EVALUATION OF THE PERFORMANCE OF ANAEROBIC BAFFLED REACTOR FOR DOMESTIC WASTEWATER TREATMENT

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In

MICROBIOLOGY

By

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2022

DEDICATION

"Dedicated to my beloved ***PARENTS & TEACHERS*** because whatever I am today could never be possible without their continuous effort, guidance, sincerity and prayers. I am heartily grateful to them."

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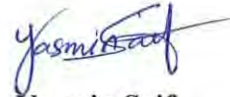

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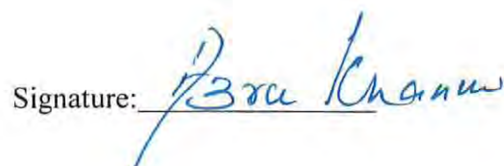

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
COD	Chemical oxygen demand
ABR	Anaerobic baffled reactor
HRT	Hydraulic retention time
FR	Flow rate
OLR	Organic loading rate
C/N ratio	Carbon nitrogen ratio
h	Hours
DNA	Deoxyribonucleic acid
RDP	Ribosomal database project
EC	Electric conductivity
TDS	Total dissolved solids
TS	Total solids
VS	Volatile solids
TKN	Total Kjeldahl nitrogen
V	Volume
Q	Volumetric flow rate
VSS	Volatile suspended solids
SRT	Solids retention time
MPN	Most probable number
L	Liter
SCOD	Soluble chemical oxygen demand
SRB	Sulphate reducing bacteria
rpm	Revolutions per minute
PCR	Polymerase chain reaction
QPCR	Quantitative polymerase chain reaction
F	Forward
R	Reverse
TAE	Tris-acetate-EDTA
TA	Thymine Adenine
SOC	Super optimal broth medium
LB	Luria-Bertani
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PCoA	Principal Coordinate Analysis
bp	Base pair
EAEC	Enteroaggregative *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*

HAdV	Human adeno virus
CRISPR	Clustered regularly interspaced short palindromic repeats
EPS	Exo-polysaccharaides
VFA	Volatile fatty acids
PAOs	Phosphorus accumulating organisms

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SUMMARY

Many countries in the world are facing some major water related hitches. Some of these are environmental contamination due to over production and mismanagement of wastewater, lack of freshwater availability and water scarcity as well as challenge of pathogens and organic matter removal to meet stringent regulations of drinking water utilities. Among number of technologies used for wastewater treatment, biological treatment (anaerobic digestion) may be an appropriate option for cost-effective and sustainable solution to clean wastewater. Anaerobic digestion is an attractive technology to mitigate a sustainable treatment of organic waste and wastewater being easy management, operation and applicable. The need is to optimize the process of anaerobic digestion by analyzing the operational parameters and microbial consortia prevailing under different conditions within the reactor to make the process economic and sustainable. Anaerobic baffled reactor (ABR) is a multiple chambered single digester/reactor which has many advantages for wastewater treatment.

The aim of the present study was evaluation of four chambered anaerobic baffled reactors, one constructed at the treatment site of Quaid-i-Azam University in terms of its efficiency for organic load removal and microbial composition with variation in different environmental conditions and two laboratory scale ABRs. Reduction in organic material present in domestic wastewater (in terms of chemical oxygen demand (COD), total nitrogen, ammonia and sulphates) evaluated at different temperature conditions. For field scale ABR, the overall efficiency gradually increased with increase in temperature preferably at temperature range 28˚C to 34˚C. Maximum COD reduction noticed was 47% at temperature 34˚C. Similarly, maximum sulphates removal was 44 % achieved at 34˚C and total nitrogen removal was 31% at 28˚C. Efficiency of ABR was significantly higher in summer season as compared to winter season.

Laboratory scale reactors were also designed similar to field scale and run at two temperatures in incubator, low temperature referred to 5˚-16˚C and high temperature as 41-45˚C. Results of lab scale ABRs indicated that organic matter and pathogens removal was more prominent and consistent at higher temperature as compared to lower temperature ABR. At high temperature ABR, in last three weeks maximum pathogens removal was observed in the effluent, it reduced from 1100 (in influent) to 150/100ml

(in effluent). While in low temperature ABR, pathogens reduction was not seen in the start period, reduction was observed in middle period from 1100 to 210/100 ml.

To investigate bacterial and archaeal communities, DNA-sequencing with marker gene 16S approach was used coupled with environmental condition (temperature) and chambers of ABR. Analysis of evolution in microbial community profile of samples was done in each alternate month throughout the year for large scale ABR shows variation of microbial community structure along the chambers. Composition of microbial consortia was dissimilar in chamber 1 to 4. Each chamber harbor specific microbial flora of the respective stage as hydrolytic microorganisms in 1st chamber, acidogens in 2nd chamber, acetogens in 3rd chamber and methanogens in 4th chamber. *Helicobcteraceae* was common family present in ABR and its member *Lithotrophicum* was leading specie. The second largest group Bacteroidetes were involved in fermentation system and break down of larger components of protein, starch etc. while the 3rd largest phylum Firmicutes mostly prevailed in anaerobic environment and are reported as to produce extracellular enzymes as lipases and proteases for breakdown of organic matter. The striking aspect about the results was rapid apparent change in microbial community composition and dominance along the chambers of ABR within month and between the months.

Similarly, significant difference in microbial composition with temperature variation, along the months, during summer and winter season was observed. Microbial evolution was seen along the months, as community structure during start months was completely different than in last months. Proteobacteria was observed as the most prevailing phylum however dominance of phyla was changed with temperature change. Results showed that temperature had more prominent effects on microbial community shift as compared to chamber wise community shift.

Significant difference in microbial community structure was observed with temperature change as compared to difference along chamber. Maximum OTUs (195279) were found in sample of April and chamber 1and 3, while minimum OTUs (20138) were found in the month of October chamber and chamber 2. Alpha rarefaction curves showed complete species saturation and richness in all the samples except two samples for the month of October chamber 1 and chamber 2. Significant difference was observed in composition of microbial community with variation in temperature, while community organization along the chamber was less variable. It was observed that some taxa were consistently leading in more than one sample.

A wide diversity of bacterial families was detected along 1st and 4th chamber of the reactor. Bacterial community organization was changed along 1st chamber showing that bacterial community composition changed over the time from February to December and new families appeared in 1st chamber of each month. Core bacterial families *Helicobacteraceae* (absent in December), *Commamonadaceae* and *Desulfomicrobiaceae* were shared in almost all months. It is concluded that analysis of microbiota of ABR in the current investigation will enhance the mechanistic understanding of the diversity and biogeography of anaerobic digestion bacterial communities within a theoretical ecology framework and have important implications for microbial ecology and wastewater treatment processes. It will support the process of optimization for future development of more effective wastewater treatment and better effluent quality.

1. INTRODUCTION

With population increase and raising standards of life water consumption has been increased. Due to which multiple problems have been raised including wastewater over production causing environmental and water pollution and on the other hand water scarcity issues (global water crises) (Wojciechowska et al., 2021). Wastewater produced by various utilities goes without receiving essential treatment and mixes into larger water bodies like rivers which serve as disposal system for polluted water, resulting in direct adverse effects on environment (Dyer et al., 2003). This practice is mainly in developing countries due to lack of proper management and treatment facilities. Another adverse effect of wastewater mismanagement is negatively associated with the human health directly or indirectly. Mixing of contaminated water with drinking water causes waterborne diseases including threat of carcinogenic diseases and even increase in mortality rate (Roushdy et al., 2012). It is estimated that 1.1 billion people are devoid of safe drinking water and 5 million people each year die with water borne diseases. Pakistan is on 80th number among 122 nations suffering with drinking water quality problems (Azizullah et al., 2011).

Wastewater is produced from industries, agriculture, household usage (domestic wastewater) etc. Domestic wastewater accounts a huge amount of contaminated water and is loaded with pathogens (e.g. faecal coli forms) and other pollutants which need to be treated before discharge into rivers (Ichinari et al., 2008). Carbohydrates, lipids, and proteins are principal organic matter (macromolecular matter) present in wastewater. Now a day's domestic wastewater management is getting main interest with central goal of treating wastewater with less energy requirement and environmental clean-up (Guest et al., 2009).

Wastewater treatment is carried out using multiple methods 1) chemical methods (photochemical process, electrochemical process, photocatalytic oxidation, Fenton, ozone oxidation) 2) physical methods (membrane separation, adsorption, and magnetic separation) 3) biological methods. Biological methods include aerobic digestion, anaerobic digestion and aerobic-anaerobic combination method. Biological method is carried out by microorganisms. Aerobic digestion is carried out by aerobic

microorganisms (utilize oxygen) to degrade organic matter into ultimate products (CO_2 & biomass). Aerobic process needs energy for its operation (Cakir et al., 2005). Trickling filter is an example of aerobic process. While anaerobic digestion is a chain of multiple steps carried out by anaerobic microorganisms (without use of oxygen) to degrade complex organic matter (fats, carbohydrates, proteins) into simpler units and then to ultimate products in the form of biomass, methane and CO_2 (Meyer et al., 2014). This technology is used for the treatment of wide range of wastewaters. Lagoons, septic tank and anaerobic baffled reactor are some examples of anaerobic digestion process (Guest et al., 2009).

The process of choice preferred for wastewater treatment must be efficient, environment friendly, sustainable, cost effective and self-sufficient. Anaerobic digestion is considered to be an efficient and feasible microbial technology for wastewater treatment as it offers several benefits including mass reduction, energy generation, pathogens removal, waste stabilization, and green image and cost-effectives (Bohutskyi et al., 2015). Anaerobic digestion chiefly has preferred goals to treat wastewater with minimum HRT (hydraulic retention time), HRT is that time which liquid remain with soluble compounds in digester (Hamawand 2015). Overall, anaerobic digestion offers an economical solution for the treatment of domestic wastewater in comparison to other treatment methods (Van Haandel et al., 2006).

Anaerobic digestion process is carried out in multiple types of reactors with multiple sets of conditions. Anaerobic baffled reactor (ABR) is a kind of reactor with multiple chambers. It consists of a series of baffles dividing the reactor into more than one compartments. Baffles (vertical baffles) are designed to force the wastewater from one compartment into other entering through series of blanket sludge (Tauseef et al., 2013). Anaerobic baffled reactor has inbuilt advantages over the single compartment reactor due to its physical configuration, circulation pattern, higher contact time of biomass with substrate and separation of solid retention time (retaining biomass) from hydraulic retention time (incoming wastewater), lower yield of sludge which enhance overall efficiency of the ABR (Hassan, 2013). ABR was introduced by Mc Carty and coworkers in 1980s at Stanford University. ABR works as multiphase system without associated

control problems. It prevents most of the biomass being exposed to low pH during shock loads and enhances reactor stability (Vossoughi et al., 2003). Anaerobic baffled reactor could be used for the treatment of a wide variety of wastewaters. Domestic wastewater treatment using ABR may be more profitable strategy because existing studies indicate strong resilience of the ABR towards loading variations and shock loads, as domestic wastewater generally shows high flow variations due to the number of inhabitants and dwellings connected to the sewer system and climate change (Reynaud et al., 2016). One of the considerable advantages of ABR is to provide favorable conditions for the two groups of microorganisms acidogenes (fast growers) and methanogens (slow growers), having ability to separate them in longitudinal direction. As failure to maintain balance between these two groups is the major cause of digester failure (Barber et al., 1999).

Within anaerobic digester, digestion is mediated by complex microbial community which is responsible for observed conversions. Food web of anaerobic digestion involves: 1) hydrolysis 2) acidogenesis 3) acetogenesis 4) methanogenesis (Zhang et al., 2018). Hydrolysis is breakdown of larger polymers (proteins, lipids etc.) of organic matter into simpler subunits (amino acids, fatty acids) by hydrolytic enzymes, secreted by relatively anaerobic hydrolytic microorganisms (Cirne et al., 2007). Acidogenesis is carried out by another group of microbes known as acidogenic microorganisms which utilize products of hydrolysis as source of substrate and convert them into CO_2, hydrogen, acetate and long and short chain fatty acids. Different microorganisms produce different volatile fatty acids (Kalyuzhnyi et al., 2000). Pseudomonas, Clostridium and Bacillus belong to acidifying group of microorganisms (Shah et al., 2014).

In acetogenesis stage of anaerobic digestion, acetogenic bacteria are involved which convert products of acidogenesis into hydrogen, CO_2 and acetic acids (Bjornsson et al., 2000). *Syntrophomonas* and *Syntrophobacter* belong to acetogens (Dhamosharan & Ajay 2014). Last stage of anaerobic digestion is methanogenesis. In 4th and last stage methanogenesis, methanogens group of microorganisms is involved which uses H_2/CO_2 and acetate as source of substrate and produce biogas (Ostrem et al., 2004). *Methanosaeta* and *Methanosarcina* are examples of methanogens. Usually, hydrolysis is the rate limiting step for breakdown of more complex polymers or recalcitrant material in

a balanced AD system, while methanogenesis is considered as a rate limiting step for those substrates which are quite easy to degrade (Karlsson et al., 2014).

To understand composition and growth of microbial population performing in digester is a key parameter for digester's better performance, as bioconversions of organic matter is dependent on harmonious activities of anaerobic microorganisms (Karakashev et al., 2005). Population composition and growth of microbial consortia within digester is affected by set of environmental conditions/factors (temperature, pH, ammonia, sulphur etc.) which should be appropriate to carryout AD. These factors have crucial effect on efficiency of anaerobic baffled reactor as they create a set of conditions for survival and better performance of microbial consortia present within the reactor. They control metabolic rate and growth of microbial community present in the reactor (De Vrieze et al., 2015).

Temperature is one of the most influential parameters which affect process of anaerobic digestion as composition of microbial community, its survival, growth and variance within anaerobic digester is highly temperature dependent. Micro biota of each anaerobic digester has different suitable temperature conditions to maintain its balance and good performance efficiency (Weiland, 2010). Intentional shifts of digester's temperature and intermittent fluctuations have significant effect on the stability of the anaerobic digester. Microbial consortia present in anaerobic reactor responds to these fluctuations and result in adaptation of pre-existing microbial community to develop unique microbiology (Wilson et al., 2008). Anaerobic digester could be operated at wide range of temperature as psychrophilic, mesophilic and thermophilic temperature (Hagos et al., 2017).

Microbial consortia are also significantly linked to pH in WWTPs (wastewater treatment plants) which affects directly the progress of anaerobic digestion by affecting microbial growth within the digester. The value of pH 6.8-7.4 has been reported as an ideal range for anaerobic digestion (Fang et al., 2002). Ammonia content in the digester also affects functioning of anaerobic digester. Concentration of ammonia change digester's pH value. Free ammonia concentration must be controlled to attain proper microbial growth, as high concentration of ammonia increases pH of digester (Dinamarca et al., 2003). Sulfur

also acts as an inhibitor of anaerobic digestion. It mostly occurs in anaerobic digestion of low COD wastewaters (Meyer et al., 2014).

Appropriate nutrients supply is also a crucial factor for growth and activity of microorganisms present in anaerobic digester. It is mandatory to supply nutrients/micronutrients in sufficient quantity at right proportions to sustain the optimal microbial (bacteria and archaea) growth (Jefferson et al., 2000). Addition of trace elements/micronutrients to anaerobic digesters treating the waste having low micronutrients concentration may have stimulatory effect on microbial growth and stabilize the reactor to avoid reactor failure (Zhang et al., 2012). They act as building blocks for microbial growth and aid in enzyme activity (as cofactor) (Demirel et al., 2011).

Understanding of microbial ecology, as community dynamics and diversity in correlation with influential factors is incomplete due to lack of knowledge about community structure which remains mostly as microbial dark matter and need to be explored. Complexity of microbial consortia and cross-species relationships make investigations more challenging (Campanaro et al., 2016; Grohmann et al., 2018). One of the key areas that need incorporation of new knowledge is relationship among structure and function of microbial communities working in anaerobic digesters. It is needed to have better understanding of microbial species composition to identify the potential key players of anaerobic digesters and their interventions. Microbiology could be studied by conventional (culturable) and molecular (unculturable) methods.

Microbial consortia working in anaerobic reactor can be distributed into 2 domains, bacteria and archaea. A balanced anaerobic reactor could be representative of four main groups; hydrolytic bacteria, acetogens (bacteria), acetoclastic and hydrogenotrophic methanogens (archaea) (Demirel et al., 2008). Conventional methods to study microbiology of anaerobic digesters are not very efficient to study whole bacterial population. Instead, molecular techniques like next generation sequencing (NGS) make available immense volume of the data about microorganisms (Ziganshine et al., 2013). Next generation sequencing resulted in greater understanding of microbial communities present in digesters and shift in microbial community with time, temperature and

substrate composition (Lebuhn et al., 2014). Certain biomarkers are developed while observing shifts in microbial populations which help to determine process stability (Vrieze et al., 2015).

The aim of the present study is to analyze reduction of organic matter and pollutants from domestic wastewater and insight into microbiology of anaerobic baffled reactor including qualitative and quantitative relationship between microbial communities of ABR. Analyses were made for a field scale ABR working at environmental/ambient temperature and two small scale ABRs working at controlled temperature: one ABR functioning at low temperature and other at high temperature. Each of 3 reactors has same design with four compartments treating domestic wastewater coming from single source.

AIM AND OBJECTIVES

The aim of the present study is evaluation of the performance of anaerobic baffled reactor and characterization of microbial community structure working in ABR for domestic wastewater treatment.

The aim was fulfilled by the following objectives of the study.

- Designing and construction of two laboratory scale anaerobic baffled reactors (four chambered each) and evaluation of their treatment efficiency (physicochemical parameters and pathogens reduction) at two dissimilar temperature conditions. Low temperature range (5-16°C) and high temperature range (41-45°C).
- Evaluation of the efficiency (physicochemical parameters and pathogens reduction) of field scale anaerobic baffled reactor working at environmental temperature.
- To characterize microbial communities and establish relationship between structure and function of identified microbial communities with respect to temperature and chambers of lab-scale and field scale ABR.

Chapter 2

REVIEW OF LITERATURE

Chapter 2 REVIEW OF LITERATURE

Water is the most essential and vital among ecological resources for the existence of life on earth. Water is needed for everyday consumption and is being used for routine activities by human. Because of change in people lifestyle and raising standards use of water is enhanced and on the other hand due to issue of water shortage more wastewater produces causing environmental problems. Regrettably, world is facing dual problems at the same time 1st rapid depletion of natural resources of water and 2nd increasing unregulated wastewater discharge to the environment (Corcoran et al., 2010).

Wastewater may be defined as, water contaminated and used by human activities. Wastewater quality is defined by its physical, chemical and biological characteristics. Wastewater includes domestic, industrial, surface runoff or storm water and septic tank inflow (Tilley et al., 2017). Wastewater is the main contributor of pollution and needs suitable management and handling (Asano et al., 2007).

Economic development and population explosion are increasing agricultural and industrial demand for water. Since World War II freshwater consumption has more than doubled worldwide and is expected to rise further 25 percent by 2030 that leads to water insufficiency (Francke et al., 2007). Estimated production of global municipal wastewater is 330 km^3 /year; it would be theoretically an adequate amount to produce biogas to supply energy for millions of households and to irrigate millions of hectares of crops. But unluckily only a little proportion of the wastewater is currently treated (Sagasta et al., 2015). WHO /UNICEF has reported the average percentage of wastewater treated by successful treatment plants is 35% in Asia. On an average, in developing countries only about 10 % of all wastewater receives treatment (Mannan et al., 2014). Due to increasing world's population day by day, billions of tons of waste will be producing which also includes wastewater (Mateo-Sagasta., 2012).

More than one-third of the world's population live in water-stressed countries and this number is expected to rise to two-thirds in 2025. Pakistan's water outline has changed

severely from being a water rich state, to one facing water stress. From 2,172 per resident to 1,306 between 1990 and 2015, water accessibility per capita declined (DAP, 2016). It was expected that in Pakistan overall production of wastewater is 4.369 x 10^9 m^3/year and 392,511 million gallons of municipal and industrial wastewater is discharged into the river which affects the water life (Murtaza et al., 2012). In Pakistan, only 8 % of wastewater is treated before discharge into a sewer system (Sato et al., 2013).

Groundwater resources are being destroyed by continuous social development worldwide. Thus, there is an urgent need to review the collective impacts of natural and enhanced anthropogenic sources on groundwater chemistry (Sisira et al., 2018). Without proper management and direct discharge of wastewater without getting essential treatment into rivers is the source of severe environmental and ecological destruction and have adverse effect on aquatic life (Venugopal, 2009, Qing et al., 2016). It was estimated by WHO, that mortality rate of people at world level is 1.7 million because of water borne diseases (Naidoo et al., 2014). Without proper treatment, if wastewater directly used for agricultural purposes, it originates life threatening infections (Baker et al., 2005). Health control and disease status (human, animals) is directly depending on water quality (Kazi et al., 2009). Studies reported the connection between severe water-borne diseases and water pollution including cholera, cryptosporidiosis, hepatitis, typhoid, diarrhea, dysentery, and giardiasis. Because of water pollution people are at the risk of and viral diseases, which contribute to cancer development (Roushdy et al., 2012). Release of pollutants causes toxic pollution, organic pollution and eutrophication. Management of wastewater should be taken as mandatory part for ecosystem management (Venugopal, 2009, Qing et al., 2016).

2.1 Composition of domestic wastewater

Domestic wastewater contains black water and sludge. Sludge consists of significant organic and inorganic solid collection. On the other hand, by weight black water composition is not just 50% of suspended and dissolved matter but also contains highly harming microbes, which perform harmful effect on one's health (Petterson and Ashbolt, 2001; Hunter et al., 2002; Ashbolt, 2004). Domestic wastewater also includes

toxic organic compounds such as heavy metals, pesticides, polycyclic aromatic compounds and inorganic non-toxic organic compounds such as calcium, silicates, aluminates and magnesium containing compounds along with important heat contents (Verstraete et al., 2009). Domestic wastewater normally has 4.7 kg or 40-50 gram of nitrogen per m^3, of which 70% comes from urine, while faecal material, kitchen wastewater and grey water each contributing roughly around 10 %. Likewise, phosphorus content in domestic wastewater is probable 0.9 kg or 10 g/m^3 of which 40-50 % originates from urine, 30 % from faeces, 10-20 % from grey water and 10 % from kitchen wastewater (Verstraete & Vlaeminckx, 2011).

According to the place, the period of the year and the pluviometric index domestic wastewater composition can differ. Wastewater contains products to be recovered such as carbon, nitrogen which are the source of nutrients (Nelson et al., 2017). In municipal wastewater the nutrient loads are diluted but because of the huge volumes generated in urban populations still add up to significant daily loads (Verster et al., 2014).

