University of Stavanger

MASTER THESIS

Curriculum: Master of Science in Environmental Engineering	Spring semester, 2019 Open
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Master thesis title: **Pathogen Removal from Domestic Wastewater using Membrane Filter**

Credits **30**

Keywords: Pathogens, Membranes, Filtration, Antibiotics, Antibiotic Resistant Genes	Number of pages: 32 + appendices/other: 13 Stavanger, 14.06.2019 date/year

Acknowledgement

I express my gratitude towards my thesis supervisor Associate Professor Krista Kaster for her constant supervision and guidance throughout the entire run of the project. I would also like to thank my friend and colleague Yaw Boateng for his immense support during the laboratory work for the research. I would also like to thank Associate Professor Roald Kommedal for providing this opportunity to work in the research and gain this experience. I would also like to thank Remya Nayar for helping with the membrane operation for the sample collection.

I am thankful to my friends and colleagues for the support and encouragement during the study period.

Finally, I am grateful for my family who are constantly wishing for the betterment and success in my life from back home. This would not be possible without them.

June, 2019

Riwaj Khanal

Abstract

The study assessed the use of membrane filter for the removal of harmful pathogens and other microorganisms from domestic wastewater. Effluent wastewater from the UASB reactor was used as feed for the membrane filter. The feed, permeate and retentate sample from the filter was collected and was analyzed for the presence of microbial community. The pathogens analyzed were Total *coliform*. *E. coli*, enterococcus and other heterotrophic organisms. Chromogenic selective agar was used as the media for total *coliform*, Slanetz-Baintley selective agar for enterococcus and Plate-count agar was used for the heterotrophs. The enterococcus colony was confirmed using Bile-Esculin selective agar for enterococcus. The process was repeated for four different samples run through three different filter operation modes: conventional micro filter, dead-end and cross-flow mode. The micro filter was ceramic tubular membrane with pore size 0.1 µm. the dead end filter was a silicon-carbide flat sheet membrane with pore size 0.1 µm. the cross-flow was a flat-sheet polymeric membrane with pore size 0.1 µm. The results from all the filter operation were evaluated for the performance based on the removal percentage of the pathogens. Overall, the membranes were very effective in the removal of pathogens with 90 % removal efficiency with most of the membranes. Out of the three operation modes, cross flow mode was found to be the most effective one.

Not only that, the membrane filters were used for the removal of the ARGs and the results were analyzed qualitatively using PCR. Sulfamethoxazole, tetracycline and erythromycin were the antibiotics chosen for the study. *sul*I, *sul*II and *sul*III were considered for sulfamethoxazole, *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*O, *tet*G, *tet*K, *tet*L, *tet*M and *otr*B for tetracycline and *erm*A, *erm*B, *erm*C and *msr*A were considered for erythromycin resistant genes. The genes above were subjected to PCR for amplification. The resulting solution was run in 1% gel for 60 minutes in 100V. The result showed the membrane was not very effective in the removal of the ARGs except for some tetracycline genes which seem to be retained by the membrane. All three filter modes were used for ARGs as well and the cross flow mode seemed to be the most effective one. Since the study was a qualitative one, the efficiency of the filter in removal of the ARGs could not be exactly reported.

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List of Abbreviations

UASB: Upflow Anaerobic Sludge Blanket

COD: Chemical Oxygen Demand

BOD: Biocemical Oxygen Demand

UF: Ultrafiltration

MF: Microfiltration

ARG: Antibiotic Resistant Genes

PCR: Polymerase Chain Reaction

SLB: Slanetz-Baintley Agar

DNA: Deoxyribose Nucleic Acid

RNA: Ribose Nucleic Acid

Taq: *Thermus aquaticus*

MRSA: Meticillin Resistant Staphylococcus aureus

HRT: Hydraulic Retention Time

OLR: Organic Loading Rate

qPCR: Quantitative PCR

ddPCR: Droplet Digital PCR

1 INTRODUCTION

Wastewater can be defined as the state of water where its physical, chemical and biological properties have been changed due to introduction of unwanted substances. (Amoatey & Bani, 2011). Water use is inevitable and much of the water consumed ends up as waste. Most of the pollution occurring in the water is due to various human activities though some occur due to natural processes. (Sonune & Ghate, 2004) Once turned into waste, the aesthetic and economic values of the water is lost. In order to maintain the water cycle going, the treatment of water is must. Wastewater treatment is a process that removes most of the contaminants that are found in the wastewater.

Wastewater can be categorized into four categories based on the source of the pollutants namely domestic, industrial, infiltration and storm water (Sonune & Ghate, 2004). Based on the source, the pollutants are different and so are the treatment methods accordingly. The treatment methods include primary treatment, secondary treatment and tertiary treatment. The primary treatment involves the use of clarifiers and settling tanks so that the effluent from a primary treatment consists of mainly dissolved and colloidal organic and inorganic solids. This is followed by secondary treatment where various microorganisms are allowed to function in a controlled environment (Sonune & Ghate, 2004).

In most treatment plants, prokaryotic microbes are the most dominant ones (Wagner et al., 2002). This study focuses on the removal of the harmful pathogens from domestic wastewater. Pathogens are the microorganisms that cause harmful diseases among the human beings. Most of them are enteric i.e., they effect the digestive system when ingested (Hai et. al, 2014). The most common pathogen in wastewater though are bacteria (Hai et al., 2014). Some of them are to be opportunistic ones that affect children and elderly under appropriate conditions (Hai et al., 2014).

One of the most commonly used biological wastewater treatment technique is a bioreactor. Upflow Anaerobic Sludge Bed or UASB reactors are the most commonly used efficient reactors for advanced secondary treatment of the wastewater. In this process, influent water travel from bottom to top in the reactor through a sludge blanket zone containing granular particles (George et. al, 2014). For more efficient pathogen removal, the UASB is coupled with a membrane filter that ensures the optimum removal of pathogens. In the applications of wastewater treatment, the membrane processes are found to be effective in elimination of microorganisms and particles (Iorhemen, Hamza, & Tay, 2016).

Another concerning problem with the effluent of a wastewater plant is the increasing antibiotic resistance genes among the bacterial population. In spite of various treatment methods taken into consideration, bacteria and the genetic material that go through the antibiotic resistance are not destroyed completely. (Macleod & Savin, 2014). Sulfonamide, Tetracycline and Erythromycin are the antibiotics taken into consideration in this study.

In this study, the experimental set-up consisted of a UASB reactor coupled with a membrane filter. The effluent from the UASB was passed through different types of membrane filters namely microfiltration (normal and dead-end) and cross-flow filtration for determining the effectiveness of the membrane in removal of the bacterial pathogens. For checking the results of the membrane process, standard plating methods were used where the feed, retentate and permeate from the membrane were cultured in media plates and incubated for colony counting. For the determination of the ARG, PCR was used for the amplification of the genes which were then separated by using electrophoresis.

2 BACKGROUND

2.1 OBJECTIVES OF THE STUDY

The following were the main objectives of the study:

- To find the efficiency of the membrane filtration method in the removal of pathogens from domestic wastewater coupled to a UASB reactor.
- To observe the efficiency of the membrane filter with different types of filter operations.
- To identify the presence of antibiotic resistant genes in the wastewater qualitatively.

2.2 WASTEWATER CHARACTERISTICS

Wastewater is the state of water that contains waterborne solids and liquids that are discharged into the sewers that represent a part of the waste of the community. It consists of organic solids: dissolved and suspended that are biologically decomposable (Sonune & Ghate, 2004). When kept untreated, the waste in the water accumulates and leads to more trouble due to the presence of urine and feces, soap and shampoo, hair, food fabrics, conditioners etc. that affect the health of the people as well as the surrounding environment (Amoatey & Bani, 2011). In addition to that, it also contains numerous harmful pathogens that pose a serious threat to human health. (George et al., 2014). Not only that, the nutrients in wastewater stimulate the growth of some aquatic plants that may contain toxins or carcinogens (George et al., 2014). Thus, the treatment of wastewater is necessary for the protection of the public health and a cleaner environment.

Wastewater treatment is a new practice although the drainage systems have been found before the nineteenth century. Before that time, the dirty soil were placed in buckets, dumped into “honeywagon” tanks and disposed over at agricultural lands. The concept of sewer was started due to this problem caused due to the transportation issues. The first modern sewerage was built in Hamburg, Germany in the year 1842 by an English engineer named Lindley (Amoatey & Bani, 2011). Over the time, the accumulation capacity of the waterbodies begin to give up and that was when the realization of wastewater treatment became more apparent (Amoatey & Bani, 2011).

