Though they were not present in the period 4 samples but that could have been due to the precipitation which caused their concentrations to be reduced that they were not detected. According to some researchers *tet* A has been seen to be present in different types of wastewater and other environmental samples compared to other tetracycline resistance genes (Aubertheau *et al*., 2017; Nordgard *et al*., 2017). Similar issue happened in removal of *tet* G resistance genes the only difference was that they were not present in both inlet and outlet samples of period 2.

This study also determined that the UASB was able to remove *tet* D whenever it was present in the inlet samples. This was seen in period 1 and period 3. This could be useful in further applications, but further study needs to be done before conclusions can be drawn though it worked this time based on the parameters the UASB was working under. The presence of *tet* L was only found in the inlet samples of period 2 but they were removed by the UASB reactor so were not seen in the outlet samples. Wherever *tet* O was present, *otr* B too was present (period 4) and when it was removed from the outlet samples by the UASB reactor (period 2) *otr* B too was removed. It was found that *tet* C was only present in the inlet and outlet samples of period 1 but were not removed under by the UASB reactor during that period.

For *tet* E it exhibited all the outcomes that were obtained during this study. Firstly, it was not seen in period 1, secondly it was seen in period 2 but was removed from the outlet samples by the UASB reactor, thirdly it was absent from the inlet samples of period 3 but was present in the outlet samples which was due to an error in PCR reaction and finally it was present in both the inlet and outlet samples of period 4. One other significant thing which happened was that *tet* K and *tet* B were the only tetracycline resistance genes under study that were never seen in any of the inlet and outlet samples which gives the realisation that *tet* K and *tet* B were absent in the wastewater that was used.

The positive controls that were available for tetracycline resistance genes were *tet* A, *tet* C, *tet* D and *otr* B which were used to confirm the results that were obtained. From the study of Özkök (2012) *tet* B was not seen in any of the activated sludge samples which was similar to what happened during this study. For Özkök's, the positive control was positive while there was no positive control during this study. For *tet* E, G and O the positive controls were negative during Özkök's (2012) study in the activated sludge but some of the results were positive which also was similar to this study but the only exception was that this study had no positive control for them (*tet* E, G and O). Finally, the positive control and results of *tet* K, *tet* L and *tet* M were positive for Özkök's (2012). But there were no positive controls available during this study even though *tet* L and *tet* M resistance genes were detected with *tet* M found in all samples except period 4.

5.5 Comparing Erythromycin Resistance Genes in various periods

According to Wen et al. (2016), there are more than 30 classes of different *erm* genes that encodes resistance for macrolides that have been identified and detected in different environmental samples and macrolides were represented by erythromycin during this study. Rizzo et al. (2013) said the occurrence of macrolides in aqueous environment at high concentrations is not to be expected because of the fact that they have low water solubility and sorption capacity to activated sludge under typical wastewater conditions (pH 7 – 8) which I agree because of the results that were obtained during this study.

But from the results, it was found that only *erm* C and *msr* A were present in the samples that were analysed. It was also found that the UASB reactor was not able to remove any of the

erythromycin resistance genes that were present and *msr* A was dominant since it was present period 2 and period 3 samples from both inlet and outlet while *erm* C was present in the inlet and outlet samples of period 1. According to Chen et al., (2016) *erm* B is known to be prevalent in many environmental samples as well as samples connected to wastewater treatment plants but from the results obtained from this study it was not present at all. Also, *erm* A was not present in any of the inlet and outlet samples for all the periods of sampling. No erythromycin resistance genes were observed in period 4 as has been explained earlier. There was the availability of positive control which was used during the study for all the erythromycin antibiotic resistance genes.

5.6 General Overview of UASB Reactor Performance

Overall the UASB reactor significantly decreased pathogen numbers with the average percentage removal of 90%,74.9% and 86.0% in period 2, 3, and 4 respectively which disagreed with the results of Keller et al. (2003), where UASB pathogenic bacteria removal was very low during the study. However, despite having the one of the longest retention times period 1 only removed 24.2%. This could be due to the fact that the reactor had only been running for 75 days at that point and perhaps the microbial community had not stabilized at that point and was thus not as effective at removing pathogens Which agrees with Musa et al. (2018) study where they said that at early stages of UASB reactor pathogen removal was decreased. Furthermore, the removal of antibiotic resistance genes by UASB reactor also corresponded with the pathogen removal efficiencies that were found in period 2 especially for tetracycline resistance genes since *tet* E, L, O and *otr* B were removed from the outlet samples. The antibiotic resistance gene such as *tet* removal agreed with Du et al. (2015) where there was a decrease in antibiotic resistance genes in the anaerobic and anoxic when they were compared with aerobic treatment. But though the removal efficiency of period 4 was high, none of the antibiotic resistance genes were removed from the outlet samples.

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The study had the main of assessing the effectiveness of UASB in removal of pathogenic microorganisms considering the effect hydraulic retention time and organic loading rate had on them removal effectiveness. The bacteria enumeration was done by using culture dependent technique of serial dilution. The antibiotic resistance genes removal was also qualitatively assessed from the UASB reactor outlet samples. Sampling was done in four periods.

From the results, it was found that period 2 had the highest removal efficiencies with *E. coli* recording 100% while period 1 had the least removal efficiency. But period 2 had the lowest hydraulic retention time with period 1 having one of the highest. The low removal efficiency of period 1 could be that it was in the early stages of reactor operation and perhaps microbial community had not stabilized as was seen in Musa et al. (2018). Removal efficiency of period 4 was also the second highest and there was precipitation that day which might have contributed to that.

Overall performance of UASB reactor in removing tetracycline resistance genes were high since *tet* E, L, O and *otr* B were removed especially in period 2 which also recorded the highest pathogen removal efficiencies. *Tet* B and K were never found in any of the samples and so as *erm* A, B and *sul* II which could be because they were low in concentration or were not present at all. Erythromycin resistance genes showed the least presence in all samples and also, none of the *erm* genes and *sul* genes were removed.

6.2 Recommendations

Future studies should also consider the effect of increasing temperatures on the UASB reactor on pathogen and antibiotic resistance gene removal. Potential bacteria growth inhibitors in anaerobic treatment plants such as NH_3 toxicity and volatile fatty acid toxicity should be considered during future studies.

It will be nice if real time PCR is used or digital droplet PCR which would allow exact quantification of genes. It will also be interesting if future studies consider heavy metal resistance genes in relation to antibiotic resistance genes since according to Baker-Austin et. al (2006), there

is a mechanism of co-selection between the two groups of resistance genes. Consideration of other antibiotic resistance genes should be performed n\on those that were not done during this study.

Study on combination of UASB reactor with an aerobic treatment should be considered for future study since according to Christgen et al. (2015), the combination helps to decrease antibiotic resistance genes by 85%.

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