2.2 Wastewater treatment technologies

The water purification techniques involve a three-step process which includes primary, secondary and tertiary treatment (Hogan, 2011). Now a days many advance and innovative method are in common practice which helps to remove contaminants from wastewater. Physical methods like sieving and filtration are very effective but along with them many chemical and biological treatments are also in general practice (Song et al., 2002; Sonaloet et al., 2004; Bouzaza et al., 2004). Among all biological treatment methods are considered as the most efficient due to their cost effective and eco-friendly nature (Shalaby, 2008).

Biological methods are further categorized into two types a) aerobic process b) anaerobic biological processes. In aerobic process, aerobic microbes degrade organic material of wastewater in the presence of oxygen. Product of aerobic process is carbon dioxide and biomass. The process needs energy for its operation (for aeration) and enhance process cost as compared to anaerobic process (Gasparikowa et al., 2005). While anaerobic processes are generally considered as self-sufficient process in terms

of energy needs and is favourable technology to treat waste (Verstraete et al. 2005 and 2009).

Anaerobic treatment is one of the maintainable and suitable wastewater treatment technology for developing countries and is recognized as simple and cost-effective technology for treatment of a wide range of wastewaters (Alptekin, 2008). Activated sludge, anaerobic digestion, trickling filters and constructed wetlands are few of the well-acknowledged biological treatment processes. Biogas sludge reduction are important benefit of the anaerobic digestion process depending on the substrate composition and solid retention time. Reported production of sludge by AD process is between 0.04 and 0.2 kg DW/ (kg COD removed) in anaerobic process as compared to some 0.3 to 0.4 kg DW/(kg COD removed) in typical aerobic treatment (Verstraete, 2011). Domestic wastewater treatment is now an extremely mature technology from the point of view of human health, and environmental impact. In an anaerobic process, organic matter of wastewater is degraded by microbes in an anoxic condition. The product of anaerobic process is treated wastewater and biogas which is also used as energy source. Anaerobic process is beneficial as compared to aerobic process as in anaerobic process energy production in the form of biogas occurs which fulfills the energy requirements (Amit et al., 2004). Many researchers concluded that for resources recovery from the municipal wastewater anaerobic digestion (AD) is crucial technology and needs to be applied on large scale instead of conventional aerobic processes (Zeeman et al., 2008).

Domestic wastewater treatment is carried out by primarily using biological methods due to their costs effectiveness as compared to chemical methods. In biological treatment of wastewater AD is the most suitable, economical and effective solution than the conventional aerobic system (Birol et al., 2011).

2.3 Anaerobic digestion

Anaerobic digestion is a naturally occurring biological process in which organic matter is remediated by microorganisms without use of oxygen. It involves degradation and stabilization of organic matter which leads to formation of biogas and biomass (microbial) as end products and significant reduction in the amount of sludge produced

and greenhouse gasses. Bacteria and archaea are involved in this process (Ward et al., 2008, Divya et al., 2014, Okpara, 2015; Wang et al., 2020). Anaerobic digestion is considered as an efficient technology for wastewater treatment because of cost effective, easy handling and minimum generation of waste sludge (Wieland et al., 2010, Zwain et al., 2014, Shoener et al., 2014, Sun et al., 2017). It is aimed for the reduction of hazardous effects of waste on the environment (Frac et al., 2012). Generally, the process comprises of liquefaction and hydrolysis of the insoluble compounds and then gasification of the intermediates. This comes with partial or complete mineralization and humifaction of the organic substance (Skiadas et al., 1999). It is process of energy metabolism during which energy transfers and transformed using several metabolic pathways mainly methanogenic pathways (Nan et al., 2018). Energy is produced in the form of biogas containing methane which is a renewable source of energy and is a promising mean to fulfill global energy needs (Chen et al., 2014, Haberl et al., 2012). It is an attractive technology for digestion of organic waste having potential to deal with environmental pollution and energy demand. It is environmentally sustainable, economically affordable and socially acceptable and is excellently applied to treat wastewater of each sector (Meyer et al., 2014). Another advantage of AD is removal of heavy metals by reductive precipitation, which limits the discharge of heavy metals into aquatic system (Deublein & Steinhauser, 2008). Digestate of anaerobic digestion does not require cost of landfilling or other disposal as it could be used as fertilizer, because digestate has better availability of nitrogen and is valuable to use as short-term fertilizer (Weiland, 2010).

2.4 Reactors used for Anaerobic Digestion

Anaerobic reactors are used to carryout process of anaerobic digestion. Particular reactor design is very crucial due to having strong influence on entire treatment efficacy and its capital cost. With increasing deterioration of water resources of the world, it needs to configure technical & economically viable recycling and wastewater treatment technologies, to satisfy growing complexity of wastewater which has become a great challenge. Environmental regulation of wastewater has become great challenge over the past decades. Some examples of anaerobic reactors are up flow anaerobic

sludge blanket (UASB), anaerobic membrane bioreactors (AnMBRs), expanded granular sludge bed (EGSB) and anaerobic baffled reactor (ABR).

2.4.1 Up flow anaerobic sludge blanket (UASB)

The system comprises with minimal investment, stable operation and high efficiency with decent quality adaptability for a wide range of wastewater treatment. It shows strong resistance to the compounds which are recognized as toxic compounds (e.g., additives, dyes etc) in medium, the UASB digester is been established an effective for treatment of wastewater produced from textile printing & dyeing (Wijetunga et al., 2010; Senthilkumar et al., 2011). Among various anaerobic reactors UASB reactor has been recognized as common anaerobic reactor and used for the treatment of several types of industrial wastewaters (Qiu et al., 2013). Wijetunga et al., (2010) reported that if HRT (hydraulic retention time) of 24 h while with OLR (organic loading rate) of 5.6 kg COD/m^3/day, optimum COD removal efficiency obtained was of 53.1%. Presence of some reducing enzyme in reactor's anaerobic conditions lead to the breakdown of chromogenic groups like azo bond. It is studied by Somasiri et al., (2008) that >90 % decolorization (colour elimination) for the industrial textile wastewater using UASB reactor. Studies suggest that some refractory components that could be further degraded with appropriate HRT. For example, 90 % COD removal was reported by Bras et al., (2005) at HRT 96 h and influent COD 3000 mg/L by utilising bench-scale digester (UASB) for the produced synthetic dye wastewater. Nevertheless, investment expenses regarding to the capital anaerobic digester construction will be certainly increased with the prolonged HRT.

2.4.2 Expanded granular sludge bed (EGSB)

EGSB represents third-generation anaerobic reactor, which was established on basis of UASB reactor. EGSB digester has advantages of low occupation, high OLR and biogas production and improved shock resistance in comparison to the UASB. Traditional anaerobic reactors like UASB, does not show efficient removal efficiency in case dealing with refractory compounds. To tackle present problem, a concept of the EGSB design digester involves effluent reflux, due to which toxic ingredients get diluted in ultimately discharge and then enhances mass transfer and encourage biodegradation.

Textile printing process and dyeing discharge can undergo treatment effectively by EGSB digester. A few available applications of digester EGSB are mostly focused on the pharmaceutical wastewater treatment, alcohol wastewater, starch, sugar containing wastewater. It mainly applied in the developed countries as United States and Europe (Xu et al., 2018).

2.4.3 Anaerobic membrane bioreactors (AnMBRs)

Anaerobic membrane bioreactor (AnMBR) is known to merge anaerobic technology to the membrane separation technique. Their advantage of bearing high organic load, generation of biogas, less energy utilization and also high-rate interception (Lee et al., 2014). That's why, it permits greater application promises to treat discharged wastewater from various industries. Based on various combinations of AnMBRs modules, they are divided into 2 categories: as external and submerged type bioreactor. Division is based on combinations of the membrane modules. External AnMBRs, belong to most commonly used type of AnMBRs. Spagni et al., (2012) examined treatment of dyeing wastewater by use of submerged type of AnMBR showing efficient COD removal. Its decolorization competence for dyeing wastewater was more than 99%.

2.4.4 Anaerobic baffled reactor

Anaerobic baffled reactor is modified form of septic tank consisting more than one chamber. Several vertical baffles divide the reactor into compartments through which wastewater moves from one compartment into the next in an up flow/or down flow manner which enhances solid retention time to increase process efficiency. Anaerobic baffled reactor consists of two to ten compartments. These partitions or compartments separate bacterial groups performing in the process of anaerobic digestion (Wang et al., 2004; Krishna et al., 2007; Chang et al., 2008). In some literature 3-10 compartments are mentioned for ABR (Ozdemir et al., 2013). Furthermore, unique shape of ABR is more advantageous because of its phase separation of the microbial consortia of digester (as acidogenesis & methanogenesis) in single digester/unit. This technique of phase separation provides protection to microbes of digester against inflowing toxic materials and fluctuating environmental situations. Besides, configuration of ABR is

very easy with no need of the support material, like stirring and also liquid–gas separation system (Yang et al., 2018; Elreedy et al., 2015; Malakahmad et al., 2011). In ABR process, baffles direct the flow of wastewater in up-flow mode in a series of sludge blanket reactors (Nachaiyasit et al., 1997). The ABR reactor behave more like a plug flow regime with axial dispersion rather than being a mix flow one (Ghaniyari et al., 2010). ABR has resemblance with up-flow anaerobic sludge blanket due to having serially arranged up-flow & down-flow baffles that assist water to flow through the compartments from first to last compartment (Langenhoff et al., 2000; Tian et al., 2011). Compartmentalization of ABR is responsible for separation of different phases of anaerobic digestion. Two stages of AD (acidogenesis and methanogenesis) have very different population and need to be separated for efficient performance of the process which are separated in ABR (Barber and Stuckey, 1999; Plumb et al., 2001; Uyanik et al., 2002). ABR is one of those preferred system for wastewater treatment, which scientists are using now a days (Liu et al., 2009).

ABR was 1st discovered by McCarty and the co-workers in 1981 at Stanford University. They noticed while in Rotating Biological Contractor (RBC), the majority of biomass was in the suspended form and discs were separated from RBC, which resulted information of ABR (Wang et al., 2004; Dama et al., 2005). Original ABR structure was presented by Bachmann et al., in 1985. Configuration of ABR allows for naturally happening, three-dimensional separation of microorganisms that accomplish chronological steps of the hydrolysis, acidogenesis, and methanogenesis, for transformation of the complex organics to methane. ABR's can also treat larger volume of solids without related problems of clogging and segregation. ABR also work as a promising technology for biogas production with higher concentration of methane in downstream process (Wang, 2004). To improve efficiency and reliability of ABR to treat waste, some modifications were made in its modelling and configuration by scientist, in few past decades. In 1993, the distillery wastewater was treated using baffled (vertical) anaerobic sludge bed as baffled reactor with the combined function of anaerobic contactor reactor, anaerobic filter and UASB simultaneously (Li et al., 2001). Some modifications were projected from (PABR) periodic anaerobic baffled reactor, (SFABR) split-feed anaerobic baffled reactor and (CABR) carried anaerobic baffled reactor to the advanced highly efficient systems such as modified anaerobic baffled

reactor (MABR) and hybrid membrane-aerated biofilm reactor (HMABR) (Bodkhe, 2009; Faisal and Unno, 2001; Hu et al., 2009; Skiadas and Lyberatos 1998). In 1998, (PABR) periodic anaerobic baffled reactor developed with the latest modification of ABR. Significant role of the PABR is having arbitrary procedures for obtaining optimum treatment efficiency (Skiadas and Lyberatos, 1998). Carrier anaerobic baffled reactor (CABR) developed with characteristics of the biofilm reactor that is valued to enhance bacterial activity for degradation, by ensuring high-rate contact between the cell and substrate to be degraded. A short summary of main alterations made in anaerobic baffled reactor was presented by the Barber and Stuckey in (1999).

ABR has wide range of applications to variety of wastewater i.e., municipal, industrial, domestic, low strength and high strength wastewaters ABR produces less amount of the waste sludge (Fayza et al., 2008; Krishna et al., 2009). Compartmentalized design of ABR enhances the solid retention time (SRT). It prevents the risk of blockage as well as it is very stable to organic and hydraulic shocks. Initiation of anaerobic digestion process in ABR requires months because anaerobic microbes are slow growers and require time for establishment (McKeown et al., 2009). Current challenges associated with reactor design optimisation require several well-monitored long-term and broad-scale reactors investigation. Different investigations about existing ABRs yield promising results and supporting implementation of ABR for waste treatment. ABVR supporting that the ABR could be one solution answering the global call for robust low-maintenance treatment systems (Reynaud and Buckley, 2016). ABR configuration is advantageous in preserving steady active form of biomass which helps to make the process more resilient (Putra et al., 2020).

In the literature, performance of ABR in terms of COD removal was evaluated for the treatment of low strength wastewaters e.g., domestic and synthetic wastewater. Reactor was operated at 10 & 48 HRT/h. COD removal efficiency observed was 74±5 % and 0.36 v/v/d gas production, respectively (Krishna et al., 2007). Similarly, lab-scale and pilot-scale ABR was studied to treat high strength wastewater as Palm oil mill wastewater and whiskey distillery wastewater with COD removal efficiency of 77.3 and 96.1%, respectively (Faisal et al., 2001, Akunna et al., 2000). It suggests that anaerobic digestion for primary sludge may subsidize to almost 78 % of total potential

recoverable energy in the WWTPs, although mere 40 % of the total COD in the domestic wastewater is taken from primary settling tank (Zhao et al., 2016). Several studies evaluated feasibility of ABR application for treatment of textile printing and dyeing wastewater. Ozdemir et al., (2013) reported 98 % COD removal efficiency and 93 % sulphate removal efficiency at HRT of 48 h, using ABR for the treatment of synthetic dyeing wastewater. That suggested that ABR can provide favourable environment for growth of different microorganisms involved in anaerobic digestion. Goel (2010) also evaluated treatment of textile printing & dyeing wastewater using ABR and 67% COD removal was obtained. Another study conducted by Hui Xu et al., 2018 on wastewater treatment by ABR for 70 days demonstrated that start-up is completed and reached stabilization during this period and average COD removal observed was up to 78%. A recent study conducted to treat fishmeal wastewater by ABR shows 94% and 98% COD removal efficiency (Putra et al., 2020).

In another study, performance of conventional ABR and a new configuration of the hybrid ABR for treatment of thin stillage was studied. Hybrid ABR achieved COD removal, sulphate removal and yield of methane 97–94%, 94–97% & 294–310 ml CH_4 g^{-1} $COD_{removed}$, respectively at the organic loading rate (OLR) of 1–3.5 kg COD m^{-3} d^{-1}. While from conventional ABR COD, sulphate removal and methane production were 75–94%, 67–76% and 140–240 ml CH_4 g^{-1} $COD_{removed}$, respectively at the OLR range of 1.1–1.8 kg COD m^{-3} d^{-1} (Farid et al., 2018).

A lab scale experiment conducted to investigate treatment of lipid-rich (solid) wastewater using ABR and combined with down-flow hanging sponge (DHS) reactor. Experimental phase of the study was divided in three phases in order to explore effective treatment of solid and lipid present in wastewater influent. In ABR, >90% of influent COD was removed while >70% of COD removed was converted into methane under steady-state condition during every phase (Fujihira et al., 2018).

ABR can naturally separate hydrolysis, acidogenesis and methanogenesis between the sequential chambers of reactor and improved the process of methane production (Hahn et al., 2015, Sarathai et al., 2010). Krishna et al., (2020) applied bench-scale ABR to treat a low-strength soluble wastewater at two different HRTs (8 &10 h) and different

OLRs (1.5 and 1.2 kg COD/m^3d). More than 90% of the removal of COD was achieved. Furthermore, Langenhoff and Stuckey, (2000) observed ABR performance while treating dilute wastewater (500 mg/L COD) at lower temperature and concluded process feasibility. Approximate removal observed was 95% COD at 35°C, 70 % COD removal at 20°C & 60% COD removal at 10°C.

Sarathai et al., (2015) treated synthetic wastewater having a COD concentration lower than 1000 mg/L in an ABR with an HRT of 48 h and achieved approximately 85% removal of COD and 90% removal of TSS. Other researchers tested the ABR for a range of HRTs and organic loading rates (OLRs) and found good adaptability of the reactors to an HRT of 8 h with the removal efficiency reaching 90% for COD and 94% for TSS.

Performance of ABR used to treat landfill waste leachate was assessed by Amin et al., (2016) at different HRT and varying concentrations of landfill leachate for 52 days. Results indicated reductions in content of COD, TKN, nitrate, and total dissolved salts from 55 to 86 %, from 42 to 92.4 %, from 41 to 96.6 %, and from 20 to 64 %, respectively. In another study SCOD removal by ABR was observed up to 60 % in 90 days at controlled 37°C temperature and HRT 24 h. Further hydrolysis and acidogenesis were observed gradually within ABR. It was noticed that maximum COD removal occurs within first chamber of ABR. In addition, concentrations of easily biodegradable organic matter (BOD to COD ratio) have been increased up to double through the reactor (Abolghasem et al., 2015).

2.5 Factors affecting ABR efficiency

Different factors affect efficiency of ABR. It includes temperature, pH, ammonia and micronutrients. Temperature has central role in wastewater treatment e.g., microbes involved in process of anaerobic digestion produce some enzymes negatively affected by temperature due to which biogas yield is affected (Enright et al., 2007; Dobre et al., 2014). Studies showed that temperature is the most influential parameter for the microbial community variance (Siggins et al., 2011). Anaerobic digestion for wastewater treatment mostly occurs at three different temperature including low temperature (4-20ºC), mesophilic temperature (20-40ºC), high temperature (40-60ºC)

(Bidik et al., 2000). Microbial activity change with change in temperature, even a degree in temperature has significant effect on microbial performance. From past few decades, interest is developing to carry out AD at low temperature because of its low treatment cost. It also does not require external energy source to heat the system, which ultimately increase interest in AD technology of waste and wastewater (McKeown et al., 2009; Lettinga et al., 2001; McHugh et al., 2006). Microorganisms are present in many low temperature environments even in psychrophilic ecosystems which are involved in global nutrients cycle. Different studies reported that anaerobic degradation of organic waste is observed at temperature 2ºC (Nozhevnikova et al., 1997). Successful operation of anaerobic reactors with good methanogenic activity is reported in studies which may be alternative to mesophilic treatment (Lettinga et al., 2001; Collins et al., 2003; McHugh et al., 2003; Collins et al., 2006; O' Reilly et al., 2009). It was observed that 4 chambered ABR give more effective wastewater results in treatment at low temperature having removal efficiency of 90% TSS, 65% COD, 60% BOD5 (Kennedy et al., 2005). Due to compartmentalization of ABR, its SRT (solid retention time) is increased and microorganisms get maximum time to degrade organic material from wastewater. Hence ABR is effective technology in low temperature wastewater treatment also (Ji et al., 2011). Another research conducted at low temperature range from 12ºC to 23ºC using four chambered ABR showed quite good efficiency at low temperature as removal of TSS 90% and COD 65% was noticed (Hahn et al., 2015). Further investigation is necessary for overall low temperature AD process, its biochemical pathways, and microbial interactions (Metje and Frenzel, 2007).

Mesophilic and high temperature (thermophilic temperature) treatment of wastewater through anaerobic digestion are well documented comparatively in studies.

2.6 Stages of anaerobic digestion

Anaerobic digestion process consists of four steps, hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.1; Sebola et al., 2003; Christy et al., 2014).

2.6.1 Hydrolysis

Hydrolysis is the 1st step of anaerobic digestion process accomplished by decomposition of insoluble compounds into soluble. In this step obligate or facultative anaerobic microorganisms hydrolyse complex organic matter of the wastewater into small components. Carbohydrates, proteins and lipids are broken down into monosaccharides, amino acids and long chain fatty acids respectively by secretion of enzymes (extra-cellular) including cellulases, proteases, lipases etc. (Veeken et al., 2000; Cirne et al., 2007; Verstraete, 2011). Hydrolysis is rate limiting step in anaerobic process and depends on size of particles, enzyme production, pH, diffusion & adsorption of particles to be degraded (Read et al., 2008). Hydrolysis is carried out by a group of relative anaerobes bacteria of genera like Streptococcus and Enterobacterium (Shah et al., 2014).

2.6.2 Acidogenesis

In acidogenesis 2nd step of AD, acidogenic microbes produce carbon dioxide, hydrogen, acetate, volatile and long chain fatty acids by fermentation during process of anaerobic digestion (Chang et al., 2004). This step is also known as acidification in which acidogenic bacteria convert water-soluble compounds into short chain fatty acids. Example of acidifying bacteria is Flavobacterium (Appels et al., 2008; Asad et al., 2014).

2.6.3 Acetogenesis

In acetogenesis, acetogenic microbes are strict anaerobes and consume the products of acidogenic microbes (Arno et al., 2002). Hydrogen forming acetogenic bacteria are able to degrade the short chain fatty acids into hydrogen, carbon dioxide and acetate. Homoacetogenic as well as alcohol producing bacteria form acetate from CO_2 and H_2 (Bjornsson et al., 2000). The acetogenes are responsible for the conversion of end products of acidifying phase into acetates and H_2 which are then directly used by methane producing bacteria. For example, *Methanobacterium suboxydans* convert pentanoic acid into propionic acid and *Methanobacterium propionicum* convert propionic acid to acetic acid. Acetates are the key intermediate products which depict

the efficiency of biogas production because acetate reduction produces 70% methane. During the acetogenic phase almost 11% H_2 and 25% acetates are produced which are key intermediates for biogas production. First three steps of anaerobic digestion together are called acid fermentation. In AD process, organic matter is not removed from the liquid phase but converted to substrate for beyond process of methanogenesis (Dhamodharan and Ajay, 2014).

2.6.4 Methanogenesis

Methanogenesis accomplished by 3 dissimilar pathways named as a) hydrogenotrophic (use H_2/CO_2 for the synthesis of CH_4 b) acetoclastic (involve transfer of the methyl group from acetate to tetrahydrosarcinapterin & then to coenzyme M (CoM)) c) methylotrophic methanogenesis (production of methyl CoM by using methyl group from methanol & methylamines (mono-, di-, and trimethylamine). These 3 pathways converge at one common step in which finally methyl CoM is then converted to methane by the enzymatic complex universal present in all the methanogens e.g.methyl coenzyme M reductase (Borrel, 2013). Methanogens consume products of acetogens and produce methane/biogas and sludge as end products (Ostrem et al., 2004; Duin et al., 2008). Acetoclastic process is dominant than hydrogen utilizing microbes because hydrogen is present in limited amount during AD and former have 70% contribution in methane production. Co-enzymes M and F420 have major role in process of methane production. It converts format and CO into methane. Another enzyme co-enzyme M also perform important function in transformation of acetate and carbonyl during mechanism of methane metabolism (Hussain et al., 2016). Biogas produced as product of methanogenesis contain larger methane content and can used as natural gas after its purification (Appels et al., 2008). Another product of the process slurry/ digestate contain some important nutrients e.g., nitrogen and can be used as fertilizer for agricultural purposes (Tambone et al., 2009).