The main objectives of the wastewater treatment were i) the removal of suspended and floatable materials ii) treatment of biodegradable organics and iii) the removal of harmful microorganisms. The treatment processes were focused mainly in reduction of the suspended

solid contents, oxygen-demanding materials, dissolved inorganics and harmful microorganisms (Sonune & Ghate, 2004). With advancement in times, the objectives of wastewater treatment have been emphasized in treating wastewater for minimizing the long-term health effects and long lasting environmental impacts. (George et al., 2014).

Based on the source of pollution, the wastewater can be categorized into the following types:

Domestic: wastewater originating from common households, institutions or similar locations

Infiltration/Inflow: Infiltration is water entering through indirect or direct means through joints, cracks or such. Inflow in storm water entering the sewer through foundations or basement drains.

Storm water: runoff from flooding due to rainfall.

Industrial: wastewater originating form industries comprising mainly of chemical and other harmful constituents. (Sonune & Ghate, 2004)

The contaminants present in the wastewater are categorized into physical, chemical and biological. The presence of the contaminants are ascertained by the measurement of certain indicators which include some physical properties like electrical conductivity and presence of solids (dissolved and suspended), chemical properties like BOD, COD and presence of inorganic (Nitrogen and Phosphorus) and biological properties like presence of coliform and other related microorganisms (Amoatey & Bani, 2011).

2.3 WASTEWATER TREATMENT

2.2.1 Treatment Methods

The treatment processes for the wastewater is chosen on the basis of the constituents to be removed. The methods are individually classified as physical, biological and chemical unit processes. Physical unit processes are carried out with the application of physical forces. Some examples of physical unit processes include mixing, screening, coagulation, flocculation, sedimentation and filtration. Chemical unit processes include the application of chemical reaction with addition of chemicals. The processes include adsorption, disinfection, and precipitation. Biological unit processes are the ones in which microorganisms are used for the conversion of the colloidal or dissolved organic into escapable gases or cell tissues that accumulate into biomasses (George et al., 2014).

For an efficient waste removal from the wastewater, the physical, chemical and the biological unit processes are coupled. The above processes worked together make up for primary, secondary and tertiary treatment processes. These are termed often as various levels of treatment that needs to be applied for achieving the required degree of treatment (Amoatey & Bani, 2011).

Preliminary treatment: screen, grit chambers that remove coarse and grits before entering the main treatment process to avoid operational and maintenance problems (George et al., 2014).

Primary Treatment: removal of settlable inorganics and organics by process of skimming. Also some heavy metals, organic nitrogen and phosphorus also removed (Amoatey & Bani, 2011).

Secondary Treatment: effluent from primary treatment are further subjected to further treatment of the residual solids. Also biodegradable organic matter is removed using biological treatment processes. The methods include trickling filters, activated sludge methods or anaerobic treatment methods like oxygen ditches (Amoatey & Bani, 2011).

Tertiary Treatment: residual solids from secondary treatment removed. Disinfection using chemicals also carried out at this stage (George et al., 2014).

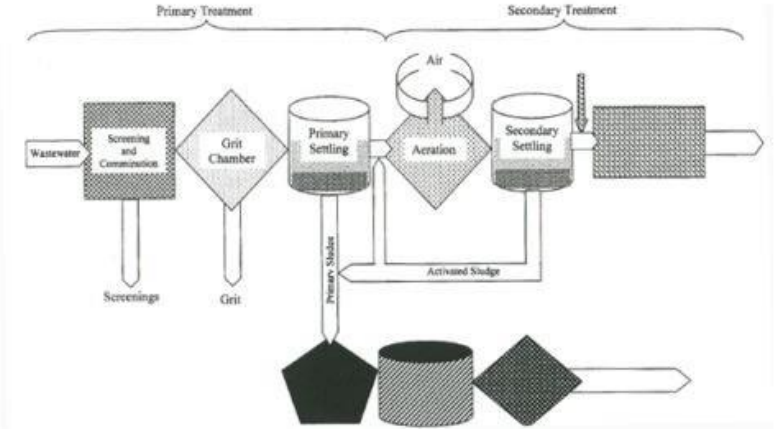

Figure 2.1 The unit processes in a Wastewater Treatment Plant (Amoatey & Bani, 2011).

2.2.2 Up flow Anaerobic Sludge Blanket (UASB)

The most commonly used biological wastewater treatment method is the aerobic one but various advancements and improvements in the anaerobic processes opened up alternate technologies for treating wastewater biologically. Lower biomass yield, less nutrients required, higher volumetric loadings are some of the advantages that anaerobic processes pose over the aerobic processes. (George et al., 2014)

The advancements in treatment processes lead to the development of anaerobic up flow filter process. This represented a very high stride in the field of wastewater treatment as the filter was capable of trapping and maintaining a high concentration of biological solids. This would allow a long Sludge Retention Time (SRT) ensuring a more effective removal (Bal & Dhagat, 2001). UASB is one of the types of up flow filter which works at high loading capacity. The influent wastewater is distributed at the bottom of the anaerobic reactor and it travels in upward direction through a sludge blanket containing dense granular mass particles (George et al., 2014). Also, UASB is known for the simplicity of the design. It comprises of both physical and biological processes. The physical process separates the solid and gases from the liquid while biological unit is for the decomposition of the organic matter anaerobically (Bal & Dhagat, 2001).

The main operation of the UASB depends on the preparation and maintenance of the dense granular bed that results in high biomass concentration in the reactor. This ensures high loading rate operation of the reactor. The granular sludge particle size is generally in the range of 1 to 2 mm but may vary depending on the waste treated and hydraulic and gas shear. Particle densities are in the range of 1 to 0.05 g/L and have settling velocities 15 to 50 m/h (George et al., 2014).

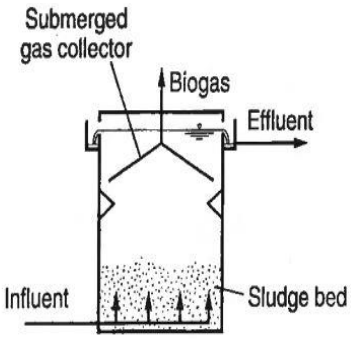

Figure 2.2 A UASB Reactor (George et al., 2014).

2.2.3 Membrane Filtration Processes

The bioreactor is followed by a filtration process for the production of a better quality effluent. The suspended and dissolved solid particles that manage to escape the bioreactor are retained in the membrane ensuring clear effluent.

A membrane is a material that selectively resists the transfer of different particles in a liquid thereby ensuring separation. The separation usually refers to solid particles separated from liquid or gas but the application of membranes is extended further to separate dissolved solids as well (Cheryan, 1998). The membrane is made up of a material with a reasonable strength capable of producing the desired flow-through at a high degree of selectivity. The physical structure of the membrane material is based on a sheer layer of the material with a small range of the pore size and high porosity of the surface (Visvanathan et al., 2000).

The water supplied to the membrane is called as *feed water*, the liquid that passes through is called *permeate* and the fraction of liquid that does not pass is called *retentate* (George et al., 2014).

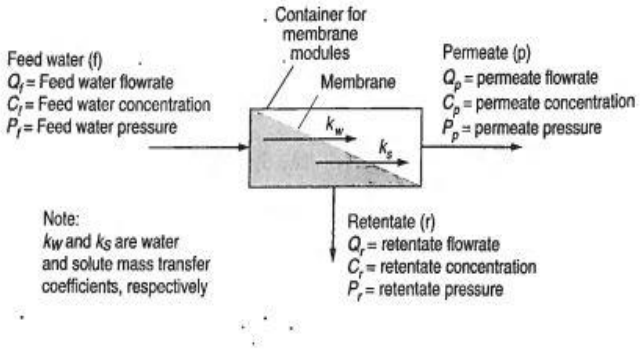

Figure 2.3 Separation process through membranes (George et al., 2014)

The types of membrane processes include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO) and electro dialysis (ED). The processes are classified according to the following mechanisms; Membrane configuration, nature of driving force, material that makes up the membrane, mechanism of separation and size of the permeate achieved (George et al., 2014).

Table 2.1 Membrane size Perspective (Cheryan, 1998)

Size	Example	Membrane Process
100 µm	Pollen	
10 µm	Starch	
1 µm	Blood cells Bacteria	Microfiltration
1000 Å	Albumin,	
100 Å	Vitamin B-12, Pepsin	Ultrafiltration
10 Å	Glucose	Nano filtration
1 Å	Water, NaCl	Reverse Osmosis

The particle separation in MF and UF occurs by the mechanism of sieving. In case of the NF, the separation mechanism is sieving accompanied by diffusion and exclusion. RO on the other hand, uses non-porous membranes. Thus the separation occurs due to diffusion mechanism. (George et al., 2014).