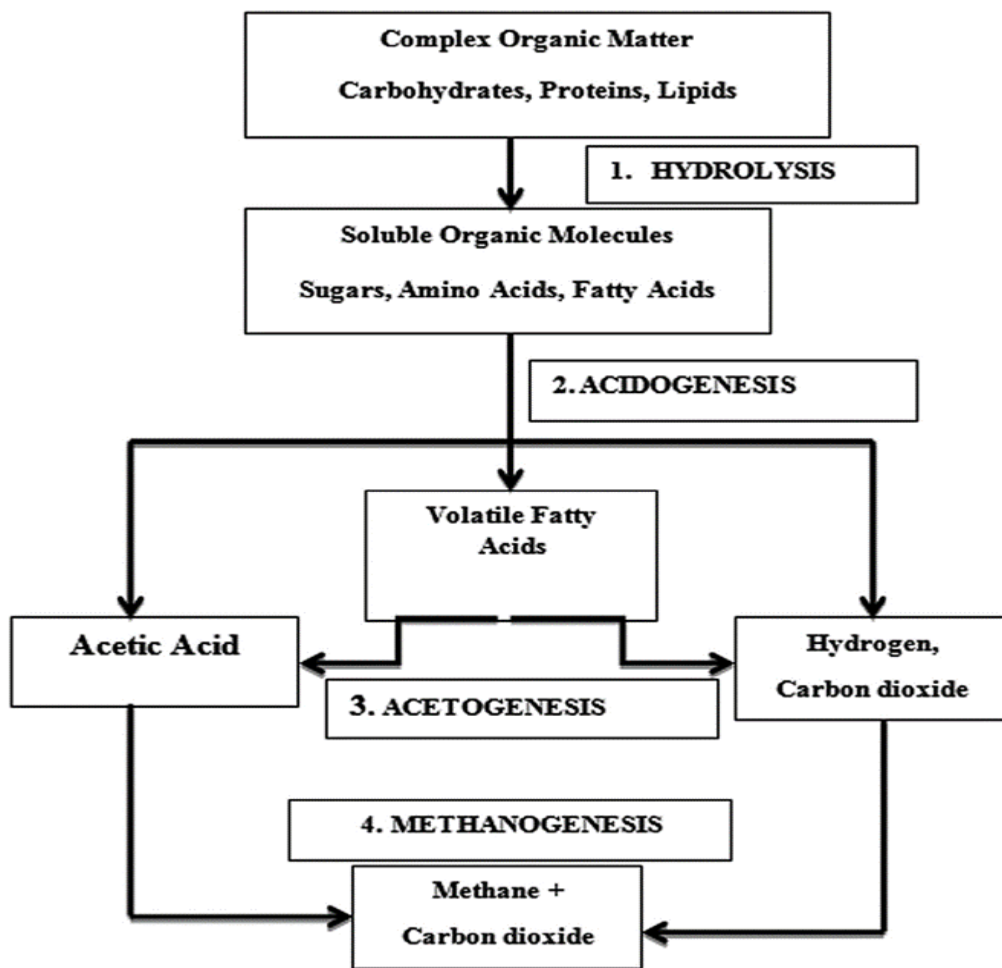


Figure 2.1 Anaerobic digestion process (Ray et al., 2013).

2.7 Microbial Ecology of Anaerobic Digesters

Anaerobic digestion is series of biochemical reactions performed by specific group of microbes within a digester. Microbial consortia of the microbial communities' act on organic waste and break down complex organic matter into simpler molecules with ultimate production of CH_4 and CO_2. Four main groups of microorganisms are involved in the process of AD: hydrolysers, acidogens, acetogens and methanogens. All these four groups have equally important role in anaerobic digestion process and interact with each other to form a chain.

2.7.1 Hydrolysers

Hydrolysers (hydrolytic bacteria) are present in 1st (initial) stage of AD, hydrolysis. They carry out degradation of complex organic compounds (proteins, lipids and carbohydrates) into simpler molecules (amino acids, long chain fatty acids and monosaccharides) by secretion of extracellular enzymes (Sevier and Kaiser 2002; Kim et al., 2003). Enterobacterium is an example of hydrolysers. Hydrolysis may be a rate limiting step in case of certain substrates (particulate matter) (Batstone et al., 2002) which can reduce process efficiency.

2.7.2 Acidogens

Acidogens also called fermenters, are present in second stage of AD, acidogenesis. This group of microorganism's carryout fermentation of the products (monomers) of previous stage and degrade them further into alcohols (methanol), short chain fatty acids (propionic acid, butyric acid etc), acetate, formate, CO_2 and hydrogen (Fang and Liu, 2002). Bacteria involved in acidogenesis are facultative anaerobes, they can utilize oxygen which is accidentally rushed in the digester and create favourable conditions for other microbes in the digester which are obligatory anaerobes like pseudomonas and clostridium (Shah et al., 2014).

2.7.3 Acetogens

Acetogens carry out 3rd step of AD, acetogenesis, in which acetogens use products of acidogenesis phase and oxidize them to acetate and/or H_2-CO_2. This group of microbes is strictly anaerobic and utilize CO_2 as final electron acceptor. Acetogens can use various compounds present in food web of anaerobic digesters like sugars, organic acids, alcohols etc. (Stams, 1994). Clostridium and acetobacterium are some examples of acetogens.

2.7.4 Methanogens

Methanogens are strictly anaerobic microbes which belong to archaea, present in final (4th) phase of AD. Archaea (methanogens) biologically produce methane by using hydrogen and acetate (Chartrain et al., 1987). They have characteristic of anaerobic

respiration metabolism, in which some methanogens (hydrogenotrophic methanogens) reduce CO_2 by H_2 to produce methane. Some methanogens (known as acetoclastic methanogens) use acetate as substrate to start methanogenesis via acetate decarboxylation (Ferry, 1999). Some methanogens are capable to use both substrates (H_2-CO_2 and acetate) to form methane for example methanosarcinaceae (Boone et al., 1993).

Microorganisms within ecosystem of anaerobic digester survive or disappear depending on their interaction (synergy or competition) to each other which decides their survival in the system (Stolyar et al., 2007). All these groups of microorganisms are dependent on each other for their survival. Optimum temperature for syntrophic interactions is 25-45˚C and pH 6.3-8.5 (Schink 2000; O'Flaherty et al., 2006).

Some other types of microbes including nitrifying bacteria, Sulphur oxidizing and sulphate reducing bacteria are also present in AD and perform different role in organic matter degradation and removal. For example, sulphate reducing bacteria are involved in hydrolysis (McInerney et. al., 1988) and acetogenesis (Dolfing et al., 1988) and biodegradation of different environmental pollutants (Ensley et al., 1995).

Studies have conducted to explore structure and function of microbial consortia present in ecosystem of different reactors and conditions to enhance the process efficiency by maintaining healthy and efficient environment to grow digester's microbes and perform better. Different changes in environmental conditions as change to temperature, pH, ammonia and substrate shape the microbial community structure in anaerobic digester (Saunders et al., 2016). Microbiology of anaerobic reactors is not completely studied and identified due to its complex structure and composition and is named as black box. Detailed description of microbial population is necessary to better understand AD process and function of microorganisms performing in the reactor (Khelifi et al., 2009, Gannoun et al., 2013). Advances in molecular techniques has enabled us to detect, quantify and identify bacterial population present in the digester to improve understanding about complexity of microbial ecosystem treating organic waste.

2.8 Investigation of microbial ecology

2.8.1 Culture dependent methods

In culturable technique microorganisms are studied by culturable methods. Initial study of microbiology evolved from culture dependent methods (example; plate count) whereby microbes grow in laboratory conditions. Staining and microscopic methods proved crucial in microbial (bacterial) classification and their identification (Ben-David and Davidson 2014). Biochemical and morphological approach is a useful tool to explore initial diversity of microbial flora while molecular characterization is beyond the doubt proven as best tool to completely evaluate microbiology (Nazir et al., 2019). Culturing of microbial isolates is challenging for many reasons including microbial interdependency and limited information about specific conditions they require (Muyzer and Smalla, 1998). Yet it proved valuable and has provided sufficient amount of knowledge about water, soil and air microbial population (Hmeed et l., 2018). Microbial culturing needs complex media provided with different ingredients as micronutrients, vitamins etc., specific to the species to be grown. Importance of culturable methods in field of microbiology cannot be denied, but this technique represents only one percent approximately, of all the microorganisms that actually exists (Amann et al., 1995; Alain and Querellou 2009; Lewis et al., 2010). That's why investigation of true microbial diversity in any ecosystem sample requires some non-culture methods.

2.8.2 Molecular techniques to investigate microbial ecology

Continuous revolution and development of modern tools for bacterial detection has improved laboratory work by usage of smart apparatus in recent decade. Journey was begun with culture dependent methods for enumeration which now has been evolved in some cultural independent techniques, which are more accurate and sensitive to microbial detection. It covers the gap in the microbial study as only an insignificant fraction is cultivable (Hameed et al., 2018). This led to generation and development of molecular microbial ecology. In present time metagenomics approach gives information about functional genes of DNA from microbiome. It is used for environmental microbial study as well (Phulpoto et al., 2020). It enabled the scientists

to bypass the previous shortcomings of culturable techniques. Next generation sequencing (NGS) techniques help to achieve more detailed/complete analysis of complex microbial flora in less time as compared to fingerprinting methods (Picot et al., 2020). Large number of species were identified including their evolutionary history, metabolic chain of community, genotype, and phenotype. This all was possible because of multiple techniques of molecular microbiology as denaturing gradient gel electrophoresis (DGGE), cloning, temperature gradient gel electrophoresis (TGGE), fluorescent *in-situ* hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP), stable isotope probing (SIP), quantitative polymerase chain reaction (qPCR) and high throughput/next generation sequencing (NGS) (Leclerc et al., 2004; Sousa et al., 2007; Malin and Illumer, 2008; Kushwaha et al., 2020) gyrB, 23 rRNA genes, 16S rRNA genes, dnak, ARDRA (Nazir et al., 2020). Some techniques rely to target on 16S rRNA gene of different species for identification in complex environmental sample (Woese, 1987). As 16S genes are largely used as diversity marker in a community study. These genes are capable to identify microbes (bacteria) because of 16S region hypervariability and have specific sequence for each species and helps to study microorganisms which are unculturable (Clarridge, 2004).

These second-generation techniques depend on DNA (deoxyribonucleic acid) fragment library construction involving clonal amplification of DNA fragments which are to be sequenced. Instead, Ion torrent and 454 pyrosequencing techniques base on proton when nucleotide incorporated by the enzyme DNA polymerase and pyrophosphate detection respectively. While Illumina technique uses fluorescently labelled nucleotides which results in high base accuracy because of fact that at a time only one nucleotide is added (Reckem et al., 2020).

2.8.2.1 DGGE/TGGE and T-RFLP

DGGE/TGGE methods have common use in microbial diversity analysis and mutational analysis. Both of these methods are extensively used to study microbial complexity. 16S rDNA fragments are studied in DGGE, which are previously amplified by PCR (Muyzer et al., 1993). DGGE, TGGE & T-RFLP techniques are useful for the purpose to screen the microbial community and its pattern recognition

(Theron and Cloete, 2000). DGGE and TGGE have high rate of detection due to high sensitivity. The only difference between DGGE and TGGE is that in TGGE temperature gradient across gel & species will melt at the different points according to the sequence.

T-RFLP relies on the size of polymorphism measurement of terminal restriction fragment from the PCR product. T-RFLP is a fingerprinting method same as DGGE and TGGE (Liu et al., 1997). It gives higher taxonomic resolution and better reproducibility. It is cost effective in comparison to cloning. It includes DNA extraction from environmental sample and PCR using primers fluorescent labelled, with next step of digestion with restriction enzyme and ultimately result in terminal restriction fragment. TRF fragments size is measured by electrophoresis platform. It tells relative abundance of each fragment in environmental sample (Cotton et al., 2014).

2.8.2.2 Cloning

Clone libraries are widely used to study microbial community analysis of an ecology. Cloning includes a chain of process, DNA extraction, PCR (polymerase chain reaction) amplification of 16S marker gene to study microbial diversity of environmental samples as (wastewater) (Sanz and Kochling 2007, Riviere et al., 2009, Leigh et al., 2010, Shah 2014). Stapes of PCR are repeated for amplification until satisfactory results are obtained. Then amplicon clean-up and subsequent insert preparation is performed in a plasmid vector and transformation is done in *E. coli* competent cells and then screened. In last stage sequencing is performed for identification of clone (Krober et al., 2009).

2.8.2.3 FISH

Fluorescence *in situ* hybridization is useful for microbial study, it is simple and have ability to rapidly quantify microorganisms (Weiland, 2010), help in localization and detection of microorganisms (Lima et al., 2020). It helps to explore complex microbial system by determining physiological properties and spatiotemporal dynamics of methanogens in an environment. It is culture independent method and does not encounter problems related to culturing of anaerobes. It identifies nucleic acid

sequences with use of probe that is labelled fluorescently and hybridize to specific complementary target sequence.

FISH includes following steps, sample cells fixation and to prepare sample, hybridization, unbound probe wash-off followed by mounting, visualization and in final step is results documentation. Common target which is mostly used by fish is 16S rRNA. It is helpful to investigate diversity of microbial consortia in wastewater samples. It works for both archaea and bacteria (Sirohi et al., 2010, Leahy et al 2010).

2.8.2.4 High throughput/ Next generation sequencing (NGS)

Sequencing techniques are of great importance in microbiological studies and been used from last three decades at the time of identification and discovery of markers genes 5S, 16S & 18S rRNA in prokaryotes (Head et al., 1998, DeSantis et al., 2006, Eckburg et al., 2005). It is comparatively cost effective than Sanger sequencing. It generates short reads (150 bp) and long (multikilobase) reads by use of some new platforms which make it able to reference independent genome assembly nd generation of long-range haplotype (McCombie et al., 2019). These are culture independent microbial techniques whose level of accuracy is decided by choice of primer (Klindworth et al., 2013). Some universal primers are also established for 16S rRNA gene (Caporaso et al., 2012). Mostly used 16S and 18S rDNA includes data bases as SILVA database (Pruesse et al., 2007), RDPII (Cole et al., 2007), green genes (DeSantis et al., 2006). Mutation is detected by NGS sequencing complete region of interest gene. NGS based polymorphism can detect novel mutations of the potential relevance. NGS has extensive use in medical field for detection by genotyping-by-sequencing of genetic markers of the human diseases and in mutations which make pathogens resistant to used drugs (Delye et al., 2020)

2.8.2.5 Sanger sequencing

Sanger sequencing helps to identify and classify microorganisms. It involves dye labelled normal deoxynucleotides (dNTPS) which are mixed to dideoxy-modified dNTP (which terminated the process during elongation phase of PCR) in a PCR reaction. Gel electrophoresis separates the DNA strands (Goodwin et al., 2016).

Currently Sanger can take 96 sequences for single run having almost 650bp length and thus used as phylogenetic marker while NGS is more efficient as compared and can produce huge (millions) of sequences or varying length in parallel (Escobar- Zepeda et al., 2015).

2.8.2.6 Illumina sequencing

It is fast and more popular sequencing technology having low cost with high yield. Principle of illumine technique is, it synthesizes DAN fragments with fluorescently labelled nucleotides which undergo reversible termination sequencing. It includes flow cell technique where DNA pieces get attached & distributed just after the addition of labelled nucleotide. Laser excites fluorescent molecule to relay signal to machine. After that fluorophore gets detached and next nucleotide incorporated. DNA molecule can be sequenced on both ends creating up-to 300 bp read length (Bennett, 2004, Metzker, 2010). This technology has wide application including anaerobic digesters. Illumina is only 2nd-generation sequencing technique which allows paired end sequencing in reliable way which ensures higher accuracy of base (Reckem et al., 2020). It can produce huge number of data and has capability of simultaneous detection on a large number of mutations in massive number of samples (Delye et al., 2020).

2.8.2.7 Analysis of sequenced data

NGS platforms generate huge datasets which require high and more complex level of bioinformatics and large amount of the storage & computational power to analyse the data (Logares et al., 2012). As one run of Illumina HiSeq2500 can generate 600 gigabases data (Scholz et al., 2012). Species diversity analysis includes alpha and beta diversity which relates community organization of different samples (Lemos et al., 2011). Alpha diversity describes richness and evenness of species in a community within the sample (Lozupone and Knight, 2008). Alpha analysis is performed in terms of rarefaction curve, Shannon and Simpson. Beta diversity relates the species between the samples (Anderson et al., 2011, De Juan et al., 2013). It distinguishes between two or more communities.

2.9 Productivity and Efficiency of ABR

In addition to provision of treated water and sanitation to the community, anaerobic process is also efficient in terms of its productivity i.e biogas production containing good methane content which could replace use of fossil fuels. Pakistan have developed infrastructure for natural gas utilization which could be easily used. Biogas may have adverse effects if it gets discharged to the environment or not properly collected from the digester.

Anaerobic digestion is cost effective and easy operation process which in turn gives energy in the form of biogas, which in turn could be used for multiple purposes including the operation of anaerobic digestion process, which makes anaerobic digestion self-sustainable process. Biogas consists of methane, carbon dioxide and other inert gases in the reactor. Its composition is dependent on type of feed stock and organic loading rate. Methane content should be maximum for good quality of biogas. Methane content per kg of COD varies according to the feed composition (Mel et al., 2015). For example, 1 mole of sugar/glucose ($C_6H_{12}O_6$) gives 3CH4 + 3CO2. The amount of methane produced is 0.373 NL/g glucose. In turn 6 moles of oxygen are required for simple oxidation of 1 mole of glucose.

Methane is the key component of natural gas as 70-90% (Mel et al., 2015). We can improve methane content of biogas by controlling some parameters as if more the fat content in the feed is incorporated, more will be methane content as compared to CO2 in the biogas (Jorgensen et al., 2009). Including feed composition some other factor to closely monitor are temperature, pH and OLR which CH4/CO2 ratio as these factors influence the process of methanogenesis and ultimately control growth of methanogens responsible for methane production (Mel et al., 2015). Anaerobic baffled reactor is efficient for wastewater treatment as it gives more than 80% COD removal efficiency (Bwapwa 2012), which gives good biogas production as consumption of each gram of COD produces a specific mount of biogas. For example, 1 mole of sugar/glucose ($C_6H_{12}O_6$) gives 3CH4 + 3CO2. The amount of methane produced is 0.373 NL/g glucose. In turn 6 moles of oxygen are required for simple oxidation of 1 mole of glucose. One cubic meter biogas can produce 6-7 hrs of 60-watt energy, generate 1.25 kW of electricity, can cook 3 meals and can operate 1 hp motor for 2 hrs.

ABR could be used for treatment of wastewater at large scale with good efficiency in terms of organic matter removal and is comparatively stable to shock loads and other factors contributing to reactor failure. ABR could be used in the low-income communities for wastewater treatment (Foxon et al., 2005).

Chapter 3

EFFICIENCY ANALYSIS OF ANAEROBIC BAFFLED REACTOR WITH TEMPERATURE VARIATION

3.1 INTRODUCTION

Global challenges including climate change, urbanization, growing societies and water crisis are increasingly becoming critical in terms of their impact on life quality. Wastewater overproduction, lack of resources and a sustainable cost-effective solution for wastewater treatment are major concerns seeking attention. Wastewater treatment in Pakistan is not preferred task resulting into huge amount of contaminated water which is directly discharged into larger/freshwater bodies without receiving essential treatment. Conventional wastewater treatment technologies currently prevailing in the world are expensive and unaffordable for developing countries, which make it unfeasible for authorities to establish wastewater treatment plants and their operation. Present part of the study is aimed to evaluate the efficiency of a cost-effective wastewater treatment technology (anaerobic digestion) using anaerobic baffled reactor (ABR) with no use of external energy source. For this purpose, three ABRs were operated, two laboratory scale ABRs and one field scale ABR at different temperatures, working as continuous type of reactors. Four chambered anaerobic baffled reactors were used in the present study as it is considered as one of the high-rate reactors (Jin et al., 2012).

Removal rate of organic matter in a reactor depends on operation conditions of a reactor (Fan et. al., 2012) e.g removal of COD from wastewater is affected by multiple factors, one of which is change in hydraulic retention time (HRT) (Zhang et al., 2013). Pathogens removal from wastewater is another concern related to water treatment. Wastewater effluent if not properly treated, may contain pathogens. To meet the effluent quality standards, anaerobic digestion process of wastewater should be efficient and balanced. Several factors are involved in maintenance of anaerobic digesters as temperature, pH, OLR (organic loading rate), HRT (hydraulic retention time), type of reactor, type of waste etc.

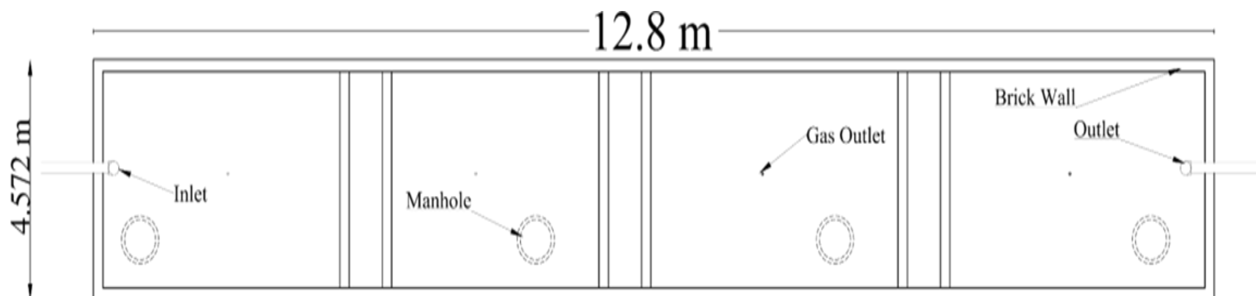
3.2 MATERIALS AND METHODS

3.2.1 Unit design and experimental setup

Three ABRs (one field scale and two laboratory scale) each with four chambers, were constructed with the same design. The field scale ABR was constructed of bricks while two laboratory scale ABRs having equal volume (28 Liters each) were fabricated using 25 mm soda-lime glass sheets.

The volume of the field scale ABR and its design were based on the number of households to be treated at Quaid-i-Azam University (QAU) residential colony and the flow of wastewater, which was ~ 110 m^3/day, calculated by the float method of Grand & Dawson. The site of the field scale ABR was chosen following survey of three different existing drain locations and the calculated footprint requirement. The ABR was constructed of brick masonry with a plaster render with dimensions 12.8 m × 4.572 m × 2.75 m (L×W×H) and included four digester chambers and six baffled walls, three vertical and three hanging, each of 0.23 m thickness. In the last chamber a columnar filter 0.9 m radius and 2.08 m high and containing 1-2 cm aggregate was constructed. The vertical baffles contained eight horizontal 0.30 m x 0.15 m (L x H) openings at the top whereas the hanging baffles had four openings 0.30 m × 0.60 m (L x H) at the bottom. Each digester chamber was fitted with an exhaust gas collection pipe of 0.05 m diameter and the ABR was connected to the existing sewer system via an inlet pipe at a height of 2.13 m and an outlet at 2.08 m relative to the ABR base. The entire ABR was made airtight with manhole covers (Figure 3.1). A pre-screen brick-built chamber of dimensions 1 m x 0.5 m x 0.5 m which contained crisscross steel bars with a spacing of 0.02 m preceded the inlet to remove solid particles/plastic bags and avoiding choking.

While approximate dimensions of each laboratory scale ABR were 58 cm × 20 cm × 40 cm (L×W×H). Length of 1st inner baffle of the reactor was 40 cm, 2nd baffle was 20 cm from bottom and 3rd baffle was 24 cm from bottom. Inlet hole of the reactor was located in 1st wall of reactor 30 cm above the bottom while outlet hole was located 26 cm above the bottom of reactor. Sampling ports were positioned on top lid of the reactor. Peristaltic pumps were used to feed ABRs with wastewater and for sampling different chambers (3.2)



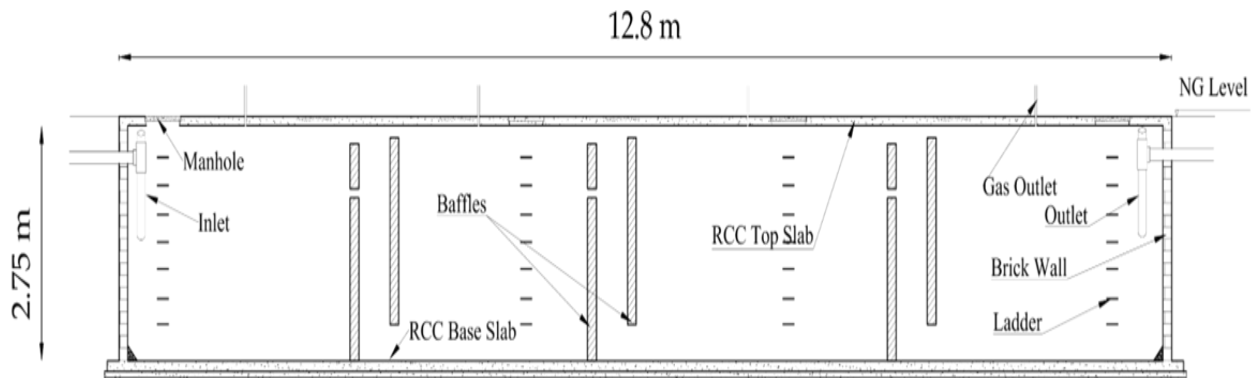


Figure 3.1 Layout design of anaerobic baffled reactor (ABR) as primary treatment unit for D-type colony a) plan view b) cross sectional view

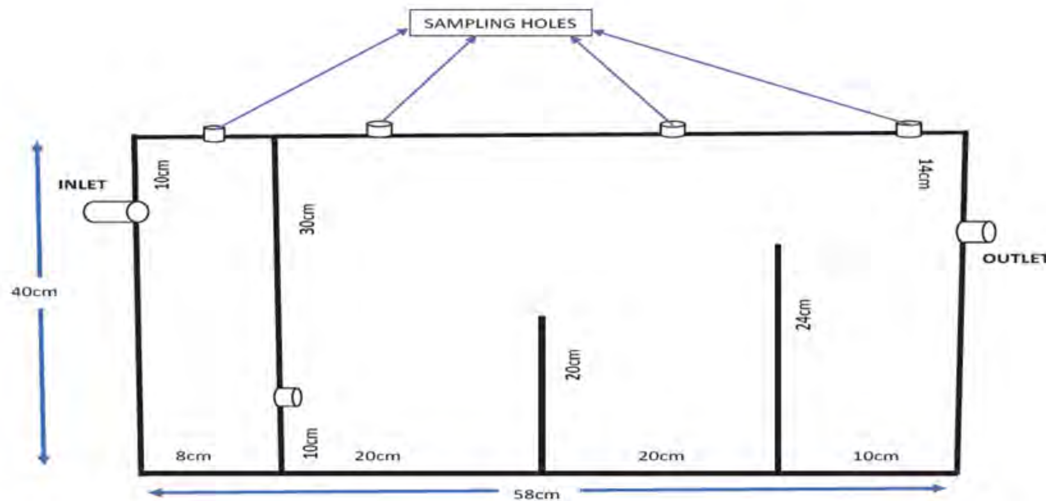


Figure 3.2 Schematic diagram of laboratory-scale ABR

3.2.2 Startup and operation of field scale ABR

Field scale reactor was operated for one year January-December. The average hydraulic retention time (HRT) was ~20 hrs. resulting in a working volume of 92.15 m^3 and an organic loading rate (OLR) of 5 kg BOD_5/m^3 per day. The sludge volume of the designed ABR was 10 %, with sludge

removal intervals of 18-24 months, calculated based on the solid retention time equation of Droste. The temperature varied throughout the year from 13-34 °C and the pH range was 6-8.0.

Eq. 3.2.1

HRT=V/Q (Eq. 3.6)

V= Volume of ABR, Q= Volumetric flow rate

Typical HRT value for ABR was almost 10 hr. (Barber and Stuckey, 2000). SRT calculated by mass of sludge in reactor divided by mass removal rate of the sludge from reactor (Droste, 1997).

$\Theta x = VXv/QwXw$ (Eq. 3.7)

Qw = volumetric flow rate of waste solids from system, Xv =Average concentration of VSS in reactor, Xw =VSS concentration in Qw, V =Volume of reactor (Droste, 1997).

3.2.3 Sampling and storage of wastewater

The field scale ABR operated at ambient temperature and grab samples were collected at ~10-day intervals in triplicate from the inlet (influent) and outlet (effluent) in 250 ml sterile bottles from January to December during the years 2015 and 2017. Samples were stored in the ice box and analysis was done on the same day in the Applied and Environmental Lab., Quaid-i-Azam University, Islamabad. Standard analysis of COD, SO_4, PO_4, TKN and pathogen removal was done for each sample.

3.2.4 Startup and operation of laboratory scale ABR

3.2.4.1 Inoculum development

Inoculum used as source of seed sludge/starter culture in laboratory scale ABRs was developed using fresh cattle manure, collected from local farms located near Quaid-i-Azam University, Islamabad. Total solids (TS) & volatile solids (VS) of cattle manure were analyzed in triplicates using standard methods of 2540B (APHA, 2005) for TS and 2540G (APHA, 2005) for VS.

Calculations for TS & VS were made using equation 3.1 and 3.2 respectively:

Eq.3.1:

% of Total Solids = (Weight of dried residue + crucible) – (Weight of crucible) x100
(Weight of wet sample + crucible) – (Weight of crucible

Eq. 3.2:

% of Volatile Solids (VS) =

(Weight of dried residue + crucible) – (Weight of residue + crucible after ignition) x100
(Weight of dried residue + crucible) – (Weight of crucible)

3.2.4.2 Reactors set-up and operation

Both reactors were operated at same conditions except temperature. One ABR was operated at low temperature range 5-16˚C and other ABR operated at high temperature range 40-45˚C. ABRs were housed in two incubators adjusted at respective temperature ranges. Same domestic wastewater was used for all the three ABRs. Inoculum to substrate (domestic wastewater) ratio was (1:4), adjusted according to measurements of chemical oxygen demand (COD) of substrate and inoculum. Digester was sealed and flushed with oxygen-free nitrogen gas to remove inner residual air. Reactor was incubated for one-month to stabilize prior to start continuous feeding of wastewater. Reactors were operated for 60 days (excluding incubation time). Hydraulic retention time for both ABRs was HRT 20 hours, organic loading rate organic loading rate was 23.15 mg COD/1.47 L/hour and flow rate flow rate was 35 L/day were constant throughout the process.

Eq. Hydraulic retention time (HRT)

Hydraulic retention time (HRT) of ABR was 19 hours. Calculation for HRT was made using equation 3.3:

Eq: 3.3: Hydraulic Retention time (HRT) = Volume of the reactor / flow rate

HRT = 28L / 1.47(L/hour)

HRT = 19 hours

Eq. Flow rate (FR)

Flow rate was 1.47/h. Flow rate (FR) calculated using equation 3.4:

Eq.3.4: Flow rate (FR)= Volume of the reactor / HRT

Flow rate = 28 L / 19 hours

Flow rate = 1.47 L / hour

Flow rate = 1.47 L x 24 hours

Flow rate = 35 L / day

Eq. Organic Loading Rate (OLR)

Organic loading rate was (OLR) 23.15 mg COD/hour. OLR was calculated using equation 3.5:

Eq. 3.5: Organic Loading rate = Concentration of V.S x Flow rate / Volume of reactor

Organic Loading rate = 300 mg COD x 1.47 L / hour ÷ 28 L

Organic Loading rate = 15.75 mg COD / L / hour

While our flow rate is 1.47 L / hour. So, 15.75 x 1.47

Organic Loading rate = 23.15 mg COD / 1.47 L / hour

3.2.5 Samples collection

Influent and effluent wastewater samples were collected every week from laboratory scale ABR. pH of influent and effluent was monitored at the time of sample collection and then the samples were stored at 4˚C for further analysis.

3.2.6 Physico-chemical analysis

Physicochemical analysis was performed for all the three ABRs. Chemical oxygen demand (COD) of influent and effluent was determined by kit method, low range 14560 and high range 14541CSB/COD kits (Merck Co.) and absorbance of the digested samples were taken using Spectroquant (Pharo 100 spectroquant R Merck, Germany). Nitrates were determined using 4500 NO_3-N method and sulphates were determined using 0375 Barium Chrometery according to (APHA, 2005) standard methods. Total nitrogen (TKN) 2-150 mg/L (100683) and ammonia 4.0-80.0 mg/L (114559) were measured by kit method by Merck Co. Plastic ware and glassware used throughout the process was cleaned with deionized water and soaked in 10 % HNO_3, rinsed again with deionized water prior to use. Daily analysis of pH was done by PCS tester.

3.2.7 Pathogens reduction analyses

To measure pathogens (total coliforms) in influent and effluent wastewater the most probable number (MPN, multiple tubes test) method was used. Three steps of the process were performed as presumptive test, confirmed test, and completed test as explained in APHA, 2005. Media and chemicals used in MPN method were from Fluka Granite CH-947 Bushes, BDH Laboratory Chemical Division, DIFICO Laboratories, ICI America 9211 street of North Harborgate Portland, UK Oxoid Chemicals Company and Sigma Chemicals Cooperation and E., Merck, St. Louis. Media preparation for culturing was prepared according to manufacturer instructions.

3.3 RESULTS

3.3.1 Efficiency of Laboratory scale ABR at two different temperatures

Two laboratory scale ABRs were operated at different temperature (one at temperature 5-16ºC and other at 41-45ºC) to analyze their efficiency for domestic wastewater treatment. Source of wastewater was same for both ABRs. Hydraulic retention time (HRT), organic loading rate (OLR) and flow rate (FR) were the same and constant for both reactors. Removal of COD, soluble COD

(SCOD), ammonia, total nitrogen and sulphates was observed throughout the operation period of the reactors after month incubation time was provided to the reactors for adjustment of microbial flora present in the inoculum used to the environment of reactor.

3.3.2 pH and Electrical Conductivity (EC)

Reactor pH is an important factor that affects process of anaerobic digestion. pH of the substrate (untreated wastewater) used in the present study was not adjusted as its pH was in the range 6.5 to 8.5, suitable for anaerobic digestion process and as also within pH range of water suggested by WHO. All samples of treated (effluent) and untreated (influent) wastewater of both the laboratory scale ABRs have pH within said range as shown in (Fig. 3.3)

Electrical Conductivity (EC) is measure of the presence of metal ions in the water. Conductivity is related to salinity as it is index measured by number of ions relative to salinity. It is linked to anaerobic digestion because of significant effect on microbial structure determination in the digesters (Lozupone et al., 2007). It is expressed in micro-Siemens per centimeter. EC was measured for influent and effluent of both laboratory scale ABRs on daily basis. EC values for influent and effluent of both ABRs were within range 150-400 µs. For high temperature (41-45˚C) ABR, initially higher values of EC were recorded in effluent as compared to influent. Decrease in EC values in the effluent were noted during 29-45 and 51-60 days with insignificant variation of 10-50 µs. For low temperature ABR (5-16˚C), EC reduction during initial period was minimum with range 2-5%. Later, in 5th week and last three weeks maximum EC reduction in effluent was noticed with value 13-28% (Fig. 3.4).

3.3.3 Total dissolved solids (TDS)

Total dissolved solids (TDS) are measure of the presence of all types of dissolved solids in water. According to WHO, range of TDS in water should be less than 1000 mg/L. Treated and untreated wastewater samples of both laboratory scale ABRs showed TDS within the range as described by WHO. TDS values of influent and effluent lies in range 60-300 ppm for both lab scale ABRs. For low temperature ABR, TDS reduction in effluent was noticed from the initial period. In start two weeks reduction was very low 1-9%. In 3rd week to onward TDS reduction in effluent was 16-25%, while maximum TDS reduction occurred in last two weeks 28-30%. For high temperature ABR, peak value of TDS in influent and effluent was 256 and 253 respectively. In start period (3rd and 4th week) TDS values of effluent were higher than influent. After that a slight reduction of

TDS (nearly 9-20 ppm) was observed in effluent. And then in last week (5 days) again TDS reduction was observed in effluent (Fig. 3.5).

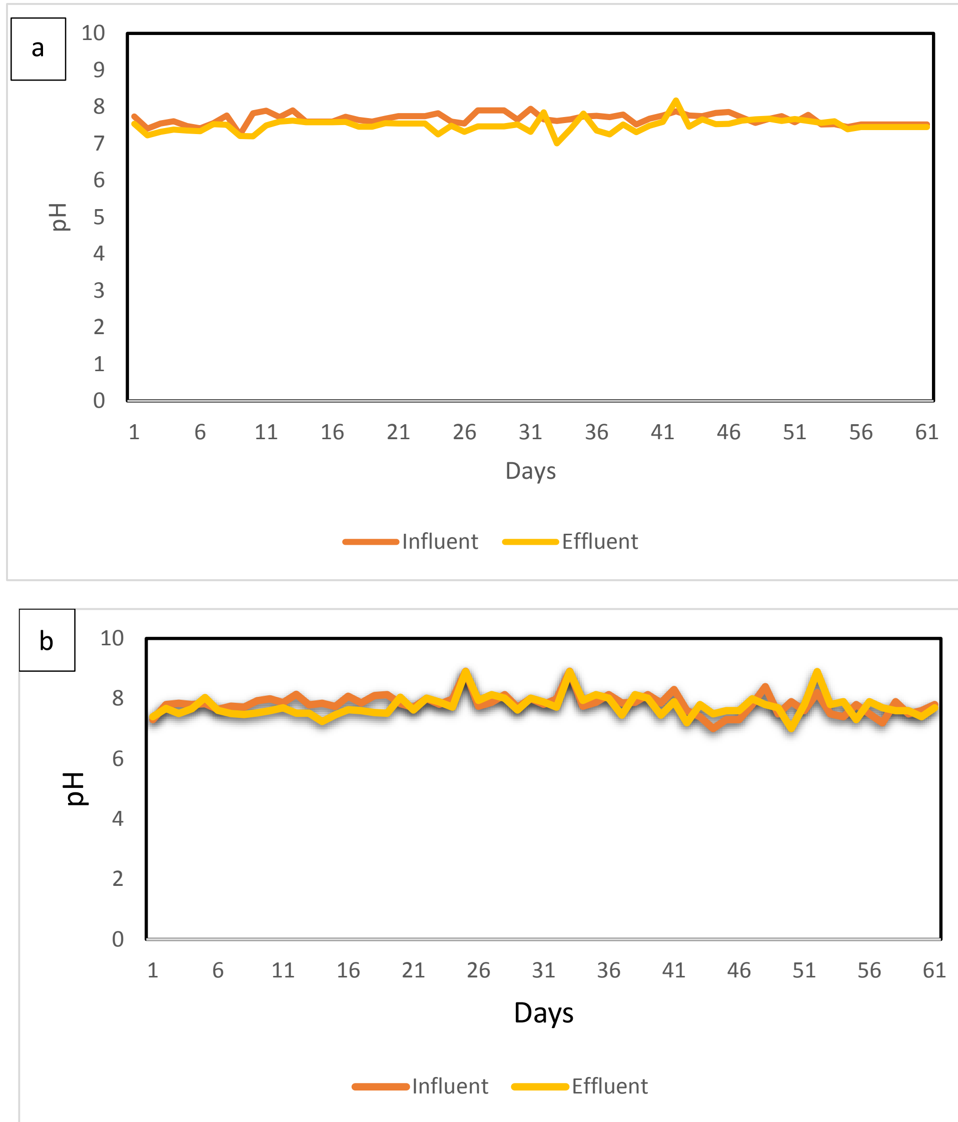


Figure 3.3 The values of pH of influent and effluent of laboratory scale ABR during the course of treatment, (a), ABR at low temperature (5-16ºC) and (b), ABR at high temperature (41-45ºC).

Figure 3.4 The values of EC of influent and effluent of laboratory scale ABR during treatment, (a), ABR at low temperature (5-16°C) and (b) ABR at high temperature (41-45°C).

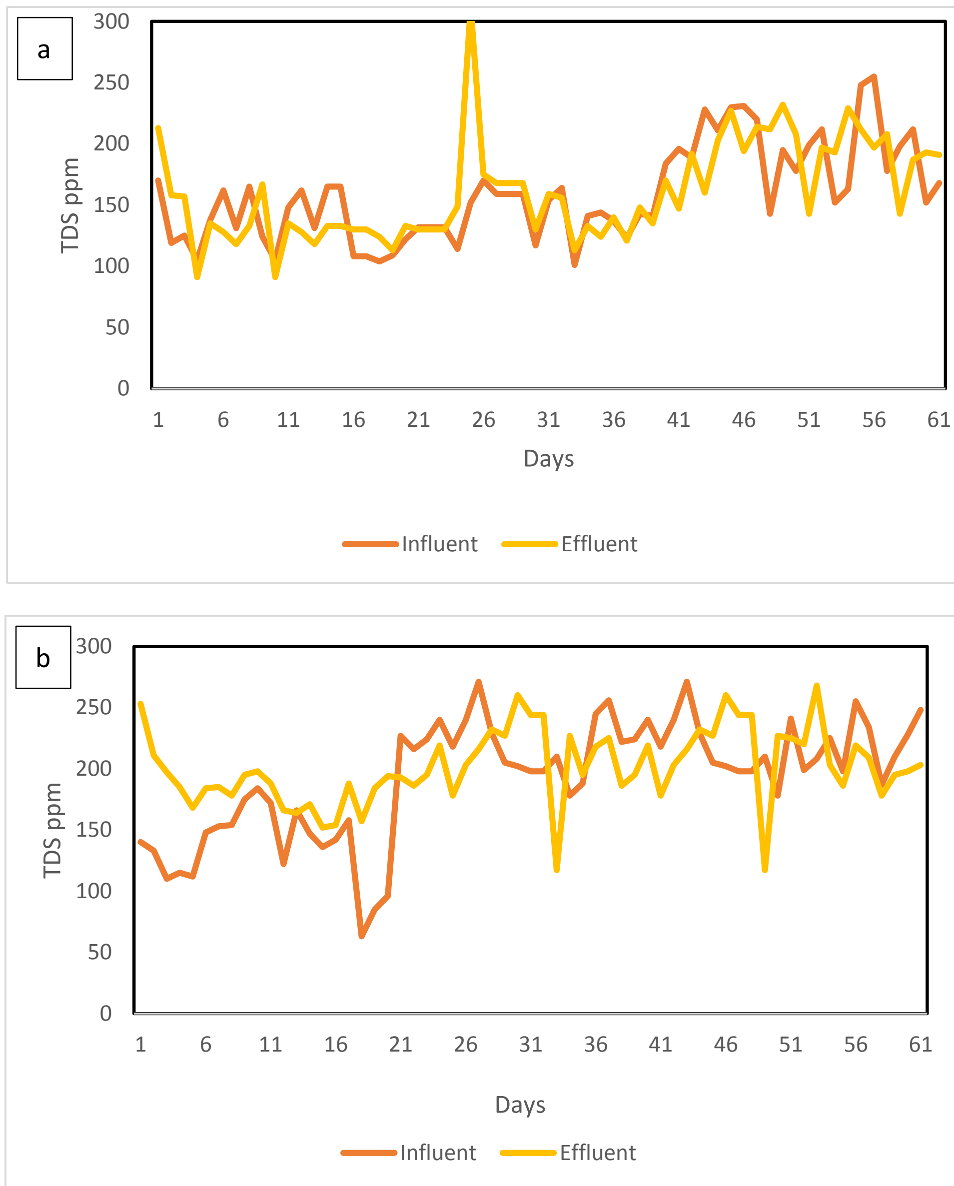


Figure 3.5 Changes in TDS of influent and effluent of lab scale ABR working at low temperature (5°C-16°C) a; and at high temperature (41°C-45°C) b.

3.3.4 Salinity

Anaerobic biological process is affected by elevated concentration of salts in the substrate used for digestion. Microbial consortia present in anaerobic digestion are sensitive to salinity fluctuations. Salinity of all wastewater influent and effluent samples of both ABRs was below the toxic level.

Salinity of low temperature ABR was below 250 ppm salts concentration and for high temperature ABR was below 200 ppm salts concentrations. Salinity of high temperature ABR was almost same for influent and effluent (70-90 ppm). In start 3 weeks, salt values of effluent were higher than the influent with approx. variation 10-60 ppm. Then in next two weeks salinity of effluent was reduced as compared to influent with small variation. For low temperature ABR, salinity reduction in effluent was 4-6% initially and then reduction was enhanced especially in 3rd, 7th and last week up-to 30%. In other weeks reduction was 8-22%.

Studies showed that salinity affects the process of anaerobic digestion and is an important factor which is involved in regulation of bacterial structure and function within the habitat (Lui et. al., 2008; Mohamed et al., 2011) (Fig. 3.6).

3.3.5 Chemical oxygen demand (COD) removal

Chemical oxygen demand prevails mainly in two forms in anaerobic digesters; one is total chemical oxygen demand (COD) and the other is soluble chemical oxygen demand (sCOD). Influent COD range was 51 mg/L to 421 mg/L during the whole operation time, which is indicative of diluted domestic wastewater, while effluent COD range was 55 mg/L to 285 mg/L for low temperature ABR. Low COD removal was observed in 1st week (2.27%) at 12˚C, while in 2nd, 3rd, and 7th week COD increase in effluent was noticed. Significant COD removal efficiency of ABR was observed at low temperature with maximum removal 64 % in 11th week at temperature 7˚C. Sudden increase in COD concentration was again observed in last week (15th) at 5˚C. This increase in COD in effluent may be due to presence of previously retained suspended solids and their biodegradation in hydrolysis phase. Results showed that no consistency was observed in COD removal, throughout the reactor operation (Fig 3.7a).