The aforementioned processes generally operate in pressure-driven conditions. The main characteristic of these processes is that the solvent is a continuous phase and the solute concentration is relatively low. The membrane properties like pore size is determined by the molecular size and chemical properties of the solute particles (Mulder, 1996). The pressure applied acts as the driving force allowing the solvent and various solute molecules to pass through the membrane while the rest of the solute are rejected depending on the structure of the membrane (Mulder, 1996).

Filter configuration

Based on the arrangement of the membrane on the filter apparatus, the filter modules can be categorized into the following types:

Plate and frame module: In this configuration, two sets of membranes are placed in sandwich like fashion with the feed side facing each other (Mulder, 1996).

Spiral wound module: In this type of model, two membrane layers glued to either sides of a permete spacer are wound around a tubular collection pipe (Mulder, 1996).

Tubular module: Tubular membrane models are not self-supporting, hence they are placed inside a porous stainless steel, ceramic or plastic tube (Mulder, 1996).

Hollow fiber module: AS the name suggests, hollow fiber modules have hollow fibers wrapped around by membrane. The feed solution can enter either inside the fiber or outside (Mulder, 1996).

Materials used for filter manufacture

The materials used for manufacturing filter membranes can be categorized as organic and inorganic. The types of organic membrane used are:

Cellulose acetate: the raw material used in this type of membrane is cellulose, the polymer of β-1,4 linked glucose units and it is prepared from cellulose by the process of acetylation (Cheryan, 1998).

Polyamide membranes: These materials have presence of amide bond in their structure (CONH) and they are associated with wider pH tolerance range, high biofouling tendencies and their worse chlorine tolerance (Cheryan, 1998).

Polysulfone membranes: These membranes have diphenylene sulfone repeating units in their structure. They have high degree of molecular immobility, have high rigidity, creep resistance and heat deflection temperature (Cheryan, 1998). The types of polysulfone membranes used are Polysulfone and polyethersulfone.

The inorganic membranes are ceramic or mineral membranes. They are prepared by baking the paste of the desired raw material and coating them by slip casting with final grain powder. They don't react with many common solvents and chemicals, have wide temperature pH and pressure limits, higher lifetime and backflushing capacity. On the other hand, they have some disadvantages like brittleness, limited pore sizes, and choice of pumping materials (Cheryan, 1998). Some of the inorganic membranes include aluminium oxides, ceramics, silicon carbides etc.

The pressure-driven processes: microfiltration and ultrafiltration, operate on two different operational modes namely dead-end mode and cross-flow mode.

Dead-end mode: In this mode of operation, the feed liquid stream is perpendicular to the membrane so that all of the solvent applied passes through the membrane as shown in Figure 5.

The particles that cannot pass through the membrane are retained in the membrane. Hence, this mode is best applicable when the solute concentration in the solvent is relatively low (George et al., 2014).

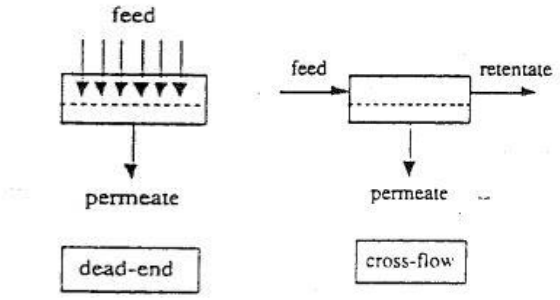

Figure 2.4 Dead-End and Cross-flow Filter Operation (Mulder, 1996)

Cross-flow mode: In cross-flow configuration, the feed water is forced tangentially to the membrane. The differential pressure across the membrane causes some of the solvent to pass through the membrane while the force of the inlet liquid velocity regulates the matter retained on the filter (George et al., 2014). The schematic of the operation can be observed in Figure 2.4.

Cross-flow operation is preferred for industrial and other applications as it has lower fouling tendency compared to the dead-end mode (Mulder, 1996). In cross-flow, the feed flux is parallel to the membrane while in dead end, the feed is fed directly on the membrane. This causes the deposit on the membrane i.e. the cake layer to grow with time causing a decline in the flux. This decline in flux can be controlled in the cross-flow mode by the adjustment of proper module choice and cross-flow velocities (Mulder, 1996).

The major problem encountered in membrane filtration process is membrane fouling. The fouling includes inorganic, organic and biofouling (Nguyen, Roddick, & Fan, 2012) . Biofouling is a major issue with the membrane process as microorganisms multiply over-time and even though most of them are removed, the remaining ones are still enough to grow in the membrane. The process of biofouling starts with the attachment of microbial cells to the membrane surface thereby forming a biofilm layer comprising of a population of variety of microorganisms. The attachment of microorganisms to the surface of the membranes are affected by factors such as membrane materials, roughness of the membrane surface, membrane surface charge and hydrophobicity (Nguyen et al., 2012).

2.4 WASTEWATER MICROBIOLOGY

Municipal wastewater not only contain organic matter and nutrients, but also is host to a large variety of microorganisms. They exist in the influent as well as the effluent of a wastewater

treatment plants (Bitton, 2005). Prokaryotic microorganisms are the most dominant species observed in any wastewater treatment plant (Wagner et al., 2002). The composition and presence of the microorganisms govern the efficiency of wastewater treatment process. Of all the microorganisms that dominate the wastewater, the main concern is related to the pathogens as they are capable of causing disease outbreak and consequently potential health risks.

2.4.1 Pathogens

Pathogens are the group of microorganisms that can cause various diseases; from mild ones to really fatal ones (George et al., 2014). The study by (FAO, 1993), as cited in (Olaolu et. al., 2014), suggested that the major pathogen groups that are of importance to wastewater are either bacteria, viruses, fungi or protozoa.

Viral Pathogens: Viruses enter the human body via mouth, multiply within the host body and are excreted in large number via feces. They are very infectious as they are very resistant to treatment and they can infect easily at a very small dose (Hai et al., 2014). Many of the viruses cause non-apparent infections which are very difficult to detect. They are present in generally small numbers in the wastewater (Bitton, 2005). Some of the major virus groups found in wastewater are Enterovirus, coxsackie A and B, poliovirus, hepatitis A and C etc (Hai et al., 2014).

Protozoan Pathogens: Protozoans are single celled microorganisms that cause variety of diseases like cryptosporidiosis, dysentery, giardiasis etc. what makes them more dangerous is that they can survive extreme of conditions outside of their hosts by transforming themselves into cysts by a process called encystment (Bitton, 2005). Major protozoans found in wastewater are *Cryptosporidium*, *Giardia*, *Entamoeba* and *Microsporidia* (Hai et al., 2014).

Bacterial Pathogens: They are the most common microbial pathogens in the wastewater (Hai et al., 2014). Some fecal matter may contain up to 10^{12} bacteria per gram (Bitton, 2005). They are introduced to the water mostly via fecal contamination (Sharma, 2013; Olaolu et al., 2014). Most of the bacterial pathogens reside in the gastrointestinal tract of the host. The most common bacterial pathogens found in wastewater are *Escherichia coli*, Vibrio, Salmonella, Shigella that cause a variety of diseases (Hai et al., 2014).

The bacteria found in wastewater belong to either of the following groups are Gram-negative facultative anaerobic(*Vibrio*, *Shigella*), gram-negative aerobic (*pseudomonas*), gram positive spore forming (*Bacillus sp*.) and non-spore forming gram-positive bacteria (*Enterococcus*, *Arthrobacter*) (Dott and Kampfer, 1988; (Bitton, 2005). Most of these bacteria are eneric and cause diseases like typhoid, fever, cholera etc.

2.5 ANTIBIOTIC RESISTANCE GENES

2.5.1 Antibiotics

According to the WHO, antibiotics are the medicines used to prevent and treat bacterial infections. They are considered to be “pseudo persistent” contaminants due to their regular

introduction into the environment (Richardson et. al, Hernando et.al; Gulkowska et al., 2008). They are poorly absorbed by human body, thus they are transferred via urine or feces unharmed or transformed (McArdell et al; Gulkowska et al., 2008). Majority of the antibiotics are disposed unchanged into the environment. The main concern is about the residue of these antibiotics and its potential impact in the environment (Sarmah et. al, 2006; Wright, 2007, Kemper, 2008;Zhang et. al, 2009).

Another major concern is about the propagation of antibiotic Resistance genes throughout the bacterial population in the environment. They carry a wide range of resistances to the drugs like β-lactams, tetracyclines,, solfonamides, erythromycins and many others (Macleod & Savin, 2014).