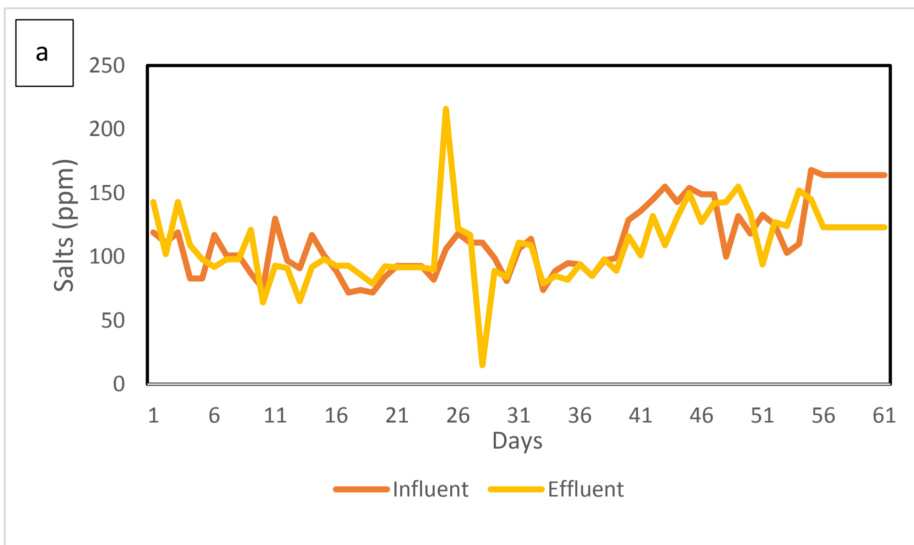
While in ABR working at high temperature (41-45ºC), overall COD removal efficiency was 64 %, observed in 7th week at 44ºC, and 61 % reduction in week 9,11 and 12 at temperature 43ºC, 44ºC and 44ºC, respectively. In 13th week (last week) COD reduction observed was 53%. COD concentration of effluent was increased in start phase of reactor for 3 weeks. Influent COD concentration ranged between 40-552 mg/L. An increase in the COD concentration in effluent was observed in initial weeks especially in week 2 the COD concentration increased to 306 mg/L while it was 206 mg/L in the influent, this odd result could be some experimental error or presence of solids in the sample and could be neglected. At later stage from week 7 to 15 of the reactor running more than 50 % reduction in COD was noted (Figure 3.7 b). In 9th week COD concentration

increased, which may be due to sludge deposition in the reactor. In majority samples COD range was same.

3.3.6 Soluble Chemical Oxygen Demand removal

SCOD for low temperature reactor was 22 mg/L to 1039 mg/L in the influent and in effluent its concentration starts from 85 mg/L. In low temperature ABR, increase in effluent SCOD was observed during start period for four weeks. In 9th week significant SCOD reduction was observed, 47% at 16 ˚C. Again, in week 12, 13 increase in SCOD was observed at temperature 9°C, 6°C, which may be due to higher solid retention time (SRT) of ABR. 46 % reduction was observed in 15th week (last week), shown in Figure 3.8 (a).

Influent of SCOD of the reactor performing at temperature 41-45°C, ranged between 53 mg/L to 466 mg/L and effluent ranged 54 mg/L to 981 mg/L. In start phase, increase in SCOD level was observed in effluent for two weeks, maximum increase was observed in 1st week. Maximum SCOD reduction observed in effluent was 66% in 7th week at 44°C while in 9th week 54% reduction was noticed at 43°C and 50% in 11th week at 44°C. In 13th week 40% reduction was calculated at 45°C. Minimum reduction was 4% observed in 3rd week at 43°C. Overall SCOD reduction observed may be affected by SRT and conversion of COD into SCOD. Longer SRT allowed hydrolysis of accumulated VSS resulting into extra SCOD generation, which could not be eliminated completely (Figure 3.8)



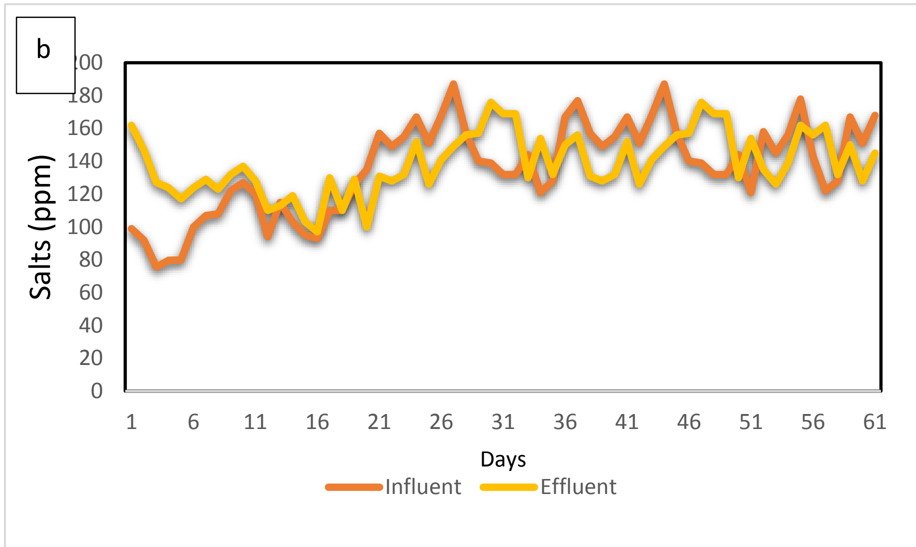
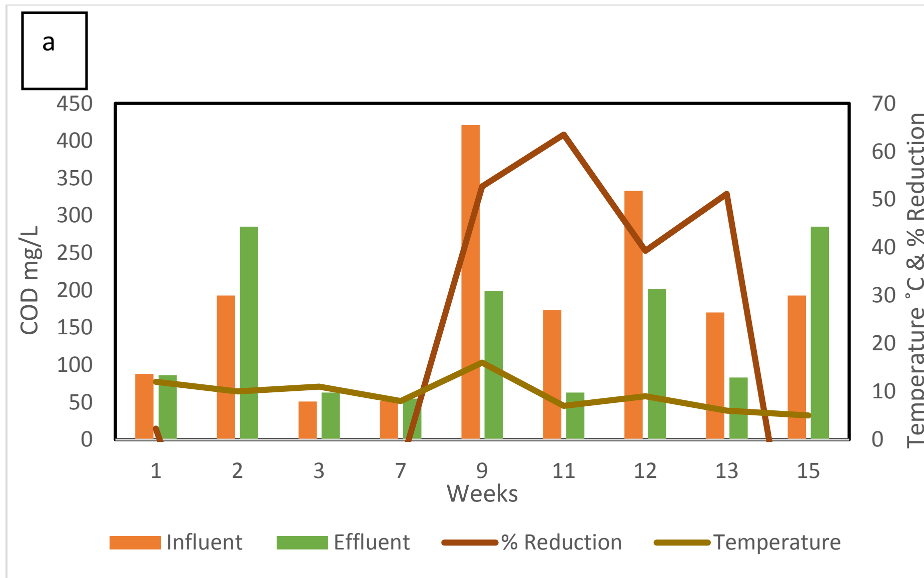


Figure 3.6 Changes in values of salinity of influent and effluent of ABR working at low temperature (5˚C-16˚C) a; and at high temperature ABR (41˚C-45˚C) b.



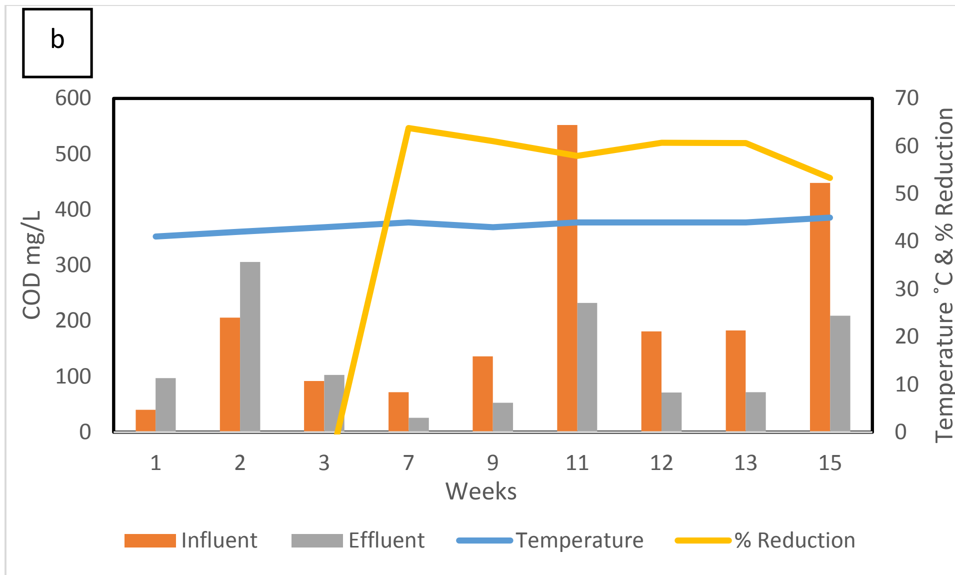
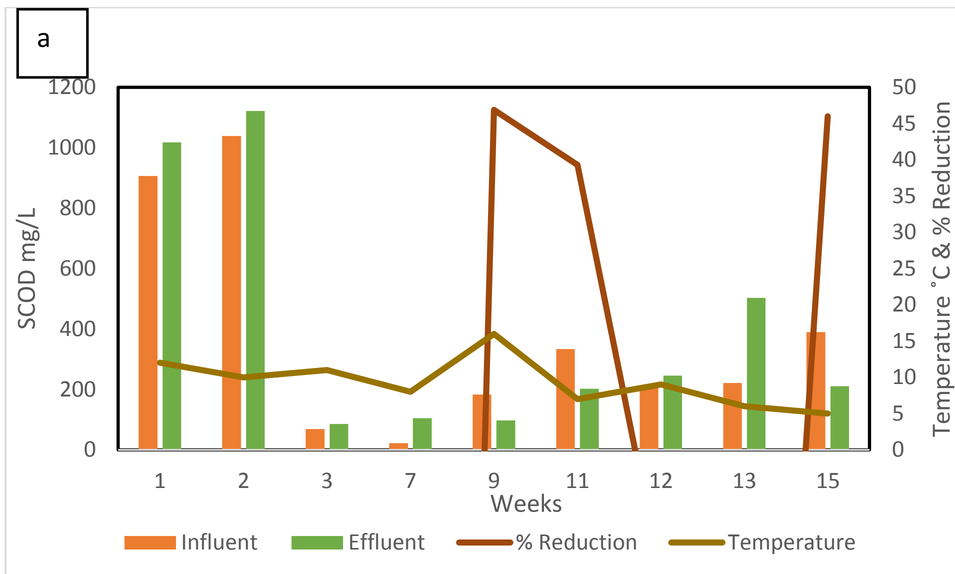


Figure 3.7 COD removal efficiency in laboratory scale ABR at low temperature (5-16°C) a, and at high temperature (41°C-45°C) b.



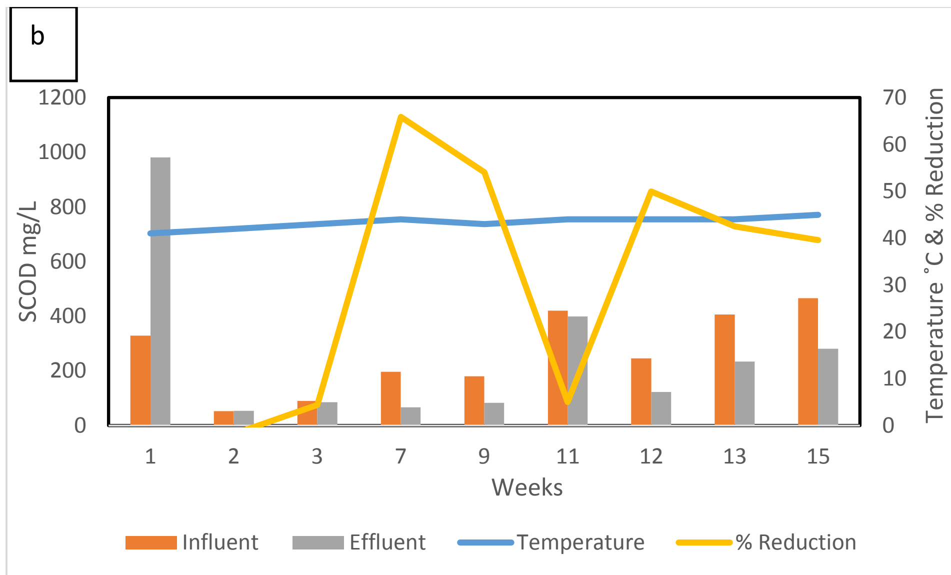


Figure 3.8 Soluble COD removal efficiency of ABR in laboratory scale ABR at low temperature (5-16°C) a) and at high temperature (41-45°C) b.

3.3.7 Total Nitrogen removal

At low temperature ABR, concentration of total nitrogen during the study ranged 27 to 59 mg/L and 24 to128 mg/L in influent and effluent respectively. Concentration of total nitrogen seemed to increase in effluent in the start 3 weeks. The total nitrogen concentration was increased in 12th week again. In 11th week % reduction observed was 33% at 7°C which was (maximum removal). In 15th week at temperature noted and the efficiency of reactor to nitrogen removal was 31% at (Figure 3.9 a).

Total nitrogen concentration in influent of high temperature ABR was 22-68 mg/L and in effluent was 27-50 mg/L. Overall efficiency of ABR for total nitrogen removal at high temperature was observed 36% in 13th week. In 9th week 31% reduction at 43°C, and in last week reduction was 33% (Figure 3.9 b). Overall, no effect of temperature on nitrogen removal was observed.

3.3.8 Ammonia removal

For low temperature ABR, ammonia concentration in influent ranged between 16 to 72 mg/L, while in effluent was 14 to 56 mg/L. Overall ammonia removal efficiency was 49 %, observed in 15th week at 5°C. Minimum reduction was 5% at 11°C in 3rd week while 49 % (maximum) reduction was observed in 13th week (last week) at 5°C (Figure 3.10 a).

Range of ammonia in influent wastewater for high temperature ABR was 19 to 161mg/L, while in effluent was 22 to 85 mg/L. During start phase ammonia concentration was increased in effluent. Overall reduction noticed was 47 % in 15th week (last week) at 45 ˚C. Minimum reduction was 15% obtained in 3rd week. Gradual increase in reduction of ammonia in effluent was observed as temperature increased from 43°C to 45°C (Figure 3.10 b).

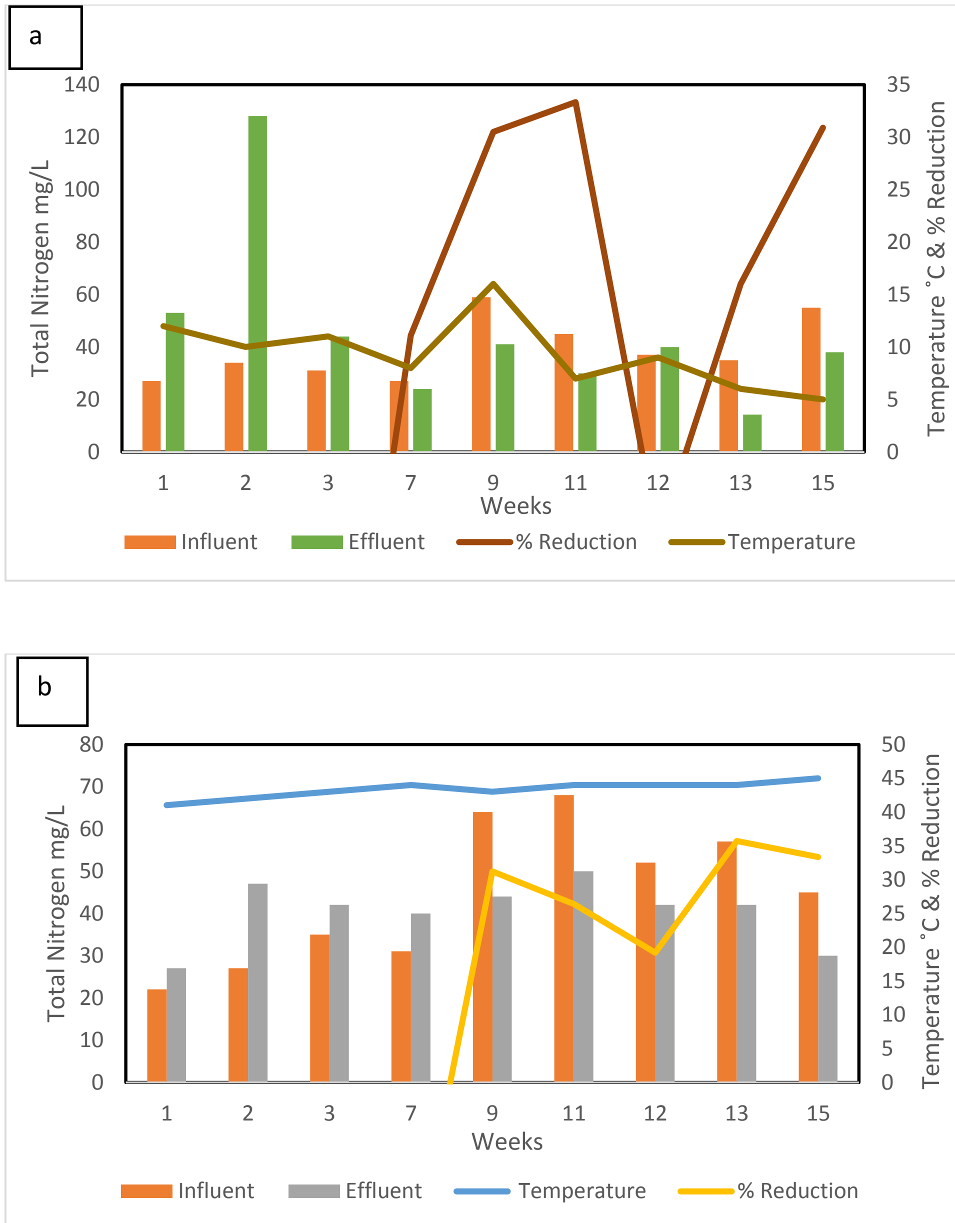


Figure 3.9 Total Nitrogen removal by laboratory scale ABR at low temperature (5-16°C) (a), at high temperature (41-45°C). (b).

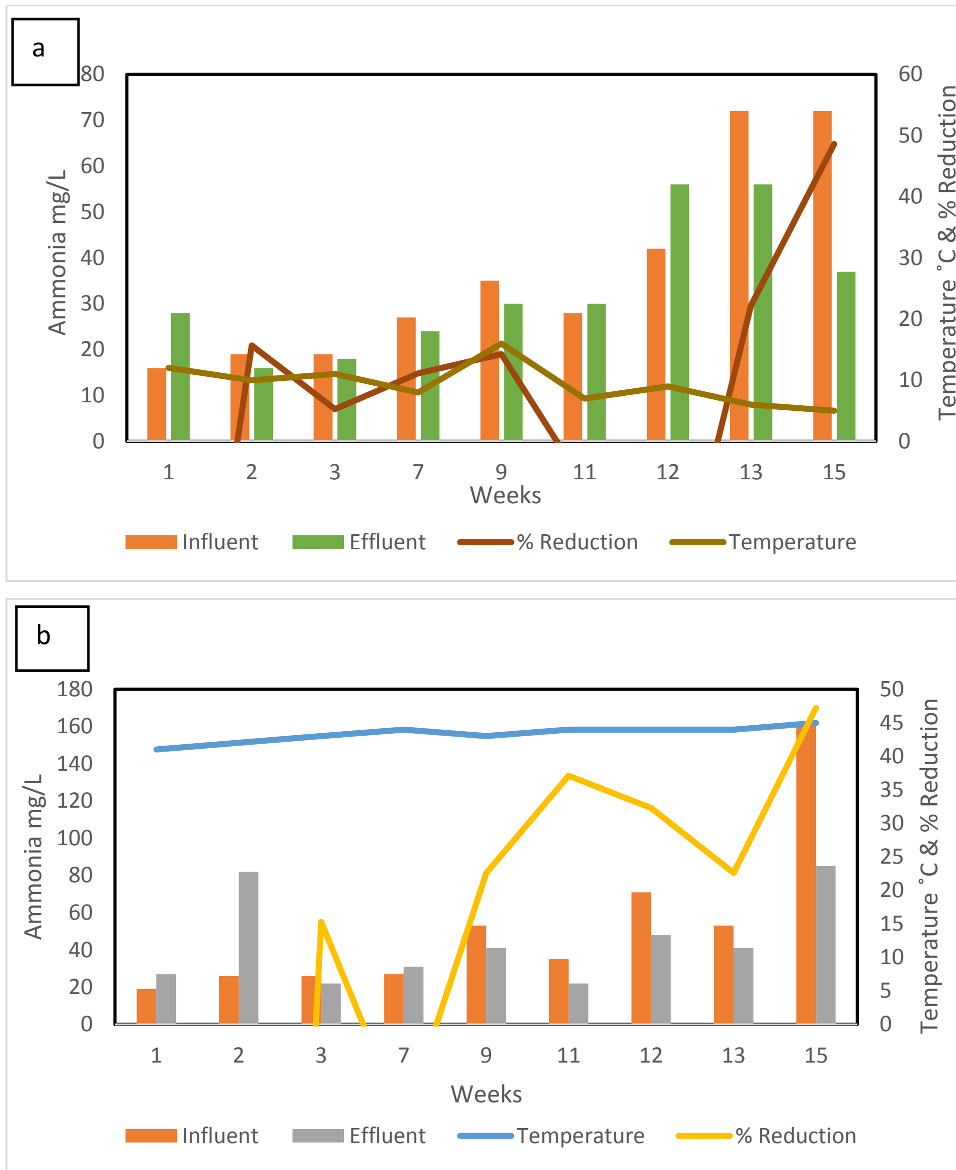


Figure 3.10 Ammonia removal efficiency of laboratory scale ABR at low temperature (5-16°C) (a) at high temperature (41-45°C) (b).

3.3.9 Sulphates removal

Sulphates in wastewater influent during the study ranged from 15 to 44 mg/L and in effluent ranged between 21 to 40 mg/L. Discharge of sulphates was within limits by national discharge standards, but their reduction was studied to analyze efficiency of ABR to remove sulphates at different temperatures. Sulphates removal efficiency was 27 % in 11th week which was maximum (Figure 3.11 a).

When sulphate concentrations were monitored in reactor operating at high temperature the values for influent were 16 to 48 mg/L. In the start phase sulphate concentration increased in effluent for

1st and 2nd week. In 3rd week 10 % sulphate reduction was noticed at temperature 43°C. Maximum reduction observed was 47% in 13th and 15th week at 44°C and 45°C (Figure 3.11 b).

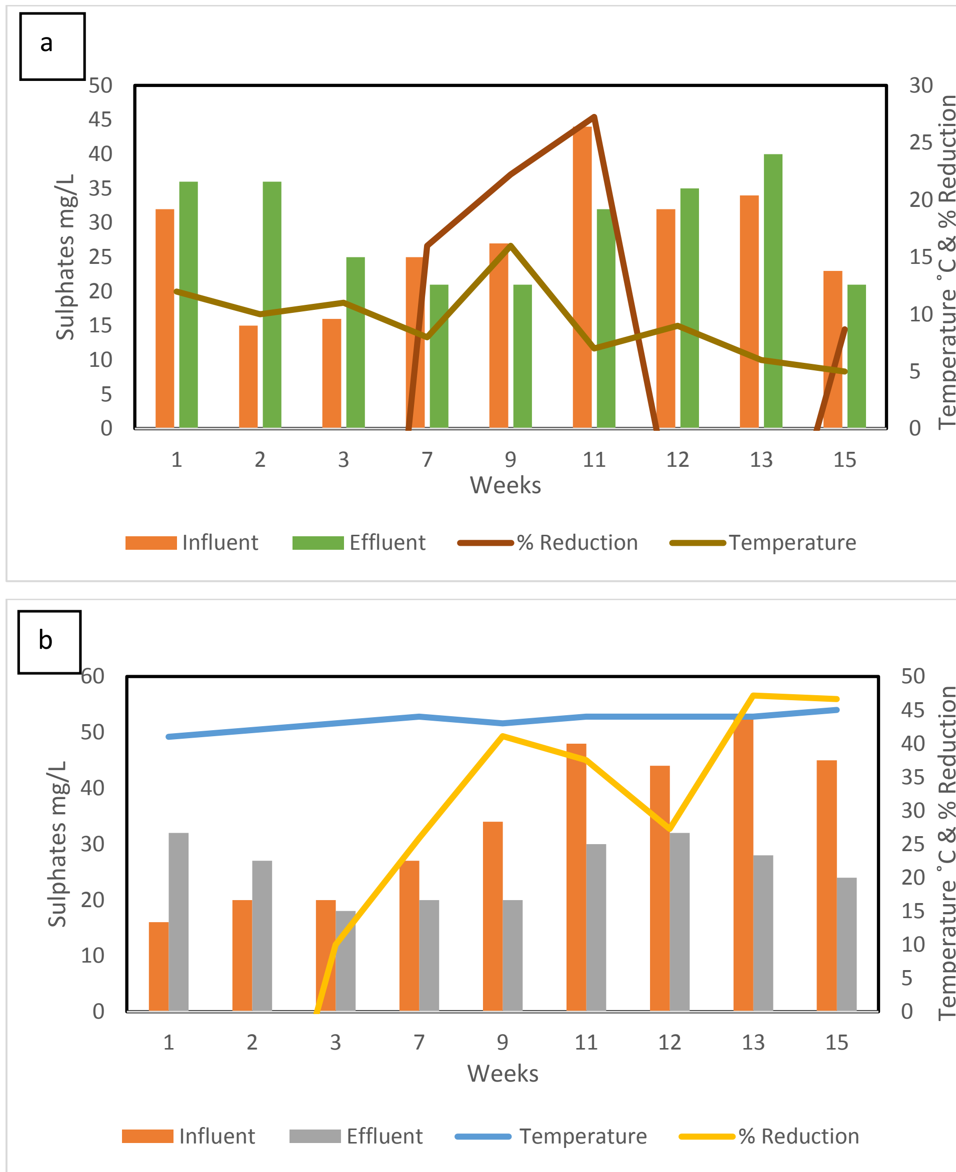
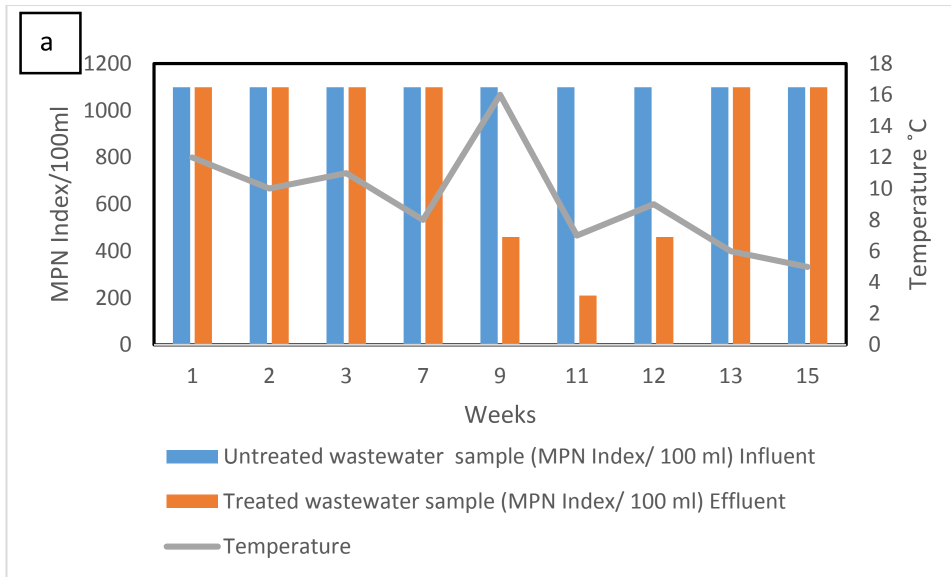


Figure 3.11 Sulphates removal efficiency of laboratory scale ABR at low temperature (5-16°C) (a), at high temperature (41-45°C) (b)

3.3.10 Pathogens reduction analysis

Pathogens reduction in effluent was observed by MPN method for both low temperature ABR and high temperature ABR. MPN index/100ml of influent and effluent was measured. Pathogens reduction was more prominent at high temperature ABR (41-45˚C). Less pathogens removal was

observed at low temperature (5-16˚C) as compared to high temperature. At high temperature, initially for start weeks pathogens concentration was same in influent and effluent. Then in 9th week pathogens number started decreasing in effluent and was reduced from 1100 to 460 and maintained this reduction for three weeks. Then pathogens concentration was further decreased in effluent with passage of time up-to 210. In last three weeks maximum pathogens removal was observed in the effluent, it reduced from 1100 to 150 which a is significant removal achieved. While in low temperature ABR, pathogens reduction was not seen in the start period and in 9th week pathogens reduction in effluent observed was from 1100 to 460 (at 16˚C) and continued to further decrease 210 in 11th week at 7˚C, which was maximum reduction. After that again pathogens concentration started to increase in the effluent and their removal was not so efficient. And in last two weeks no pathogens removal was observed in effluent. These results indicate that pathogens removal at higher temperature is significant and consistent but at low temperature slight removal was observed for limited time.



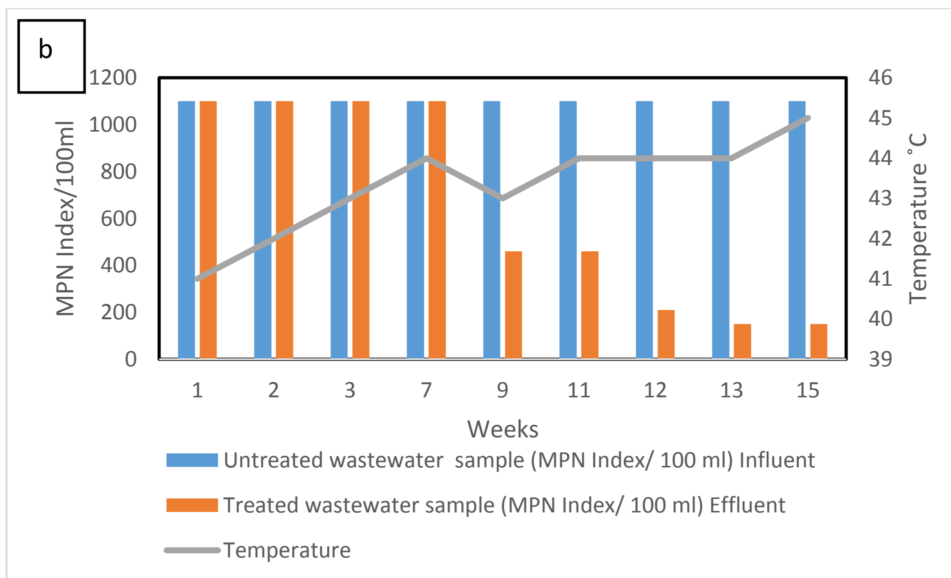


Figure 3.12 Pathogens reduction investigation by MPN method representing (a) at low temperature and (b) at high temperature.

3.3.11 Performance evaluation of Field/Lage scale ABR

Effect of seasonal temperature was evaluated on efficiency of a four chambered field scale anaerobic baffled reactor for the removal of organic matter (COD, SCOD, total nitrogen, sulfates) and pathogens from domestic wastewater during January-December 2017. The reactor was performing at ambient temperature varied throughout the year from 13-34°C (Fig. 3.15 a). The pH values of influent and effluent wastewater were daily examined, and an average of pH was taken in each month. pH range for both influent and effluent wastewater was 6.0-8.0 during whole

process (Fig. 3.15 b). Which is within the recommended range suitable for the process of anaerobic digestion. Intracellular pH of microorganisms is mostly with in pH unit of the neutral. When pH of the outside environment deviates significantly, it imposes stress on microbial cells and affects overall microbial diversity and composition within digester (Fierer et al., 2006).

3.3.12 Chemical oxygen demand (COD) reduction

COD of influent domestic wastewater range 163-371 mg/L while that of effluent from 167-266 mg/L. It was noticed that efficiency of ABR gradually increased with temperature and reached to maximum COD removal 47 % at 34°C. Gradually with decrease in temperature COD removal was decreased. At temperature 14°C COD removal observed was 36 %. Figure 3.13 showed linear relation between COD and temperature. It shows the effect of temperature on microbial flora working inside the ABR.

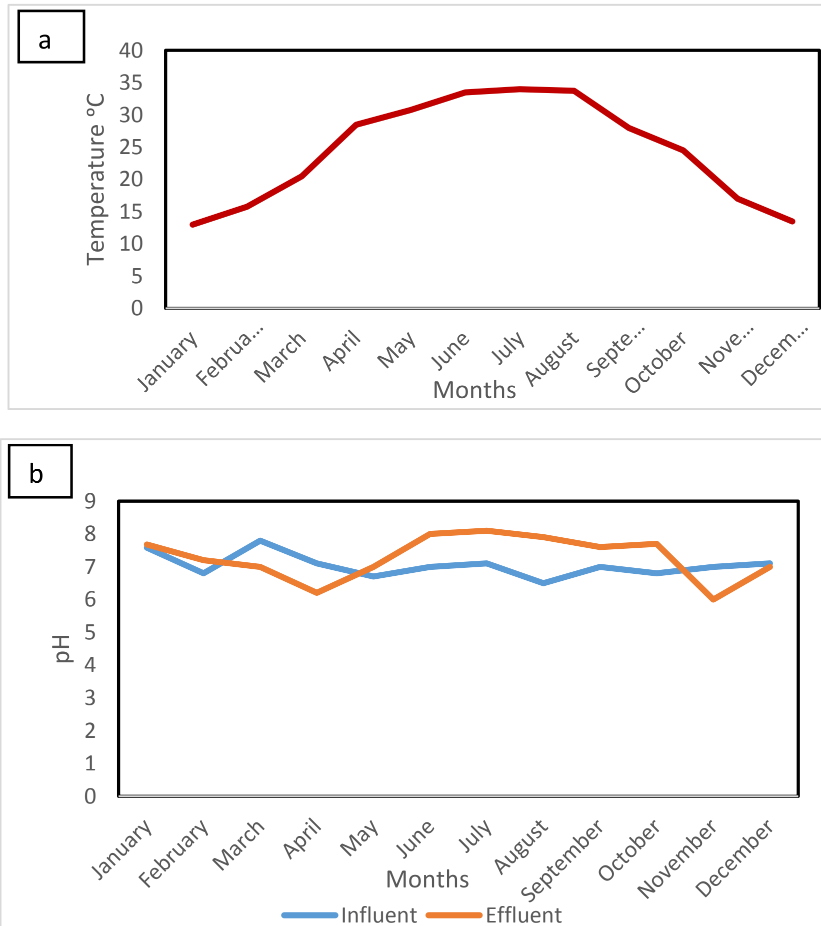


Figure 3.13 Variation in (a) Ambient temperature (b) pH of influent and effluent during operation of Field scale ABR at environmental temperature

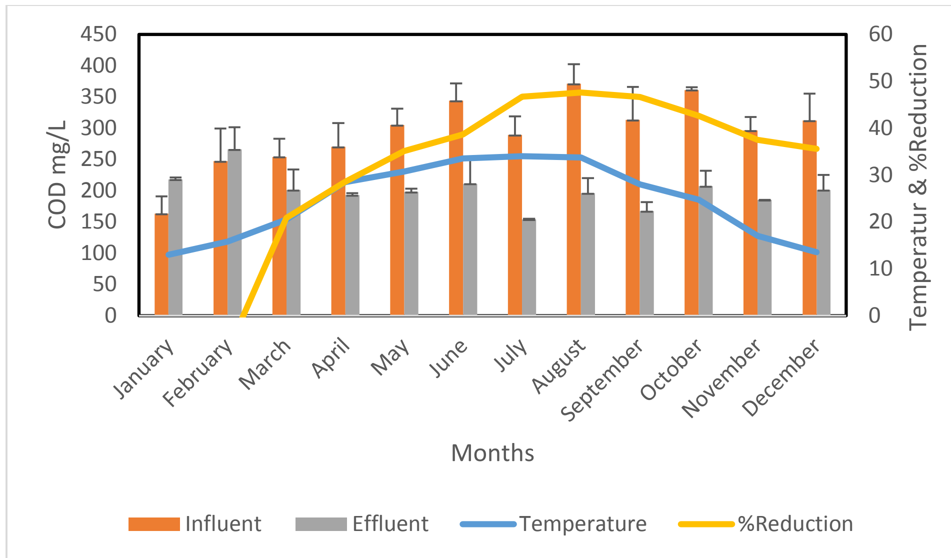


Figure 3.14 COD removal efficiency of large scale ABR at ambient temperature

3.3.13 Soluble COD reduction

During start phase of reactor negative soluble COD (SCOD) removal was observed showing decomposition of complex organic matter. Reduction in SCOD was observed to increase gradually with increase in temperature and reached to maximum 42 % at 34°C for the months of July and August. SCOD removal was also observed at 25°C in October (Figure 3.15).

3.3.14 Sulphates reduction

Concentration of sulfate was also monitored throughout the year from influent and effluent of ABR to analyze the efficiency of ABR for sulfate removal. Sulfates concentration of influent ranged from 18 to 52 mg/L. Maximum removal of sulphates was 44 % achieved in August in 34°C. Increase in removal efficiency was gradual with increase in temperature. It was noticed that efficiency of ABR was increased at temperature ranged from 28-34°C. Sulfate removal at this stage may be attributed to removal by sulfate reducing bacteria and mainly to precipitation with metals in the anoxic conditions (Figure 3.16).

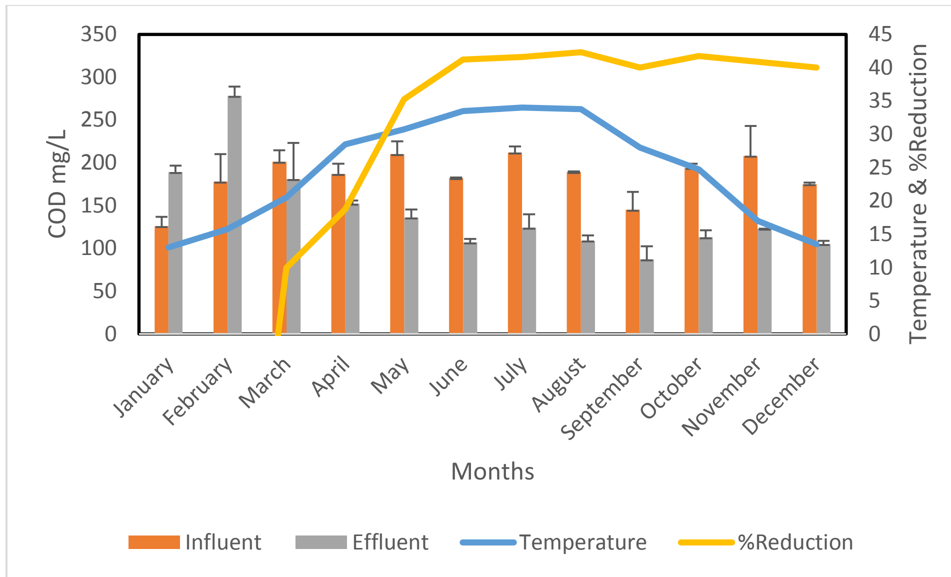


Figure 3.15 Soluble COD removal efficiency of large scale ABR at ambient temperature

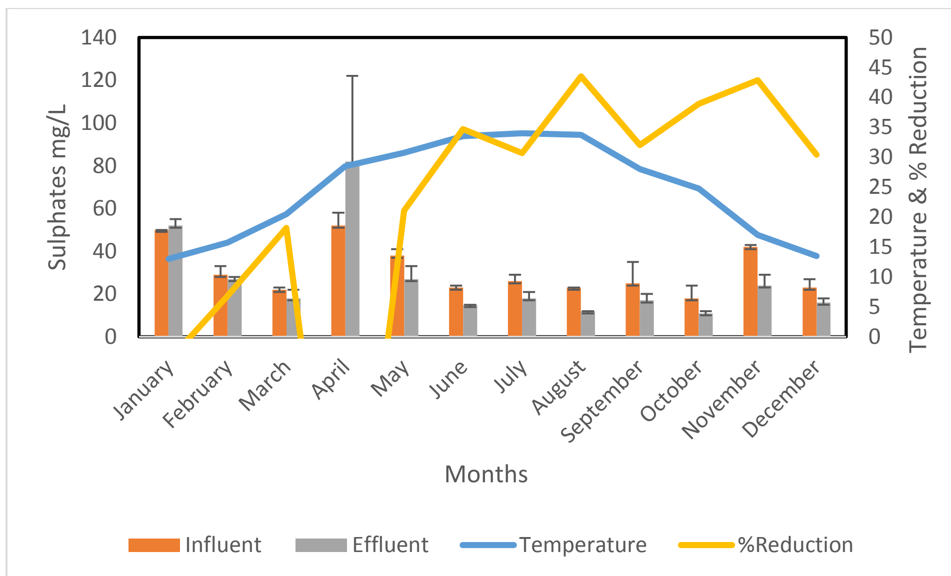


Figure 3.16 Sulphates removal efficiency of large scale ABR at environmental temperature

3.3.15 Total nitrogen reduction

Nitrogen removal occurs by nitrification and denitrification. It was noticed that total nitrogen efficiency of ABR was increased at temperature range of 28-34°C. Influent range of total nitrogen was 13-31 mg/L. Removal efficiency gradually increased with increase in temperature from 28-34°C. Maximum removal obtained was 31% at 28°C. Studies showed that temperature greatly affects nitrogen reduction during anaerobic digestion. High temperature enhances microbial metabolic activities, which on the other hand makes protein hydrolysis strong (Figure 3.17).

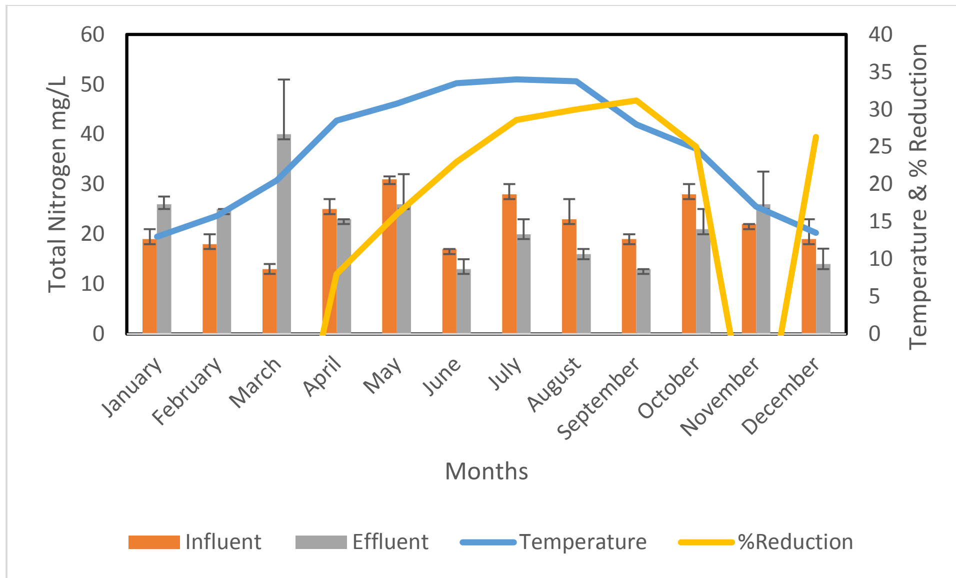


Figure 3.17 Total Nitrogen removal by large scale ABR at environmental temperature

3.3.16 Pathogens reduction

Pathogens reduction from the effluent was analyzed by using most probable number (MPN) technique. Pathogens index present in all samples of influent (untreated) wastewater was more than 1100/100 ml sample. During the start period, in 1st and 2nd month (January, February) pathogens count remained same in the effluent (treated) wastewater. Pathogens number started to reduce in 3rd month (March) at temperature 21˚C, observed reduction was up-to 460/100 ml, this value remained same for effluent of April also when temperature increased up to 29˚C. Pathogens number in effluent gradually reduced up to 93/100 ml in the month of August at 34˚C temperature. After which pathogen number in effluent increased again with decreasing temperature in September at 28˚C. Then in October at 25˚C pathogens count dropped to minimum, 75/100 ml in effluent. In last two months November and December when temperature fall 17-13˚C, pathogens count in effluent increased up to 260/100 ml (Fig 3.18).