Some of the most commonly used antibiotics in the world are listed in the table below:

Table 2.2 Major Antibiotics currently in use

Class	Group	Sub-Group	Examples	
β-lactams	Penicillins	Benzyl-penicillins	Phenoxypenicillin	(Kümmerer, 2009; Özkök, 2012)
			Oxacillin	
		Isoxazolylpenicillins	Amoxicillin	
			Carbenicillin	
		Aminopenicillins	Piperacillin	
		Carboxypenicillins		
		Acylaminopenicillins		
	Cephalosporins	Cefazolin group	Cefazolin	
		Cefuroxim group	Cefuroxim	
		Cefotaxim group	Cefotaxim	
		Cefalexin group	Cefprozil	
	Carbpenems		Meropenem	
Tetracyclines			Doxycycline	
Aminoglycosides			Gentmycin 1c	
Macrolides			Erythromycin A	
Glycopeptides			Vancomycin	
Sulfonamides			Sulfomethoxazole	
Quinolones			Ciprofloxacin	

Out of the drugs mentioned in Table 1, three of them were chosen for the representation of the major drugs being used in the world presently to study their behavior in the wastewater treatment system. The ones chosen for study were erythromycin, sulfamethoxazole and tetracycline.

Erythromycin: Erythromycin belongs to the class macrolides and are used for the treatment of many human diseases as an alternate to penicillin. They are used largely as antibacterial but consumption in large units might be harmful (Louvet et. al, 2010).

Sulfamethoxazole: Sulfamethoxazole belong to the family of sulfa drugs. About 20% of the antibiotics for the human requirements come from this group (Göbel et. al, 2005). It is nowadays used in combination with trimethoprim.

Tetracycline: Tetracycline belong to class tetracycline and is used as an antibacterial agent against variety of bacteria and protozoa. Their strong chelating properties add to their antimicrobial properties (Chopra et al, 1992, Blackwood, 1985; Chopra & Roberts, 2001).

2.5.2 Antibiotic Resistant Gene Analysis

The overuse of antibiotics in medicine and agriculture has rendered them less effective against many of the microbial infection (Osinka et al, 2017). World Health Organization has stated that the increase in antibiotic resistance among bacteria is one of the most important global problems. Within the last few years, cases have shown a considerable increase in the rates of antimicrobial resistance (Macleod & Savin, 2014). As stated in (Koch, 1981), the appearance of resistant organisms appeared quickly after the widespread use of toxic substances. According to the (Norwegian Ministry of Health and Care Services, 2015), Norway ranks 11th among the 31 countries surveyed for the use of various antibiotic drugs and among the drugs studies on this research, the mostly used one is tetracycline followed by macrolides and sulfonamides.

According to (Hawkey, 1998), in antibiotic modification, the resistant bacteria retains the same sensitive target as antibiotic sensitive strains, but the antibiotic is prevented from reaching it. The development of the antibacterial resistance is shown to have occurred by four mechanisms. The first one is called target by pass where antibiotics cannot target the enzyme due to mutational changes (Zhang et al., 2009). This can be observed in the case of MRSA where alternate penicillin binding protein is produced in addition to the normal penicillin binding protein (Hawkey, 1998). The second way is by antibiotic inactivation where direct deactivation of antibiotic molecule occurs; (Zhang et al., 2009) the example of which can be observed in case of β lactamase, where the four membered β lactam ring is cleaved, rendering the antibiotic inactive (Hawkey, 1998). In some cases the antibiotic resistance occurs by target modification where modification of action sites of antibiotics happens (Zhang et al., 2009). This phenomenon can be observed in case of Enterococci where it is resistant to antibiotics where the enzymes responsible for protein synthesis have low affinity for them (Hawkey, 1998). Finally, the fourth mechanism is the use of efflux pumps where the reduction of intracellular concentrations of antibiotics occurs by structural alteration of cellular membranes (Zhang et al, 2009). The mechanism can be observed in the case of *Pseudomonas aeruginosa* (Hawkey, 1998).

Erm genes show resistance to erythromycin antibiotics in case of some Gram-positive and certain Gram-negative pathogens that infect humans. The genes cause resistance by methylating rRNa at the active site, decreasing the ability of the macrolide antibiotics to bind the ribosome (Weisblum 1998; Vester and Douthwaite, 2001; Choi et al., 2018). Four erythromycin genes have been considered: *erm*A, *erm*B, *erm*C and *msr*A.

The resistance to tetracycline is generally contributed to one or more of the following factors: the acquisition of mobile genetic elements carrying tetracycline specific resistance genes, mutation within ribosomal binding site or chromosomal mutations leading to increased expression of intrinsic resistance mechanisms (Grossman, 2016). The following resistant genes for tetracycline are observed *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G, *tet*K, *tet*L, *tet*M and *otr*B.

The resistance gene for all sulfonamide drugs is triggered by the mutations occurring in the highly conserved regions of DHPS genes (Sköld, 2000). The resistant genes occur due to the mutations in the *sulI* gene and are spread through mobile genetic elements (Antunes et. al, 2007; Houvinen, 2001; Özkök, 2012). Three resistant genes are observed for sulfamethoxazole: sul1, sul2 and sul3.

Antibiotic resistance is a topic of concern due to the fact that it has a very high probability if being transferred among the pathogenic and non-pathogenic bacteria. This phenomenon is possible because the /resistance genes are located on the mobile genetic elements such as plasmids which appear commonly in bacteria rich areas such as WWTPs (Ziembinska-Buccynska et al., 2015). From the treatment plants, they are directed to the water tracts that causes a major threat for public health.

2.5 MICROBIAL COMMUNITY ANALYSIS

2.5.1 Polymerase Chain Reaction (PCR)

PCR is a DNA replication technique that can copy fragments of DNA up to billionfold, in a process called amplification (Madigan et al., 2015). In this process, DNA polymerase is used that naturally copies the DNA molecules. Then, artificially synthesized nucleotide primers initiate the DNA synthesis. Whole DNA is not copied actually but the stretches are actually amplified up to a thousand base pairs (Madigan et al., 2015).

The reaction follows following steps:

DNA denaturation: The double stranded DNA dissociates into two separate strands at high temperature.

Primers Annealing: At low temperature, the target DNA fragment anneals to the artificial nucleotide forward and reverse primers that flank the target DNA.

Amplification: The primers are stretched with a thermostable DNA polymerase, the enzyme that causes DNA replication in cells (Bitton, 2005).

The aforementioned process is carried out in a thermocycler that controls the temperature necessary automatically, for every step required. Some of the environmental applications of PCR technology are detection of specific microorganism environmental monitoring of Genetically Engineered Microorganisms and detection of indicator and pathogenic microorganism (Bitton, 2005).

PCR is a simple technique to use and understand, and it produces rapid results. In addition to that, it is a highly sensitive technique where billions of copies of a specific product is produced for the purpose of sequencing and analysis within a very short time period (Bolognia et al, 2008; Garibyan & Avashia, 2013).

Despite of the value and usage of the PCR technique, there are some drawbacks of using this process. Any form of contamination in the sample can produce very misleading results. (Bolognia et al, 2008; Smith & Osborn, 2009; Garibyan & Avashia, 2013). Not only that, primers designed for PCR need a prior sequence data hence PCR can only be used for the identification of the presence or absence of a known gene (Garibyan & Avashia, 2013). This method also fails to give an indication of the variability of the pathogens and parasites detected in the samples.

2.5.2 Indicator Organisms

Indicator Organisms are the group of microorganisms which, if present in wastewater suggest the presence of pathogens (Olaolu et al., 2014). An indicator organism must continuously and totally be related to the source of pathogen and must be abundant enough for the exact enumeration of the pathogen (Olaolu et al., 2014). The indicator organisms themselves may not be pathogens (Hai et al., 2014). The widely used indicator is the detection of coliform bacteria, either as total coliform or fecal coliform (Hai et al., 2014). Other bacterial indicators are fecal streptococci (*Streptococcus* and *Enterococcus*) and *clostridium* (Krauss and Griebler, 2011; Olaolu et al., 2014). Indicators for protozoa include aerobic spores and anaerobic spores. Similarly, bacteriophages are widely considered to indicators for enteric virus removal (Hai et al., 2014).

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL SET-UP

3.1.1 UASB

The rector setup was done in a fume hood in the university lab premises. The reactor was made up of polyethylene and was constructed by Ytre Vanntank (ID 350x8). The net reactor volume was 3L. It consisted of an external cooling jacket kept at 16^0C by a thermo-heating circulator. The effluent was continuously fed from the refrigerator kept at 8^0C from 25L batches. The feed was pumped by using an flow adjustable peristaltic pump (ISMATEC ISM4408). The effluent produced was stored in another 25L container stored at a second fridge at 8^0C that was used as feed to the membrane filters.