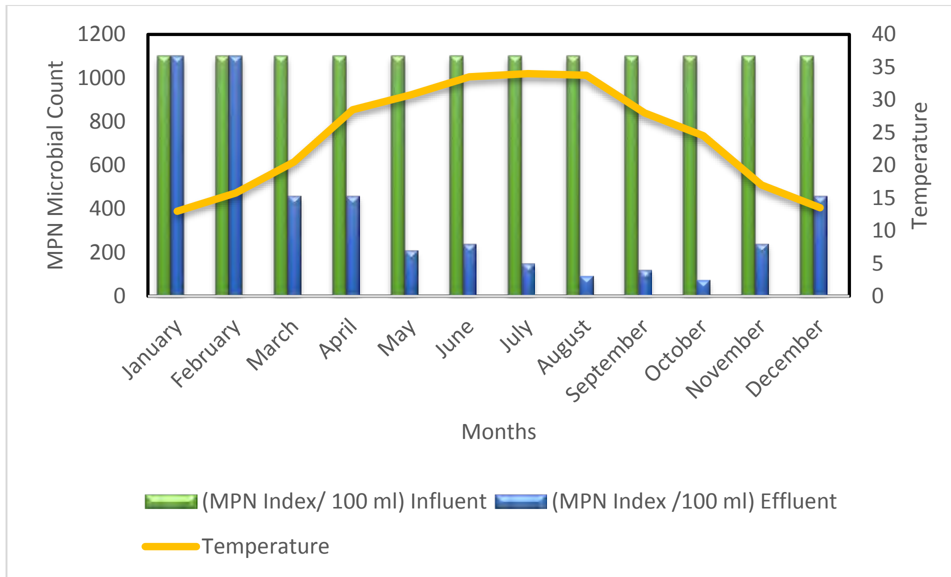


Figure 3.18 Pathogens count/100 ml in influent and effluent of large scale ABR at ambient temperature

3.4 Discussion

3.4.1 Chemical Oxygen Demand

COD removal efficiency of laboratory scale ABR at low temperature shown was up to 64% in 11th week (at 7°C) indicating that reactors microbial flora responsible for carrying out anaerobic digestion was established and adjusted to the provided environment conditions to give their maximum efficiency. Studies showed good organic matter removal efficiencies in anaerobic digestion plants even at low temperature as at 5˚C (Alette et al., 2000). Overall efficiency of the reactor at low temperature was not consistent, and it varied with temperature change which shows shift in microbial community structure of anaerobic digester with temperature shift. Significant COD removal at low temperature may also be attributed to ABRs compartment system as studies conclude that low strength wastewater can be treated efficiently at low temperature in ABR due to its compartmentalized/ (chamber) structure which can enhance hydrolysis of recalcitrant material in 1st phase because of having comparatively lower pH. (Langenhoff et al., 2000).

While in other laboratory scale ABR working at high temperature (41-45°C), COD removal efficiency was 64 %, in 7th week at 44°C. Maximum removal efficiency for both ABRs was same 64%, but steady state was achieved in less period for high temperature ABR than at low temperature ABR. The other difference between efficiency of two ABRs was that for high

temperature ABR, fluctuation in percentage reduction was higher throughout the duration and consistent. COD removal efficiency was quite good in almost all the weeks at high temperature. It means that microflora of digester at high temperature was more efficient and stable and the reactor's stability was higher than at low temperature as digestion process is strongly influenced by microbial activity in reactor which make the reactor stable for efficient degradation (Lauterbock et al., 2014). Studies show that average COD removal efficiency at HRT 18 hours is mostly up to 80% (Mottetan et al., 2013). But in some cases, more than 85% COD removal was achieved using ABR (Yuttachai et al., 2010).

For field scale ABR, in start weeks COD concentration in effluent was increased for all the reactors which may be due to breakdown of suspended particles of organic matter or due to biomass production in the reactor. Efficiency of ABR gradually increased at temperature range 28°C to 34°C and maximum removal observed was 47% at 34°C. Decrease in temperature, efficiency of ABR for COD removal was decreased showing direct effect of temperature on COD removal. A linear relation between COD removal and increase in temperature increase was observed. It shows the reduction of microbial activity with decreasing temperature. Despite significant COD removal, effluent wastewater has high number of COD, which reflects the presence of non-biodegradable organic material present in effluent like humic substances and lignin (Fenghao et al., 2011).

Increase of SCOD in effluent of low temperature ABR was observed during start period for two weeks for all ABRs which may be due to degradation of organic matter into soluble organic matter and then reduction in SCOD in effluent, maximum up to 46% was noticed. For high temperature ABR's SCOD reduction was 66% in 7th week at 44°C. For large scale ABR's SCOD reduction was 42 %. SCOD reduction observed may be affected by SRT and conversion of COD into SCOD. Longer SRT allowed hydrolysis of accumulated VSS resulting into extra SCOD generation, which could not be eliminated completely. Feng et al, in 2008 reported that carrier anaerobic baffled reactor treating sewage have SCOD removal efficiency affected by microbial biomass washout.

3.4.2 Nitrogen removal

Nitrogen comes from degradation of proteinaceous material during anaerobic digestion and prevails in two forms in anaerobic digesters as total nitrogen and ammonia. Nitrogen is present in reduced oxidation state in anaerobic digesters. Most of organic nitrogen is converted into

ammonium-nitrogen in anaerobic digesters because it is easier for biological nitrogen removal (Fenghao et al., 2011).

Ammonia concentration increased in effluent in start period for both lab scale ABRs. Increase of ammonia in start period in effluent shows increase in degradation of organic matter as studies showed that higher release of ammonium in effluent indicate improved degradation under thermophilic conditions (Ge et al., 2011). In present study ammonia increased in effluent even at low temperature ABR, depicting good degradation at low temperature. A study was conducted in the temperature range of 40-55ºC and reported that ammonium concentration tends to increase during the anaerobic digestion (Kim et al., 2006). For low temperature ABR, overall ammonia removal efficiency was 49% in 15th week. For high temperature ABR ammonia reduction noticed was 47% in 13th week. Protein acidification process produces high ammonium and concentration of ammonium in the effluent water increases at high temperature that is dependable on variable degradation efficiency of protein (Yua et al., 2003).

Nitrogen removal was not much prominent in both ABRs (at low temperature 33% and at high temperature 36%) under anaerobic conditions as the nitrification is an aerobic process and hydrolysis increases the soluble form of nitrogen (Hahn et al, 2015). It was noticed that efficiency of ABR was increased at temperature range of 28 to 34°C for total nitrogen reduction. Studies showed that temperature greatly affects nitrogen reduction during anaerobic digestion. High temperature enhances microbial metabolic activities, which makes protein hydrolysis strong. Nitrogen removal by microorganisms progress slowly due to slow growth of nitrogen eliminator microorganisms in the anaerobic digestion (Khin et al., 2004).

3.4.3 Sulphates removal

Sulphur is present in reduced oxidation state in anaerobic digesters (Fenghao et al., 2011). Enhanced sulphates removal is noticed at increased temperature as in high temperature lab scale ABR as compared to low temperature ABR. Increase in removal efficiency was gradual with increase in temperature. Sulphates reduction in effluent at high temperature increases due to enhanced growth of SRBs (sulphate reducing bacteria). Sulphates presence in anaerobic digestion led to SRBs growth (Barrera et al., 2013). Temperature has great impact on function of SRBs in anaerobic digestion. SRBs are dominant at high temperature as at 37˚C (Colleran and Pender 2002). Sulfates removal may also be linked to precipitation with metals in the anoxic conditions which is also influenced by temperature.

3.5 CONCLUSIONS

- ABR as a primary treatment unit removes significant organic material and pathogens from wastewater.
- Efficiency of ABR fluctuates with seasonal variations and comparatively better performance observed during summer season.
- ABR better acclimatize with seasonal temperature variation. It could be scaled up and installed preferably in warmer areas.
- Efficiency of ABR was increased with increasing temperature and reactor was more stable at high temperature.
- ABR as a primary treatment unit removes significant organic material and pathogens from domestic wastewater at each temperature range but prominent at higher temperature.
- Removal of sulphates and total nitrogen was lower which may be due to overall low concentration of these in the influent.
- ABR at temperature 41-45˚C is comparatively more stable than at 5-16˚C temperature.
- ABR could be used in cost effective and sustainable way for onsite primary treatment of domestic wastewater.

CHAPTER 4

INVESTIGATION OF MICROBIAL DYNAMICS AND INTERPLAY WITHIN FOUR CHAMBERED ANAEROBIC BAFFLED REACTORS AT DIFFERENT TEMPERATURE CONDITIONS

4.1 INTRODUCTION

Anaerobic digestion (AD) is natural, multistage and complex process which involves conversion of complex organic matter to simple units by multiple steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) the action of microbial consortium. A known fact is that microbial consortia catalyzing anaerobic digestion consists of multiple type of microorganisms and composition and activities of these microorganisms can vary over the time owing to substrate type fed to the anaerobic system (Angelidaki et al., 2009). Objective of the present part of the study is to characterize microbial (bacterial & archaeal) diversity and organization at dissimilar temperature conditions in anaerobic baffled reactor treating domestic wastewater. Dominant bacterial population in different chambers of ABR with different time points was evaluated and compared the similarity and uniqueness in composition of microbial community. In addition, variation in microbial population and community composition of three ABRs working at different temperature was assessed. Culturable microbial investigation method was used only for samples taken from two laboratory scale ABRs. While molecular investigation approach Sanger and Illumina is used for microbial analysis of samples collected from field scale ABR and also from two laboratory scale ABRs.

Culture based methods of microbial study are biased as they involve selection of species which does not represent real dominant structure of the digester. Molecular technique is required to fulfil the gaps in study and incomplete knowledge about microbial composition in wastewater treatment plants (He et al., 2011). Microbial community of AD is little known about their composition, dynamics and their function in digester and their response to varying conditions, that’s why anaerobic digester remains as black box. Knowledge about key players of AD and their role is mandatory for improved understanding and role of different microbial groups performing in AD. To investigate about microbial community of digester is challenging because of knowledge gap about dozens of species and their complex interplay within digester (Campanaro et al., 2016; Anja et al., 2018).

4.2 MATERIALS AND METHODS

4.2.1 Microbial diversity analyses of ABR using culturable techniques

Microbial population of ABRs was examined using culturable technique (using growth and isolation media, colony morphology, microscopy, and biochemical characterization) and unculturable techniques (molecular microbial analysis using Sanger sequencing and Illumina technique). Culture dependent technique was used only for microbial identification in laboratory scale ABRs while molecular microbial technique was used for all the three ABRs (laboratory scale and for field scale ABRs).

4.2.2 Samples collection and preparation

Wastewater samples for culturable bacterial and archaeal analysis were collected from three chambers of each laboratory scale ABR, for isolation of hydrolytic microorganism sampled from 1st chamber, for isolation of acetogens sampled from 3rd chamber and for methanogens isolation sampled from 4th chamber were collected. Characterization and identification of microbial strains was performed by morphological identification, microscopy and biochemical characterization and was performed according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Chemicals used in this study were supplied by different companies and culture media was prepared using manufacturer's instructions. Media before use were sterilized in autoclave at 15 lbs. per square inch pressure, for 15 min. at 121˚C. Chemicals were provided by following companies: E., Merck, Sigma Chemicals Co., DIFICO Laboratories, BDH Laboratory Chemical Division, Oxoid Chemicals Company UK, St. Louis and Fluka Granite CH-947 Bushes.

4.2.3 Morphological description

Morphological identification of cultured colonies was done by examining cultural characteristics of the microorganisms based on that each of microbe possess its unique cultural characteristics. Colonies then characterized based on their size (large, small, pinpoint, moderate), margin (lobate, serrate, entire, filamentous, curled), pigmentation (as colony color), opacity (opaque, translucent) and elevation (convex, flat, pulvinate, raised).

Microscopic characterization of microorganisms was performed by Gram staining and microscopy. A thin smear prepared with wire loop by taking bacterial isolates was heat dried and fixed on slides. Gram's Crystal violet dye applied on smear and was rinsed after one minute. Smear was then submerged with Gram's iodine, allowed it to stand 1minute and then washed. After that rapid decolorization was performed with C_2H_3OH (95%) and rinsed decolorized slides using tap water. In next step smear was flooded with a secondary dye Safranin, allowed it to stand 45 seconds before washing again. Slides were air dried and under the microscope objective (100x) using emulsion oil. Gram negative bacteria appeared pink and Gram-positive bacteria appeared purple under microscope.

Biochemical Characterization

Identification of isolated strains by biochemical characterization was performed as mentioned in 9th Edition of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) by doing following tests: Catalase test, Citrate utilization test, Triple sugar iron test (for lactose/ glucose fermentation), Starch Hydrolysis test, Lipid Hydrolysis test and Protein Hydrolysis test.

Nutrient Agar medium was used for cultivation and isolation of hydrolytic bacteria. 50µl wastewater sample was inoculated on each agar plate using spread plate method under sterilized conditions. 24 hours incubation time was provided at 37°C.

Medium used for cultivation and isolation of acetogenic bacteria was 'basal medium'. Wastewater sample (50µl) was inoculated on each agar plate using spread plate method under sterilized conditions. 48 hours incubation was provided under anaerobic conditions as acetogens belong to anaerobes. Microscopic examination and biochemical characterization method used was same as previous for hydrolytic bacteria.

Composition of basal media per liter of distilled water is:

Ammonium Chloride…………1.0 g   Magnesium Chloride……………0.1g

Cysteine HCl…………………0.5 g   Sodium Sulphide……………….0.5 g

Sodium Bicarbonate…………7.0 g   Calcium Carbonate…………….10.0 g

Yeast Extract…………………2.0 g Vitamin Solution ………………10.0 ml

Mineral Solution………… 10.0 ml Agar…………………………….20.0 g

pH…………………………….6.7 Potassium di-hydrogen Phosphate…0.4 g

Di-potassium Hydrogen Phosphate…………0.4 g

Cultivation of methanogenic bacteria

Enriched medium was used for isolation of methanogens. Cultivation, incubation, microscopic examination and biochemical characterization was done using same method as mentioned above for acetogens. Composition of enriched medium per liter distilled water is as follows:

Sodium Benzoate…………...0.2 g Ammonium Chloride……….0.075g

Di-potassium Hydrogen Phosphate……0.04g Magnesium Chloride……….0.01g

Resazurin……………………0.0001g Sodium Carbonate…………...0.15g

Sodium Sulpfide……………...0.025 g Agar…………………………20.0 g

4.2.4 Microbial diversity analyses of ABR using unculturable techniques

4.2.5 Samples collection for Sanger sequencing

Microbiological analysis by Sanger sequencing method was performed for wastewater collected from 1st and 4th chamber of three ABRs (two laboratory scale ABRs and one field scale ABR). Samples were collected for final month from both laboratory scale ABRs. While in case of field scale ABR samples were collected in alternate months (6 months) throughout the year from 1st and 4th chamber.

4.2.6 DNA extraction

DNA extraction was performed using DNeasy PowerSoil Kit (by QIAGEN Germany) according to manufacturer instructions. Procedure briefly described as 15 ml of wastewater sample was centrifuged at 13000 rpm to form pellet. 0.25 g of pellet sample was added to power bead tube provided. Sample was vortexed gently for mixing. 60 µL of solution C1 was added in it and inverted several times. Power bead tubes were secured horizontally using a vortex adaptor tube holder and vortexed for 10 minutes at maximum speed. Tubes were centrifuged at 10,000 x g for 30 seconds.

Supernatant was transferred to a clean 2 ml collection tube and 250 µL of solution C2 was added, vortexed for 5 seconds and incubated at 4°C for 5 minutes. After those tubes were centrifuged for 1 minute at 10,000 x g. avoiding pellet, up to 600 µL of supernatant was transferred to a clean 2 ml collection tube and 200 µL of solution C3 was added, vortexed briefly and incubated at 4°C for 5 minutes. Again, the tubes were centrifuged for 1 minute at 10,000 x g. avoiding the pellet; up to 750 µl of supernatant was transferred to the clean 2 ml collection tube. C4 solution was shaken and 1200 µL of supernatant added, then vortexed for 5 seconds. 675µL of this was loaded onto an MB Spin Column and centrifuged at 10,000 x g for 1 minute and discarded flow through. This step was repeated twice until all of sample was processed. After that 500 µL of solution C5 was added and centrifuged for 30 seconds at 10,000 x g and flow through was discarded. Sample was centrifuged again for 1 minute at 10,000 x g. The MB Spin Column was carefully placed into a clean 2 ml collection tube. Splashing any solution C5 onto the column was avoided. 100 µL of solution C6 was added to the center of the filter membrane. Centrifuged at room temperature for 30 seconds at 10,000 x g. and the MB Spin Column was discarded. DNA was collected for further downstream process.

Spectral measurement was done to estimate DNA concentration (nanodrop technique). Extracted DNA was quantified by Nanodrop^{TM} 1000 spectrophotometer (Thermo Fisher scientific).

4.2.7 Polymerase chain reaction (PCR)

Standard PCRs used for the amplification of DNA sequences of interest. To perform PCRs forward (corresponds to sequence in sense strand) and reverse (corresponds to sequence in anti-sense strand) primers were designed. Universal bacterial primer pairs were used to amplify V3 (341F+534R), V4 (515FM+806RM) and V5 (515FM+926) regions. V5 did not showed amplification results, (due to longer sequence) while V4 & V3 primer pairs produced amplification results. V3 primer pairs showed similar results as for V4 but with lesser quality of product. V4 primer pair was used for further amplification after conditions optimization for all samples as it produced satisfactory results. PCR make master mix (20 µl) contained DNA 5 µl (50-100 ng total), V4 primer pair (F+R) 1µl + 1µl, PCR mix 10 µl, nuclease free water 3 µl. Bio-Rad thermal cycler was used to perform PCR with thermocycling conditions

programmed as 95°C initial denaturing for 3 minutes, 95°C for 3 seconds, annealing temperature 50°C, extension at 72°C for 1 minutes and final extension at 72 for 5 minutes, 25x cycles and final storage at 4°C.

Quantitative polymerase chain reaction (QPCR)

Quantitative PCR (QPCR) was performed for relative quantification analysis of pathogens and methanogens from chamber 1 to chamber 4 (chamber 1, 2, 3, 4) for large scale/field scale ABR. QPCR was performed with same procedure as mentioned previously, except variation in number of cycles. All the reactions have equal amount of input nucleic acid and every sample under analysis amplify with similar efficiency up to specific number of cycles. DNA amplification was monitored at each of these cycles of PCR 20 cycles, 25 cycles and 30 cycles. After completion of reaction all PCR products were run on gel electrophoresis and observed their image under G-Box to visualize DNA band.

Gradient polymerase chain reaction

Gradient PCR was performed for determination of optimal annealing temperature. It was performed for pathogens survey in samples of different chambers. 12 reactions were run in a single lane with same composition of master mix and different annealing temperature using single primer pairs. Lowest and highest temperature range chosen was appropriate for the primer used. After completion of reaction all PCR products were run on gel electrophoresis and observed their image under G-Box to visualize DNA band.

High fidelity polymerase chain reaction

Detection of viral nucleic acid was performed by using conventional PCR (using Taq. Polymerase) and high-fidelity PCR (using DNA polymerase). Detection analysis was performed for ABCDE serotypes of human adenovirus in wastewater samples. Annealing temperature was varied. On completion of reaction, agarose gel was run for PCR product and visualized DNA band under G-Box.

4.2.8 Agarose gel electrophoresis

PCR amplicon (DNA fragments) was subjected to agarose gel electrophoresis to separate DNA molecules on basis of their size. Agarose powder of Sigma-Aldrich was dissolved in TAE 1x buffer (50x :2M tris-base, 50 mM EDTA, 0.95 Mm acetic acid, diluted 1:50 in ultra-pure water) to make total concentration of agarose 1% as generally required for separation of the DNA fragments needed for screening and cloning. Mixture of agarose was dissolved using microwave boiling. After complete mixing it was allowed to cool at room temperature and Syber^{tm} safe DNA gel stain was added and swirled to mix. This mixture then poured into a tray known as gel tray having a comb used to generate sample wells in the gel. Gel allowed to cool and then shifted to the electrophoresis tank. The gel was fully immersed in the solution 1 x TAE. Loading dye was already present in PCR mix. Samples were put into wells with DNA ladder running parallel. Gel was then run for a time as required (up to 1 hour) at temperature 90°C. Resolved DNA molecules were imaged and visualized in G-box. DNA required to be cloned and process further was excised using black box.

4.2.9 Gel extraction and DNA fragments purification

DNA fragments were excised from the gel and purified using gene JET gel extraction kit (Thermo Scientific). Excised fragments of DNA within gel slice were weighed and incubated with 3 volumes of the binding buffer at temperature 50ºC for 10-15 minutes. After complete solubilization the mixture was vortexed, poured into gene JET purification column and centrifuged at 13000 rpm for 1 minute. Flow through was discarded. The DNA in the column was washed with addition of 700 µl wash buffer to the column and centrifuged for 1 minute at 13000 rpm and the flow through discarded. This step was repeated. Column was centrifuged to remove ethanol residues for 1 minute at 13000 rpm. Column was put into clean Eppendorf and 50 µl elution buffer was added to the membrane and allowed to stand for 1 minute and then DNA was eluted into 50 µl elution buffer by centrifugation at 13000 rpm. Eluted DNA was stored at 0°C.

4.2.10 Cloning

Cloning was performed using TOPO TA (Thymine Adenine) cloning kit by Invitrogen. Gel washed PCR amplicon was cloned in the desired vector.

4.2.11 Transformation

Transformation of chemically competent cells of *E. coli* with a plasmid DNA was performed for plasmid DNA amplification. For transformation process 2 µl gel purified DNA, 1 µl Topo Vector, 1 µl salt solution and 2 µl nuclease free water added in PCR tubes. Mixed gently and incubated for 30 minutes at room temperature. Then tubes containing mixture were placed into ice box for 30 minutes. After that chilled competent cell (already stored at -80˚C) were added 25 µl in each reaction mixture (with chilled micropipette tip) and maintain mixture in ice further for 30 minutes. Then heat shock was given in thermocycler for 3 minutes at temperature 42˚C. 25 µl SOC medium was added in reaction mixture and incubated for 30 minutes at 37˚C in thermocycler. Cells were removed from thermocycler and pour onto Luria Bertani agar plates (already prepared which contain X-gal, IPTG and ampicillin) using spread plate method and incubated for overnight at 37˚C.

After overnight incubation transformed colonies (white colored colonies) were appeared in abundance on plates (some blue colonies also appeared, which were not of interest). White colonies were inoculated into Luria broth (already prepared and autoclaved) containing ampicillin.10 white colonies were separately inoculated with sterile tooth picks in to 2 ml LB media in falcon tubes. Overnight incubation was done in shaker incubator at 37°C. After incubation turbidity in falcon tubes was appeared showing growth.