Table 3.1 UASB properties on specified sampling dates

Sample Number	Sampling date	UASB organic loading rate (g/l/d)	UASB operating temperature (^{0}C)	pH	HRT (h)
1	26/02/2019	4.3	16	7.8	4.8
2	01/04/2019	5.6	16	8.0	2.4
3	04/04/2019	6.4	16	7.5	4.8
4	10/05/2019	5.14	16	7.24	3.8

3.1.2 Membrane filters

Three different types of membrane filters were used microfiltration, dead-end filtration and cross-flow filtration. The membrane and the dead-end filter were located at the membrane laboratory and the cross-flow filter was located in the microbiology lab.

Sample 1 and Sample 2 were run through a conventional micro filter membrane made of ceramic material. The arrangement of the filter membrane was tubular with pore size of 0.1 µm. the filter was manufactured by Atech Neu-Ulm, Germany.

Sample 3 was obtained from a dead-end filter membrane arranged in a flat sheet configuration. The membrane was a silicon carbide with pore size of 0.1 µm.the filter assembly was manufactured by Atech Neu-Ulm, Germany.

Sample 4 was collected from a cross-flow filter with polymeric membrane material. The membrane was arranged in tubular module and had a pore size of 0.1 µm. The manufacturer was EMD Millipore.

Table 3.2 Filter conditions in various sampling conditions

Sampling date	Filter type used	Membrane operating Pressure (bar)	Membrane operating temperature (^{0}C)
26/02/2019	Conventional micro filter	2	20
01/04/2019	Conventional micro filter	2	21
04/04/2019	Dead-end	3	23
10/05/2019	Cross-Flow	0.6	16.6

3.1.3 Media Plates

Media plates were prepared for the viable count method to be used for the enumeration of the pathogens *coliform* and *E. coli*. The agar used for the preparation of the media and the method of preparation is described below.

Chromogenic coliform selective agar

Chromogenic agar was used for the detection of total *Coliform* and *E.coli* in water and food samples. The one used for this study was manufactured by OXOID. For the preparation of the media, 30 gm of the agar powder was dissolved in 1L of distilled water and was boiled until completely dissolved. The final pH of the media was 6.8 ± 0.2 at 25^{0}C. The media was poured into the media plates after cooling. The media resemble yellowish straw color.

Slanetz-Bartley agar

Slanetz-Bartley agar is a selective medium used for the enumeration of enterococci according to Slanetz and Bartley (1957) in water and sewage with membrane filter technique. The agar was manufactured by Sigma-Aldrich. 42 gm of the agar was dissolved in 1L of distilled water and was heated and stirred to dissolve completely. The agar when heated gave out toxic fume, so the heating was done in a fume hood and sterilization in autoclave was completely avoided. The final pH was 7.2 ± 0.2 at 25^{0}C. The agar was reddish in color after the preparation.

Bile Esculin agar

Bile Esculin agar is a selective agar for the enumeration of enterococcus. In this study, it was used for the confirmation of enterococci growth in the Slanetz-Bartley agar. Manufactured by Sigma-Aldrich, the media was prepared by dissolving 56.65 gm of the agar in 1 L of distilled water. The mixture was boiled and stirred to dissolve and was autoclaved at 121^{0}C for 15 minutes. The media was allowed to cool and then was poured in the media plates. The final pH was 7.1 ± 0.2 at 25^{0}C. The media was yellowish in color.

Plate count agar

Plate count agar was used for the enumeration of bacteria in water sample. The one used for this study was manufactured by Oxoid. The media was prepared by suspending 24 gm in 1L of distilled water and by heating and stirring to dissolve. The media was then sterilized by

autoclaving at 121^0C for 15 minutes. The final pH was 7.2 ± 0.2 at 25^0C. The media was used for pour plate technique, so the media was stored in glass bottles for storage. The media had yellowish appearance at the end of the preparation.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 VIABLE PLATE COUNT

Viable plate count method was used for the enumeration of the pathogens *coliform*, *E. coli* and enterococcus. Three water samples were taken: the feed to the membrane, the permeate through the membrane and the retentate from the membrane. For the cell culture, serial dilution technique was used. 10 Eppendorf tubes filled with 0.9 ml of peptone water (1gm peptone powder and 8.5 gm NaCl dissolved in 1 L distilled water) on each of them and labelled according to the dilution factor used. The dilution value started from 10^0 to 10^{-8}. 0.1 ml of the sample was added in the first tube labelled 10^{-1}. The process was followed for other test tubes until the dilution reached 10^{-8}, thus completing the serial dilution. The process was repeated for all three samples. After the sample preparation, spread plating method was done on the media plates for the colony growth. 0.1 ml of the diluted sample was taken from every dilution and spread on the plate. Triplicates were used for every dilution. Pour plate method was used in case of the plate count agar media. 1ml of the sample was poured along with the agar and thoroughly mixed.

The chromogenic agar media plates were incubated at 36^0C for 24 hours before the colony count. The positive colonies were brownish color for *coliform* and green colonies for *E.coli*.

The SLB agar media was incubated at 36^0C for 48 hours. At the time of the colony count, the enterococci colony was dark brownish in color over the reddish media. For the confirmation of the enterococci, the colony grown in the SLB agar media was streaked in the Bile Esculin agar media. *E.coli* was used for negative confirmation. They were incubated for 24 hours at 44^0C. The positive result showed dark brown color colonies in the Bile-Esculin agar plates.

The plate count media was incubated at 22^0C for 62 hours before the colony counting was done. The bacterial growth was observed as white color colonies.

3.2.2 Microbial Community Analysis

DNA Extraction from the wastewater samples

For the purpose of analyzing the microbial community present in the water samples, the DNA present in the samples were extracted. Firstly 100 ml of the samples were filtered and the filter papers were subjected to the extraction procedure. The DNA tool kit manufactured by Mo Bio Laboratories, Inc. was used and the protocol by the same was followed.

The procedure used for the DNA extraction is listed below

Step 1: The filter paper was cut and added to the PowerBead Tubes and was gently vortexed for mixing.

Step 2: 60 µL of the solution C1 from the extraction kit was added and was vortexed briefly followed by spin in the fast-prep for 2 minutes at 6m/s.

Step 3: The tubes wre then centrifuged at 10000 x g for 60 seconds. The supernatant liquid was transferred to a sterile 2 ml collection tubes.

Step 4: 250 µL of solution C2 was then added, vortexed and was incubated at 40C for 5 minutes followed by centrifuging at 10000 x g for 60 seconds.

Step 5: Avoiding the pellet, supernatant was transferred to a sterile collection tube, 200 µL of solution C3 was added, briefly vortexed and was incubated incubated at 40C for 5 minutes.

Step 6: The solution was then centrifuged at 10000 x g for 60 seconds and the supernatant was transferred to a sterile collection tube avoiding the pellet. 1200 µL of solution C4 was added to the supernatant and was vortexed for 50seconds.

Step 7: The sample was then loaded to the spin filter and was centrifuged at 10000 x g for 60 seconds. The flow-through was discarded and the process was repeated until all of the sample was passed through the spin filter.

Step 8: 500 µL of solution C5 was added to the spin filter and was centrifuged at 10000 x g for 60 seconds, flow-through was discarded and centrifuged again at 10000 x g for 2 minutes.

Step 9: The spin filter was placed in another sterile collection tube and 100 µL of solution C6 was added to the center of the filter membrane. The collection tube with spin filter was then centrifuged at 10000 x g for 60 seconds.

DNA Amplification using PCR

The process of DNA extraction was followed by the process of DNA amplification which was carried out by PCR in a thermocycler. A PCR reaction works in three steps. Firstly denaturation occurs where the DNA strands are dissociated. This is followed by annealing where the reverse and forward primers are bound to the strands, the final step is the elongation where a Taq-polymerase is used for copying the DNA segment. These three process comprise of a cycle and each cycle is used as the template for another cycle.

Firstly, a master mix was prepared for initiation of the reaction. The master mix consisted of a PCR buffer solution, cation solution of $MgCl_2$, deoxynucleoside triphosphates (dNTPs), a pair of primers: forward and reverse) and DNA polymerase like Taq-polymerase. Also, for ensuring the accuracy of the process, negative and positive controls were used. Negative control contained no DNA, thus it would not yield any products after amplification. So negative control was used for the detection of contamination as well. Positive controls were used to ensure that the reaction was correctly completed and the DNA were correctly amplified.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was applied for the segregation of the amplified PCR products according to the DNA size. This involves a gel medium connected to electric power such that the negatively charged DN molecules will travel from anode to cathode. The movement is such that the shorter DNA molecules will travel further. The PCR product was run in 1% gel for 60 minutes in 100 V.

3.2.3 Antibiotic Resistance Genes Determination

The qualitative determination of the antibiotic resistant genes were carried out using PCR. Different set of primers were used for different drugs. Also, a set of positive controls were used for ensuring the correctness of the amplification process.

Resistance to tetracyclines

For the determination of tetracycline resistance genes in the wastewater samples, several *tet* genes (*tet* A, B, C, D, E, G, K, L, *otr*B, M and O) were chosen. The detailed information on the primers used and the master mix ingredients is given in Table 3.3.

Table 3.3 Tetracycline Primer Genes

Gene	Primers	Sequence	Amplicon Size	Reference
*tet*A	*tet*A-FW *tet*A-RV	gctacatcctgcttgccttc catagatcgccgtgaagagg	210	(Ng et al., 2001;Özkök, 2012)
*tet*B	*tet*B-FW *tet*B-RV	ttggttaggggcaagttttg gtaatgggccaataacaccg	659	
*tet*C	*tet*C-FW *tet*C-RV	cttgagagccttcaacccag atggtcgtcatctacctgcc	418	
*tet*D	*tet*D-FW *tet*D-RV	aaaccattacggcattctgc gaccggatacaccatccatc	787	
*tet*E	*tet*E-FW *tet*E-RV	aaaccacatcctccatacgc aaataggccacaaccgtcag	278	
*tet*G	*tet*G-FW *tet*G-RV	gctcggtggtatctctgctc agcaacagaatcgggaacac	468	
*tet*K	*tet*K-FW *tet*K-RV	tcg ata gga aca gca gta cag cag atc cta ctc ctt	169	
*tet*L	*tet*L-FW *tet*L-RV	tcg tta gcg tgc tgt cat tc gta tcc cac caa tgt agc cg	267	
*tet*M	*tet*M-FW *tet*M-RV	gtggacaaaggtacaacgag cggtaaagttcgtcacacac	406	
*tet*O	*tet*O-FW *tet*O-RV	aacttaggcattctggctcac tcccactgttccatatcgtca	515	
*otr*B	*otr*B-FW *otr*B-RV	ccgacatctacgggcgcaagc ggtgatgacggtctgggacag	947	(Nikolakopoulou et al., 2005)

Each PCR mastermix for tetracyclines consisted of 2.5µl 10X Buffer solution, 1µl of 2.5mM dNTP mixture, 2µl of 25mM $MgCl_2$ solution, 1µl of each 25µM *tet* forward and reverse primers, 0.2 µl 5U/ µl Taq DNA Polymerase and 1µl of the genomic DNA. Sterile water was then added to reach up to the volume of 25µl. The thermocycler conditions are mentioned in Table 3.4.

Table 3.4 Thermocycler conditions for Tetracyclines

Gene	Thermal Cycler Conditions
*tet*A	Pre-denaturation: 9 min at 95^0C 40 cycles: 45 sec at 95^0C, 45 sec at 55^0C, 90 sec at 72^0C Final incubation: 7 min at 72^0C
*tet*B	Pre-denaturation: 2 min at 95^0C 30 cycles: 30 sec at 95^0C, 30 sec at 57^0C, 50 sec at 72^0C
*tet*C	
*tet*D	Pre-denaturation: 9 min at 95^0C 30 cycles: 45 sec at 95^0C, 45 sec at 57^0C, 90 sec at 72^0C Final incubation: 7 min at 72^0C
*tet*E	Pre-denaturation: 9 min at 95^0C 35 cycles: 30 sec at 95^0C, 30 sec at 55^0C, 50 sec at 72^0C Final incubation: 7 min at 72^0C
*tet*G	Pre-denaturation: 9 min at 95^0C 30 cycles: 30 sec at 95^0C, 30 sec at 57^0C, 50 sec at 72^0C
*tet*K	
*tet*L	
*tet*M	
*tet*O *otr*B	Pre-denaturation: 9 min at 95^0C 35 cycles: 30 sec at 95^0C, 30 sec at 55^0C, 50 sec at 72^0C Final incubation: 7 min at 72^0C

Resistance to Sulphonamides

For the determination of the sulphonamide resistance genes, *sul*I, *sul*II, *sul*III were taken. The details of the primer used are listed in Table 3.5.

Table 3.5 Sulphonamide Gene Primers

Gene	Primers	Sequence	Annealing Temperature	Amplicon Size	Reference
*sul*I	*sul*I-FW	cgcaccggaaacatcgctgcac	55.9	163	(Pei et al., 2006;Özkök, 2012)
	*sul*I-RV	tgaagttccgccgcaaggctcg			
*sul*II	*sul*II-FW	tccggtggaggccggtatctgg	60.8	191	
	*sul*II-R	cgggaatgccatctgccttgag			
*sul*III	*sul*III-FW	tccgttcagcgaattggtgcag	60.0	128	
	*sul*III-RV	ttcgttcacgccttacaccag			

Each PCR mastermix for sulphonamides consisted of 2.5µl 10X Buffer solution, 1µl of 2.5mM dNTP mixture, 2µl of 25mM $MgCl_2$ solution, 1µl of each 25µM *sul* forward and reverse primers, 0.2 µl 5U/ µl Taq DNA Polymerase and 1µl of the genomic DNA. Sterile water was then added to reach up to the volume of 25µl. The thermocycler conditions are mentioned in Table 6 below. The thermocycler condition for sulphonamides are as follows: 9 min Pre-denaturation at 95^0C, followed by 40 cycles of annealing for 45 sec at 95^0C, 45 sec at 55^0C and 90 sec at 72^0C and the final incubation for 7 min at 72^0C

Resistance for Erythromycines

The method reported by (Martineau et. al, 2000) mentioned in (Özkök, 2012) was used for the erythromycin resistance genes determination. Along with the primers, erythromycin PCR mastermix consisted of an internal control that amplified the 16S rRNA gene that resulted in a better quality PCR product ensuring the better output of the PCR product. The primers used are listed in Table 3.6.

Table 3.6 Erythromycin Primer Genes

Gene	Primers	Sequence	Amplicon Size	Reference
erm(A)	*erm*A-FW	tatcttatcgttgagaagggatt	139	(Martineau et al., 2000; Özkök, 2012)
	*erm*A-RV	ctacacttggcttaggatgaaa		
erm(B)	*erm*B-FW	ctatctgattgttgaagaaggatt	142	
	*erm*B-RV	gtttactcttggtttaggatgaaa		
erm(C)	*erm*C-FW	cttgttgatcacgataatttcc	190	
	*erm*C-RV	atcttttagcaaacccgtatt		
msr(A)	*msr*A-FW	tccaatcattgcacaaaatc	163	
	*msr*A-RV	aattccctctatttggtggt		

Internal control (16S rRNA)	FW	ggaggaaggtggggatgacg	241
	RV	atggtgtgacgggcggtgtg	

Each PCR mastermix for erythromycine consisted of 2.5µl 10X Buffer solution, 2µl of 2.5mM dNTP mixture, 2µl of 25mM $MgCl_2$ solution, 1µl of each 25µM *sul* forward and reverse primers, 0.4 µl 5U/ µl Taq DNA Polymerase and 1µl of the genomic DNA. Sterile water was then added to reach up to the volume of 25µl. in addition to that, each tube contained 16S rRNA primers with 1/10 concentration of the gene specific primers. The thermocycler conditions are mentioned in Table 6 below. The thermocycler condition for *erm* and *msr* (A) are as follows: 9 min Pre-denaturation at 95^0C, followed by 30 cycles of annealing for 30 sec at 95^0C, 30 sec at 55^0C and 30 sec at 72^0C.

4 RESULTS

4.1 MEMBRANE FILTER OPERATION

The results from the microbial colony count are presented in the tables below including the standard deviation of the mean. The samples have been collected at different time periods with different physical and chemical parameters as explained in Table 3.1.

Table 4.1 Total organisms count for Sample 1

	Total Organisms (x 10^3CFU/ml)					
	Total Coliform	E. coli	Enterococcus	Heterotrophs	Mode of operation	Pore size (µm)
Feed	181 ± 6.7	3.0 ± 2.0	6.3 ± 1.5	220 ± 91.7		
Permeate	0.003 ± 0.0075	0.0 ± 0.0	1.3 ± 0.0012	11.3 ± 0.82	Conventional micro filter	0.1
Retentate	156 ± 7.2	2.0 ± 1.0	4.3 ± 0.6	19.8 ± 0.53		
Percentage Removal	99.9	100	79.3	94.8		

Table 4.2 Total organisms count for Sample 2

	Total Organisms (x 10^3CFU/ml)					
	Total Coliform	E. coli	Enterococcus	Heterotrophs	Mode of Operation	Pore size (µm)
Feed	17 ± 3	0.0 ± 0.0	0.7 ± 1.2	13.3 ± 3.5		
Permeate	13 ± 0.003	0 ± 0.0	0.0077 ± 0.004	0.5 ± 0.7	Conventional micro filter	0.1
Retentate	24.9 ± 0.4	0.0 ± 0.0	5.0 ± 0.0	369 ± 10.6		
Percentage Removal	23.5	0.0	98.9	96.2		

Table 4.3 Total organisms count for Sample 3

	Total Organisms (x 10^3CFU/ml)					
	Total Coliform	E. coli	Enterococcus	Heterotrophs	Mode of Operation	Pore size (µm)
Feed	50 ± 14.8	1.3 ± 1.5	1.3 ± 0.6	30.3 ± 18	Dead-end	0.1
Permeate	0.0037 ± 0.012	0.0 ± 0.0	0.001 ± 0.0	19.3 ± 8.1		
Percentage Removal	99.9	100	99.9	36.3		

Table 4.4 Total organisms count for Sample 4

	Total Organisms (x 10^3CFU/ml)					
	Total Coliform	E. coli	Enterococcus	Heterotrophs	Mode of Operation	Pore size (µm)
Feed	12.3 ± 3.8	3.8 ± 0.0	0.7 ± 1.2	46.7 ± 27.8	Cross-Flow	0.1
Permeate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	22.0 ± 6.0		
Percentage Removal	100	100	100	52.9		

4.2 ANTIBIOTIC RESISTANCE GENES

The PCR product of the antibiotic resistance genes was run in gel electrophoresis and the results are listed in the tables below. The (√) sign states positive resistance and the (x) sign means negative resistance.

Resistance to Sulphonamides

Mode of operation: Conventional micro filtration

Pore size: 0.1 µm

Table 4.5 Resistance to Sulphonamide genes in Sample 1

Resistant genes	Feed	Permeate	Retentate
Sul I	√	√	√
Sul II	x	x	x
Sul III	x	x	x

Mode of operation: Conventional micro filtration

Pore size: 0.1 µm

Table 4.6 Resistance to Sulphonamide genes in Sample 2

Resistant genes	Feed	Permeate	Retentate
Sul I	x	x	x
Sul II	x	x	x
Sul III	√	√	√

Mode of operation: Dead-end Filtration

Pore size: 0.1 µm

Table 4.7 Resistance to Sulphonamide genes in Sample 3

Resistant genes	Feed	Permeate
Sul I	√	√
Sul II	x	x
Sul III	√	√

Mode of operation: Cross-Flow Filtration

Pore size: 0.1 µm

Table 4.8 Resistance to Sulphonamide genes in Sample 4

Resistant genes	Feed	Permeate
Sul I	x	x
Sul II	x	x
Sul III	x	x

Resistance to Tetracycline

Mode of operation: Conventional micro filtration

Pore size: 0.1 µm

Table 4.9 Resistance to Tetracycline genes in Sample 1

Resistant genes	Feed	Permeate	Retentate
Tet A	√	√	√
Tet B	x	x	x
Tet C	√	x	√
Tet D	x	x	√
Tet E	x	x	x
Tet G	√	x	√
Tet K	x	x	x
Tet L	x	x	x
Tet M	√	x	√
Tet O	x	x	x
Otr B	x	x	x

Mode of operation: Conventional micro filtration

Pore size: 0.1 µm

Table 4.10 Resistance to Tetracycline genes in Sample 2

Resistant genes	Feed	Permeate	Retentate
Tet A	√	x	√
Tet B	x	x	x
Tet C	x	x	x
Tet D	x	x	x
Tet E	x	x	x
Tet G	x	x	x
Tet K	x	x	x
Tet L	x	x	√
Tet M	√	√	√
Tet O	x	x	x
Otr B	x	x	x

Mode of operation: Dead-end Filtration

Pore size: 0.1 µm

Table 4.11 Resistance to Tetracycline genes in Sample 3

Resistant genes	Feed	Permeate
Tet A	√	x
Tet B	x	x
Tet C	x	x
Tet D	x	x
Tet E	√	x
Tet G	√	x
Tet K	x	x
Tet L	x	√
Tet M	√	√
Tet O	x	x
Otr B	x	x

Mode of operation: Cross-Flow Filtration

Pore size: 0.1 µm

Table 4.12 Resistance to Tetracycline genes in Sample 4

Resistant genes	Feed	Permeate
Tet A	x	x
Tet B	x	x
Tet C	x	x
Tet D	x	√
Tet E	√	x
Tet G	x	x
Tet K	x	x

Tet L	x	x
Tet M	x	x
Tet O	√	x
Otr B	√	√

Resistance to Erythromycin

Mode of operation: Conventional micro filtration

Pore size: 0.1 µm

Table 4.13 Resistance to Erythromycin genes in Sample 1

Resistant genes	Feed	Permeate	Retentate
Emr A	x	x	x
Emr B	x	x	x
Emr C	√	√	√
Msr A	x	x	x

Mode of operation: Conventional micro filtration

Pore size: 0.1 µm

Table 4.14 Resistance to Erythromycin genes in Sample 2

Resistant genes	Feed	Permeate	Retentate
Emr A	x	x	x
Emr B	x	x	x
Emr C	x	x	x
Msr A	√	√	√

Mode of operation: Dead-end Filtration

Pore size: 0.1 µm

Table 4.15 Resistance to Erythromycin genes in Sample 3

Resistant genes	Feed	Permeate
Emr A	x	x
Emr B	x	x
Emr C	x	x
Msr A	√	√

Mode of operation: Cross-Flow Filtration

Pore size: 0.1 µm

Table 4.16 Resistance to Erythromycin genes in Sample 4

Resistant genes	Feed	Permeate
Emr A	x	x
Emr B	x	x
Emr C	x	x
Msr A	x	x

5 DISCUSSION

Membrane filtration

The findings of the study suggest that membrane filters coupled with bioreactor can be an effective method for the removal of microorganisms from domestic wastewater. The samples taken over different course of times had different OLRs and HRTs. In spite of that, the microbial culture results show that the membrane filters are working at a good efficiency. In case of Sample 1 and Sample 2, the conventional microfiltration technique was used with a microfiltration membrane having a pore size of 0.1 µm. The efficiency of the membrane can be observed by the number of microbial colonies in the permeate sample as well as the retentate sample. The removal efficiency for most of the samples are well above 90 % suggesting that the removal of pathogens by the membranes is very high. It can be observed in Table 4.1 that the number of coliform in the permeate sample is very small as compared to the feed and what retained on the membrane. On the other hand, there is no E. coli colony passing through the membrane. The very low standard deviation of the means suggests that the experiments represented a reliable group of samples. Sample 1 had the OLR of 4.3 g/l. d compared to that of 5.6 g/l. d in case of Sample 2 but in both the cases the filter membrane used was the same and the filtration process was the same i.e. the conventional microfiltration. The number of pathogens in the feed sample were however different which is listed in Table 4.1 and 4.2.

Sample 3 was collected from the UASB with different OLR and HRT than that in Sample 1 and 2. The OLR in this case was6.4 g/l d; the highest of all the other sampling conditions. The filter operation mode for Sample 3 was dead-end operation with a pore size of 0.1 µm. In case of the dead-end operation, the feed flow is directed normally on the membrane surface. The filtrate matter is accumulated on the surface that causes clogging of the membrane surface. The resulting permeate has a very good quality with less solute. Yet, the flow rate gradually decreases with time until the clogging blocks all the pores unless the cleaning of the membrane is done. The results of the Sample 3 show less number of pathogens in permeate as shown in Table 4.3. The experiment showed a very high removal rate as compared to the conventional membrane operation in case of sample 1 and 2. The removal rate of the pathogens in this case is well above 90% in this case as well with an exception of the total heterotrophs count which has a negative removal value i.e. there are more pathogens in the permeate than in the samples itself as shown in tables 4.2 and 4.3. The plate count is not a selective agar hence the colonies observed were not only the pathogens but other different species of bacteria as well. Many factors could have caused that to happen. First of all, the experiment conducted might have had some contaminations during the plating or even sampling conditions. Also, that could have been result of the passing through of the pathogens through the membrane after certain time. In the experiment conducted by (Hasegawa, Naganuma, Nakagawa, & Matsuyama, 2003), they tried to figure out the passing of certain species of bacteria through micro filter membranes of various pore sizes. According to their results, four representative gram-negative and gram-positive species of bacteria passed through the filter with pore size 0.45 µm. the time taken for the process though was different for the different species. Some species even passed through 0.22 µm. They concluded that given an appropriate amount of time, the microbes trapped in the

smaller pores than their size would grow on the filter surface, infiltrate the membrane surface and pass through it. They found that that the time needed for the infiltration increased with the decrease in pore size of the membrane. The membrane used for this study had pore size of 0.1 µm, smaller than the one used in their study. Based on their conclusion, it can be said that the infiltration of some bacteria through these membranes might have occurred between the time of the first and the second sampling. The number of bacteria being more in permeate than in the feed as shown in Table 4.3 could have happened because of the same phenomenon. In a similar research, (Nakazawa et. al, 2005), studied the infiltration behavior of pathogen *Listeria monocytogens* through membrane of pore size 0.45 µm and 0.2 µmm in 6 to 24 hours and 5 to 6 days. The organism was suspended in saline. Then, 10 µL of the suspension was placed on Millipore membrane of sizes 0.45, 0.3 and 0.22 µm which was then placed on selective agar medium. After incubating them in 37^0C, the filters were removed after 6 hour interval and the agar medium was incubated for 24 hours. The later examination for growth showed that some growth had occurred. The filter was tested to be intact suggesting that the organism had in fact passed through the membrane. This study did not include the test for this phenomenon but there might have been a possibility of this. Lack of proper cleaning of the membranes right after the first sampling might have caused the organisms to be trapped in the membrane and infiltrate it. When the second sample was taken, the infiltrated species could have passed through the filter membrane that caused permeate to have more pathogens than the feed. The higher number of microbial colony in case of the plate count agar can be explained by the same phenomenon. The plate count is not a selective media. It supports the growth of various bacteria species. There is a high possibility that some species present on the sample might have stayed on the membrane and growth happened over time which caused them to infiltrate the membrane. This growth then was observed on the plate count agar media.

Sample 4 was taken from the UASB operating at 5.14 g/l. d of OLR and 3.8 h HRT. The membrane was run in a cross-flow mode with the membrane pore size of 0.1 µm. The inlet fed to the UASB had less pathogens in them which could be observed by the clearness of the sample fed to the reactor. Thus the outlet to the reactor i.e. the feed to the membrane filter had less pathogens considering the very well working condition of the UASB. When fed to the membrane filter, it can be seen from Table 4.4. Total coliform and enterococcus have not passed at all through the membrane. The result of this mode of filter operation was observed to be the best of all with 100 % efficiency on the pathogens removal and 53 % on the total heterotrophs removal.

Out of the three modes of membrane processes applied, the most efficient, in terms of the pathogen removal was the cross-flow mode of operation. The membrane run on cross-flow mode has the feed applied tangentially to the membrane surface in a manner that would avoid the deposition of solute in the membrane. This makes it very useful for filtering very highly concentrated solutions. The tangential feed water velocity prevents the clogging up of the membrane pores and prevents the occurrence of membrane fouling and in this case, biofouling.

Antibiotic Resistant genes

The results of this study showed very few antibiotic resistant genes present in the samples. To begin with, there were not much genes present in the feed water so that the membrane could remove them. According to (Boateng, 2019), the UASB that provided the inlet for the filtration process was not efficient in removal of the ARGs except for some *tet* genes like *tet* E, O, L and *otr*B. It can be observed in the case of tetracycline genes that the filters were able to retain some of them as shown in Table 4.9, 4.10, 4.11 and 4.12. In case of the sulfonamide genes, their presence in the feed water was less and the ones that were present could not be retained by the membrane as shown in table 4.5, 4.6 and 4.7. Similar was the result for erythromycin where the genes were present in permeate as well as retentate as shown in Table 4.13, 4.14 and 4.15.

The results show that the membrane filtration process was effective in the removal of some of the tetracycline genes. In a study conducted by (Le et. al, 2018), they observed the effectiveness of membrane system in removal of antibiotic resistant genes. Their study concluded that the use of micro filter membrane caused a significant decrease in the number of ARGs between the feed and permeate with an effective removal efficiency. Since theirs was a quantitative analysis, they could observe a significant decrease in the quantity of the ARGs. They also concluded that for some antibiotic groups like tetracycline and sulfamethoxazole, the individual components tended to remain in a cluster for the same group of the ARGs, rendering the filtration process some advantage. The results of this study shows some similar results to that in case of some tetracycline genes as shown in Table 4.9, 4.10, 4.11 and 4.12. In another study by (Kappell et al., 2018), they studied the use of a membrane, a cross-flow membrane with pore size of 0.05 µm, operated at 200C for the removal of *erm* B, *tet* O, *sul*I and *intl*1. Their result showed a significant reduction in the genes in the filter permeate. The use of cross-flow filter operation showed a very good result in this case as well. This can be observed in tables 4.4, 4.8 and 4.12 that show the absence of the ARGs in permeate when the cross-flow mode of filter was used. Based on these studies and the results of this study, it can be said that the filtration process is effective for the removal of ARGs from the biologically treated wastewater.

In case of some genes, it can be seen that the use of membrane has no effect on them whatsoever. They are present in the feed as well as permeate. (Feys, 2016) mentions in his research about the possibility of the movement of DNA plasmids (and thus ARGs) through the membrane. He writes about a study by Arkhangelsky et. al. (2011) where double stranded DNA plasmids with a hydrodynamic diameter of 350 nm were pushed through pores of 10nm. The membrane size is even smaller than the one used in this study which is 0.1 µm. The reason for this phenomenon is explained to be the supercoiled plasmid to be stretched out into long, hair-shaped, flexible strand as a result of the applied pressure. This can be thought of as one of the reasons for the presence of the ARGs in the permeate sample.

The samples were run through three filter operations modes: conventional microfiltration, dead-end filtration and cross-flow filtration. The cross-flow filtration mode seems to be very effective in the removal of the ARGs as well. Cross-flow filter operation has feed flow tangential to the membrane surface lowering the possibility of the membrane clogging and increasing the rate and the quality of permeate.

This study was a qualitative one. The PCR results were quantitative that meant the number of genes in the feed, retentate and permeate could not be quantified from this research. That meant that even presence of a single gene in permeate meant a positive result in the ARG analysis. For instance if there were 200 genes present in the feed that were reduced to 20 in permeate meant a 90 % removal. But the result still showed positive in permeate. Hence this study did not assure the effectiveness of the membrane for removal of ARGs.

To sum up, the membrane filter was very effective in removal of the pathogenic bacteria with a very high removal percentage of around 90. In case of the total heterotrophs though, the removal percentage was not that high which can be due to the factor that the membrane had some permeability for the bacteria and also may be due to the factor that there had been some contaminations during the experimental work. In case of the ARGs, the membrane was able to remove some and some passed through the membrane. The PCR conducted for the gene amplification was not a quantitative one, so the exact efficiency of the membrane filter could not be concluded.

6 CONCLUSION AND FUTURE PROSPECTS

Conclusion

One of the main goals of this study was to find out the effectiveness of membrane filtration processes in removal of pathogens present in the wastewater. For the fulfillment of this goal, wastewater samples treated from the UASB reactor were run through the filter membranes. Three different types of filter processes: conventional microfiltration, dead-end filtration and cross-flow filtration were used over different period of times. The results show that the use of membrane filter is very useful in removal of the pathogens and other microorganisms from the wastewater sample. The removal rate of pathogens was measured to be above 90% in most cases. The other goal of the study was to evaluate the type of filter operation best efficient for pathogen removal among the three. Different types of filter operation modes were used and the results seemed very effective when the filter was run in cross-flow mode with almost 100 % removal rate. Finally, the other goal of the research was to observe the presence of antibiotic resistant genes in the wastewater sample. The inlet samples had very less antibiotic genes samples to start with, which can be credited to the UASB membrane preceding the membrane process. These samples were run through three modes of filter operation. Based on the results, cross-flow filtration mode was very efficient in the removal of the ARGs.

Future Prospects

Overall, the pathogen removal process can be more effective with the use of different membrane process. The micro filtration process used in this process had a very effective result. Use of other membrane filters with smaller pore sizes like ultrafilter membranes for future work can produce a very effective result making the filter way more efficient. And it is obvious that with decrease in pore size, the possibility of the clogging of the membrane pores increase as well. Keeping that in mind, the best mode of operation for the filtration process would be cross-flow. Secondly the qualitative study of the ARGs was not sufficient enough for the analysis of the effectiveness of the membranes in removal of the ARGs. Use of quantifying techniques like qPCR or ddPCR amplification methods so that the analysis of the membrane operation can be done on a quantitative basis. These processes provide the exact concentration of the genes in the samples. I would recommend the use of ddPCR as qPCR is quite time consuming and tedious for optimization compared to simple optimization used in the ddPCR.

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