4.2.12 Plasmid purification and isolation (mini preps)

On following day, 2 ml cultures transferred to clean Eppendorf tubes (twice) and centrifuged at 13000 rpm for 5min. Bacterial cells were pelleted, supernatant was discarded. Plasmid DNA was extracted using pellet by gene JET plasmid mini prep kit (Thermo Scientific). Bacterial pellet was resuspended into 250 µL of resuspension solution and vortexed. Then 250 µl of lysis solution was added and mixed by inversion (6 times) and incubated for 5 minutes at room temperature. Lysis reaction was then neutralized by addition of 350 µl of neutralization solution and inversion was done 6 times for mixing. The mixture was centrifuged for 5 mints at 13000 rpm to pellet chromosomal DNA and cell debris. Supernatant containing plasmid DNA was transferred into spin column for binding of plasmid DNA. Column was

centrifuged at 13000 rpm for 1 minute and discarded the flow through. Column was washed with 500 µl of wash solution, twice. Flow through was discarded and column was centrifuged one more time for removal of remaining precipitates. Column was transferred into clean Eppendorf tubes for elution of plasmid DNA into elution buffer. 50 µl of elution buffer was added to the column and after 2 minutes incubation centrifuged for 2 minutes at 13000 rpm and stored at -20°C.

Culturing and maintenance of bacterial strains

Transformed bacteria involve positive selection screening and were cultivated using antibiotic containing medium as plasmids used contain antibiotic resistant gene(s). 1000 x ampicillin was used (prepared by dissolving ampicillin sodium salt (Sigma-Aldrich) in ultra-pure water for 100 mg/ml conc.).

4.2.13 Sanger sequencing

After cloning process completed, Sanger sequencing was done by Source Bioscience to characterize genome sequence. 10 µl sample was provided for sequencing in tube format. After receiving online results, SILVA data analysis was performed and evaluated at family level. SILVA database provides a broader collection of sequences classified down to the family level, together with sequences from other kingdoms, which are helpful to identify contaminant sequences. Since SILVA NGS generates more sequences reliably classified to the family level. SILVA database is repository for the high-quality sequences clustered to be non-redundant at a 99 % similarity level. Graphs show number of sequences with classification assigned to family level in each database.

4.2.14 Samples collection for next generation sequencing (NGS) technology

Illumina sequencing, a well reputed NGS technology was used to study microbial community structure of three ABRs. For this purpose, amplification of 16S rRNA gene sequence was performed using universal primers (bacterial/archaeal), forward primer 515f and reverse primer 806r and run-on Illumina MiSeq platform. Wastewater samples collected from 1st and 4th chamber of laboratory scale ABR working at low temperature. Wastewater samples collected from 1st chamber, 3rd

chamber and 4th chamber of laboratory scale ABR working at high temperature. These samples were collected in last week of operation for both laboratory scale reactors. The chambers selected were based on analysis of difference in microbial structure of each chamber. Wastewater samples from large scale/field scale ABR were collected during five alternate months (February, April, June, October, and December) from each of four chambers (total 20 samples from field scale ABR) of the whole year. A total of 25 wastewater samples were prepared for Illumina sequencing from three ABRs.

DNA extraction for Illumina sequencing was performed using same method as described for Sanger sequencing method.

4.2.15 Polymerase Chain Reaction and product purification

PCR performed for Illumina sequencing was carrying reaction volume 60 µl, using V4 universal primer, 30 cycles, at annealing temperature 52˚C. Purification of PCR product was performed, as gel wash is not recommended for it. PCR wash was performed using NucleoSpin Gel and PCR clean-up kit. 60 µl was reaction volume of PCR, 40 µl PCR water was added in it to make total volume 100 µl, as recommended by the kit method. Mixed it well and poured into high pure filter tube and centrifuged at 13000 rpm for 1 minute. Flow through was discarded and DNA on column was washed with 500 µl of wash buffer. After 1st wash, 2nd was performed with 200 µl wash buffer and centrifuge again for 1 minute at 13000 rpm. For elution column was placed into clean Eppendorf and DNA eluted in 50 µl dilution buffer. Method for gel electrophoresis was same as described above for Sanger sequencing.

4.2.16 Illumina (MiSeq) sequencing

DNA of 25 samples taken from 3 ABRs (one working at large scale and 2 at small scale) was sent to Source Bioscience Nottingham, United Kingdom for the amplification of gene sequence of 16S rRNA using universal primers of bacteria/archaea, of which primer pair contains forward V4 primer pair on Illumina MiSeq (762) platform. All samples were run in one lane. MiSeq generated large number of sequences. Run setting was 299 bp paired end. 16S metagenomic library kit was prepared using V3 and V4. Data output format was FASTQ Phred + 33

(Illumina 1.9). QIIME2 tool used for analysis of sequences created by Illumina MiSeq. In summary, paired raw sequences were combined and ambiguous bases were removed. As a result, sequences were produced with minimum length 240 and maximum length 300. Duplicate sequences were removed and required sequences were aligned. Source bioscience sequenced raw reads, demultiplexed and quality processed the raw reads using deionizing per DADA2 standard pipeline. OTUs (operational taxonomic units) were created by combining clusters of sequences. Sequences were organized into groups related to different taxonomic level as family, species level etc. Alpha and beta diversity analysis was performed and examined for each fraction size from each sample. Cluster analysis was performed by constructing plots. Plots for these diversities were constructed using QIIME2 analysis.

Bioinformatics analysis was done using QIIME2-2019.4 (Boylen et al., 2019) pipeline to analyze microbial biodiversity in anaerobic baffled reactors in relation to temperature variation and originating from each chamber of reactor. Samples were amplified in V3 and V4 region of bacterial 16S.

4.3 Statistical analysis

For analysis of data produced by Illumina MiSeq, statistical analysis was performed on already clean-up sequence. The pairwise PERMANOVA was applied to test the significance in the samples differences which normally explain the beta-diversity. Rarefaction analysis of samples was performed up-to depth 1000 to analyze microbial community composition. Statistical analysis was specifically conducted for alpha and beta diversity using OUT, Shannon and Faith phylogenetic diversity. The beta diversity group significance was analyzed by using Bray-Curtis-distance, Weighted-Unifrac and Unweighted-Unifrac distance. These were applied for temperature and time in chamber.

Alpha and beta diversity analysis results and group significance showing statistical comparison of alpha diversity (using different alpha diversity metrics) between the requested sample groups (time in chamber, set reactor temperature, and treatment ambient recorded temperature). Beta diversity analysis results showing statistical comparisons of the identified beta-diversity distances among groups (Time in chamber and treatment ambient recorded temperature). Feature table (abundance

table) showing amplicon sequence variants, as well as their abundance in each sample (variance's abundance table). Rarefaction plots or curve used to evaluate sample group sequencing depths in alpha diversity. While taxa bar plots showing taxonomy profile for all the samples.

The received raw reads for the 24 samples were already demultiplexed and cleaned by Source bioscience per DADA2 routine to denoize the samples. The necessary input file (manifest.csv), which is needed to export the raw sequence reads into QIIME2 artifacts was prepared. Metadata file describing the study and sample was also prepared. Below is QIIME2 pipeline procedure applied for this analysis.

Using manifest file, the raw reads pairs were loaded or converted into QIIME2 artifacts. After that data was deionized using DADA2 (Callahan et al., 2016) plugin in QIIME2 to visualize the sampling depth for each sample depending on the feature count for each sample and determining sampling depth based on metadata category of your choice in interactive manner. Assigning taxonomic classification was done for the identified sequence variants. The aim is to assign the respective taxonomic labels associated with the identified sequence variants. Phylogenetic tree (rooted and unrooted) for identified variants was generated. Rarefaction plot was generated using the abundance/feature table and rooted phylogenetic tree. The principal Coordinate Analysis (PCoA) was performed to examine the sample differences or dissimilarity in samples for beta diversity analysis using the Bray-Curtis' distance and this generated emperor plots, which is used to visualize beta diversity. Eventually the alpha and beta diversity analysis were performed. The metrics used for examining alpha diversity are Faith pd, Observed OTUs and Shannon. The Kruskal Wallis test based in these metrics was applied to test the pairwise alpha diversity significance between different groups. The metrics used to examine beta-diversity are Bray-Curtis, Unweighted Unifrac, and Weighted Unifrac.

4.3 RESULTS AND DISCUSSION

4.3.1 Investigation of microorganisms in two laboratory scale ABRs by culture-dependent method

Culture dependent microbial structure of different chambers (1st, 3rd, 4th) of two laboratory ABRs was evaluated and categorized according to Bergey's Manual of Determinative Bacteriology (9th Edition) (Holt et al., 1994) based on morphological characteristics on culture media, microscopy, and biochemical tests. Strains identified from each of the three chambers showed diversity for both laboratory scale ABRs. Detailed description of microorganisms is in Tables 4.1-4.6.

Diversity was seen in three chambers of both laboratory scale ABRs. For ABR working at low temperature, bacteria detected in 1st chamber are *Clostridium*, *Peptococcus*, *Staphylococcus*, *Enterobacteria*, *Micrococcus*, *Desulfobacteria* and Pseudomonas. *Enterobacter* was common bacteria of this chamber as it is detected three times. In 3rd chamber *Syntrophomonas*, *Citrobacter*, *Klebsiella*, *Acetobacteria*, *Proteus*, *Staphylococcus* and *Salmonella*. In 4th chamber *Pseudomonas*, *Enterococcus*, *Clostridium*, *Lactococcus*, *Lactobacillus*, *Methanococcaceae* and *Haemophilus*. No abundant microorganism was detected in 3rd and 4th chamber. Methanogens family *Methanococcacea* was detected in 4th chamber of ABR. All the chambers showed different structure of microbial flora. *Clostridium* and *Pseudomonas* were the only common bacteria in 1st and 4th chamber. All other bacteria are different in three chambers. Methanogens are observed to appear in 4th chamber of the reactor.

For ABR working at high temperature, bacteria in 1st chamber are *Enterobacter*, *Staphylococcus*, *Enterobacter*, *Bacillus*, *Micrococcus*, *Klebsiella* and Lactococcus. In 3rd chamber *Enterobacter*, *Enterobacter*, *Acetobacter*, *Clostridium*, *Vibrio*, *Campylobacter*, *Acidomonas* and microorganisms in 4th chamber are *Klebsiella*, *Listeria*, *Lactobacillus*, *Acidovorax*, *Methylococcus* and *Ethansarcina*. Another microorganism present in chamber 4 of the reactor includes *Methylobacteria*, *Methanosarcina* and *Methylococcus*. *Enterobacter* was common microorganism of 1st and 3rd chamber and dominant of these two chambers. *Methanogens* are detected in 4th chamber of high temperature ABR. *Methanogens* were detected only in 4th chamber of both ABRs. Abundance and diversity of methanogens was higher at high temperature as compared to lower temperature. At low temperature only methanogen detected were *Methanococcus* while at high temperature *Methanococcus* and *Methanosarcina* were detected. Microbial structure of each chamber varied along the

chambers in each ABR and along 1st chamber of ABR, 3rd chamber of both ABRs and 4th chamber of both ABRs.

Within anaerobic reactors various microbial populations showed tendency to aggregate to facilitate degradation of organic matter by multiple steps. Hydrolysis/fermentation is 1st step of this process considered occurring in 1st chamber of ABR. Many fermentative bacteria complete this step of anaerobic digestion, are collectively known as hydrolytic microorganisms. Hydrolytic microorganisms were detected in 1st chamber of both ABRs. *Clostridium* is hydrolytic microorganism able to carry out hydrolysis of protein, starch, lipid and cellulose (Niu et al., 2014) and are fermenters (Walter et al., 2012) forming acidogenesis products format, acetate, butyrate etc. (Li et al., 2011). *Bacillus* also have role in hydrolysis by secreting extracellular enzymes (Konsula et al., 2004). In another study *Bacillus* is reported for its function in efficient removal of COD (Affandi et al., 2014). While *Micrococcus* is identified as pesticide remover/degrader (Kanjilal et al., 2015). Acetogenic microorganisms are involved in further degradation of products of hydrolysis and form different acids in a stage known as acetogenesis. Acetogenesis is anoxic step. Acetogens detected in 3rd chamber of ABR working at low temperature are *Syntrophomonas*. It is commonly found microorganism in biogas plants. Clostridium is also an acetogenic microorganism detected in 3rd chamber of ABR working at high temperature (Ivan et al., 2019). Methanogens detected in 4th chamber of both ABRs are different from each other, which may be due to difference in temperature conditions as methanogens are sensitive to change in environmental conditions. *Methanococcus* detection at both temperature conditions showed its presence in wide range of temperature conditions in biogas digesters. *Methanosarcina* prevails in condition of high concentration of acetate in medium, as it is acetoclastic methanogen (Mahon et al., 2001).

Bacteria which were identified for pathogenic in nature are *Bacillus* (Riedel et al., 1988), *Enterococcus*, *Klebsiella* (Ohki et al., 1994) and *Pseudomonas* (Li & Chu 1991, Ruxana et al., 2005), enterobacteria (Nicola et al., 2009; Roland et al., 2006), *Campylobacter* and *Vibrio* (Asa et al., 2015), *Clostridium* (Thomas et al., 2011) and *Staphylococcus* (Angeles et al., 2005). Pathogens reduction observed along the chambers was prominent in ABR working at high temperature as compared to ABR

working at low temperature. Some microorganisms having pathogenic nature are observed to be involved in organic matter degradation. As *Pseudomonas* and some *Enterococci* are involved in nitrogenous wastes removal (Van Rijn et al., 2006).

Table 4.1 Morphological and biochemical characterization of bacteria in 1st chamber of laboratory scale ABR working at low temperature. A, acid (yellow); K, alkaline, Y, yellow; R, red; NC, no change

Samples	**Grams reaction & Morphology**	**Citrate test**	**Catalase test**	**TSI test**	**H_2S test**	**Lipid hydrolysis test**	**Protein hydrolysis test**	**Carbohydrate hydrolysis test**	**Identified bacteria**
1	– , Cocci		_	A/NC	+	+	_	_	*Clostridium*
2	+ , Rod	_	+	_	+	+	+	_	*Peptococcus*
3	+, Cocci	+	+	K/A	+	+	+	+	*Staphylococcus*
4	+, Cocci	+	+	R/Y	+	+	_	+	*Enterobacter*
5	– , Rod	+	+	A/AG	+	+	_	+	*Micrococcus*
6	– , Cocci	+	_	Y/Y	+	_	+	_	*Enterobacter*
7	– , Rod	+	+	K	_	+	+	+	*Desulfobacter*
8	+ , Cocci	+	+	A/A	+	+	+	+	*Pseudomonas*

Table 4.2 Morphological and Biochemical characterization of Bacteria in 3rd chamber of laboratory scale ABR working at low temperature.

Samples	Grams reaction & Morphology	Citrate test	Catalase test	TSI test	H_2S test	Lipid hydrolysis test	Protein hydrolysis test	Carbohydrate hydrolysis test	Identified bacteria
1	–, Rod	_	+	K/NC	+	+	_	+	*Syntrophomonas*
2	+, Rod	+	_	AG	_	_	+	+	*Citrobacter*
3	–, Cocci	_	+	K/A	+	+	-	_	*Klebsiella*
4	–, Cocci	+	+	A/A	-	_	+	+	*Acetobacter*
5	–, Rod	_	+	A/A	_	+	+	+	*Proteus*
6	–, Cocci	+	+	A/A	+	+	+	+	*Salmonella*
7	+, Rod	+	+	A/A	+	+	+	+	*Staphylococcus*

Table 4.3 Morphological and Biochemical characterization of Bacteria in 4th chamber of laboratory scale ABR working at low temperature.

Samples	Grams reaction & Morphology	Citrate test	Catalase test	TSI test	H_2S test	Lipid hydrolysis test	Protein hydrolysis test	Carbohydrate hydrolysis test	Identified bacteria
1	–, Rod	_	+	A/A	_	_	+	+	*Pseudomonas*
2	–, Cocci	+	+	AG	+	+	_	+	*Enterococcus*
3	+, Rod	+	+	A/A	+	+	+	-	*Clostridium*
4	–, Cocci	+	+	A/NC	-	+	-	+	*Lactococcus*
5	–, Rod	+	-	AG	-	+	+	+	*Lactobacillus*
6	+, Cocci	+	+	A	+	-	_	+	*Methanococcus*
7	–, Cocci	+	+	K/A	+	+	+	-	*Haemophilus*
8	+, Cocci	+	+	A/A	-	+	+	-	*Corynebacterium*

Table 4.4 Morphological and Biochemical characterization of Bacteria in 1st chamber of laboratory scale ABR working at high temperature.

Samples	Grams reaction & Morphology	Citrate test	Catalase test	TSI test	H_2S test	Lipid hydrolysis test	Protein hydrolysis test	Carbohydrate hydrolysis test	Identified bacteria
1	+, Cocci	-	+	Y/Y	+	+	+	+	*Enterobacter*
2	–, Cocci	+	+	R/Y	+	+	+	-	*Staphylococcus*
3	+, Rod	+	-	A/AG	-	+	-	+	*Enterobacter*
4	+, Rod	+	+	-	+	+	-	+	*Bacillus*
5	–, Rod	+	+	K/A	+	+	+	+	*Enterococcus*
6	–-, Cocci	-	+	AC/NC	-	+	+	-	*Micrococcus*
7	+, Rod	+	-	-	+	+	-	+	*Klebsiella*
8	+, Cocci	-	+	A/A	-	-	+	+	*Lactococcus*

Table 4.5 Morphological and Biochemical characterization of Bacteria in 3rd chamber of laboratory scale ABR working at high temperature.

Samples	Grams reaction & Morphology	Citrate test	Catalase test	TSI test	H_2S test	Lipid hydrolysis test	Protein hydrolysis test	Carbohydrate hydrolysis test	Identified bacteria
1	–, Cocci	+	+	K/NC	–	+	–	+	*Enterobacter*
2	+, Cocci	+	+	A/A	–	+	–	+	*Enterococcus*
3	+, Rod	+	+	K/A	–	+	–	+	*Acetobacter*
4	+, Cocci	+	–	A/A	+	+	+	–	*Clostridium*
5	–, Rod	+	+	A	+	+	+	+	*Lactobacillus*
6	+, Cocci	+	–	A/A	–	–	–	+	*Vibrio*
7	–, Cocci	+	+	A/A	–	+	+	–	*Campylobacter*
8	+, Rod	+	+	AG	–	+	+	+	*Acidomonas*

Table 4.6 Morphological and Biochemical characterization of Bacteria in 4th chamber of laboratory scale ABR working at high temperature.

Samples	Grams reaction & Morphology	Citrate test	Catalase test	TSI test	H_2S test	Lipid hydrolysis test	Protein hydrolysis test	Carbohydrate hydrolysis test	Identified bacteria
1	+, Rod	+	+	A/A	+	+	+	–	*Klebsiella*
2	–, Cocci	+	+	A/NC	–	+	–	+	*Listeria*
3	+, Cocci	+	–	K/A	+	+	+	+	*Lactobacillus*
4	–, Cocci	+	+	K	–	+	+	–	*Acidovorax*
5	–, Rod	+	–	AG	–	+	+	+	*Methylococcus*
6	+, Cocci	+	+	A	+	–	–	+	*Methanosarcina*

4.3.2 Evaluation of microbial consortia in three ABRs using Sanger sequencing technique

Sanger sequencing technique is applied to study community structure of 1st and 4th chamber and microbial shift with temperature in both chambers of field scale and laboratory scale ABRs. SILVA database was used as reference database in the present study for bacterial community analysis of all the samples from chamber 1 and 4 of three anaerobic baffled reactors (large scale ABR and laboratory scale ABR). As SILVA database produced higher percentage of sequences with higher resolution of bacterial community under study as when compared to Green Genes database. SILVA database produced several sequences classified down to the family level. Some sequences were less classified up-to family level. Microbiology for chamber 1 and 4 was evaluated on basis of family level classification. Most of the families were present in sharing for both the chambers. Ribosomal database project (RDP) was the most resistant to identify threshold changes and had highest number of classified sequences independent of the taxonomic rank.

4.3.2.1 Relative microbial populations of large scale ABR

Microbial community structure of 1st chamber and 4th chamber of ABR was analyzed in six alternate months of the year, by Sanger sequencing method. Relative population along 1st and 4th chamber was evaluated.

Microbial structure along 1st chamber of ABR

Community structure of 1st chamber along the months varied significantly and different bacteria were observed to appear in each month. Only few families were common/shared in some months along the 1st chamber, as *Sulfurovacea* was common family present in February and June (but abundant in June), *Burkholderiaceae* was common family in April and August (abundant in August), *Xanthobacteraceae* was common in April and October (comparatively more in October), *Xanthomonadaceae* was common family in June and August (comparatively more in June), *Paludibacteraceae* was common in August and December (similar number). Variation in each month observed was, only two families *Sulfurovaceae* and *Holophagaceae*, were detected in 1st chamber of ABR during the month of February, with *Sulfurovaceae* as dominant family (Fig. 4.1). *Holophagaceae* (belongs to phylum acidobacteria) belong to strict anaerobes, which indicate the existence of anaerobic environment in the digester during February. *Holophagaceae* detected in 1st chamber

during February indicates that anaerobic conditions were developed soon in the ABR. Establishment of anaerobic environment in the ABR was positive indicator for the process of anaerobic digestion.

Both families (*Sulfurovaceae* and *Holophagaceae*) disappeared in sample of 1st chamber during the month of April and a completely new structure of families appeared. *Omnitrophicaeota*, *Kiritimatiellae*, *Burkholderiaceae*, *Syntrophorhabdaceae*, *Xanthobacteraceae* and *Hydrogenophillaceae*. *Syntrophorhabdaceae* was comparatively higher in number than other families. Detection of *kiritimatiellae* in 1st chamber in April also prevails in anaerobic environment. Wastewater treatment plants having dominant population of *Syntrophorhabdacea* in initial stages are observed to have improved startup with better performance of digesters.

In next sample for the month of June 1st chamber, reduction in bacterial diversity was observed and only two families detected were *Sulfurospirillaceae* and *Xanthomonadaceae*. *Xanthomonadaceae* was dominant family (Fig. 4.1). In 1st chamber of August good diversity in microbial community structure was observed, two families detected in this month *Burkholderiaceae* and *Xanthomonadacea* were shared with previous months and some newly appeared families were *Thermoanaerobaculaceae*, Bacteriodales, *Weeksellaceae* and *Paludibacteriaceae*. *Burkholderiaceae*, common degraders were dominant family in August, while in the present study it was also detected at low temperature at 14˚C temperature. *Paludibacteraceae* was detected in high temperature (almost mesophilic) ABR, but not in low temperature.

In 1st chamber for the month of October again bacterial diversity reduced and only two families were appeared *Xanthobactertaceae* (appeared in 1st chamber of April also) and *Pseudomonadaceae* (comparatively more in count). In December comparatively more diversity was observed and *Paludibacteriaceae*, *Veillonellaceae*, *Prevotellaceae*, *Tannerellaceae*, *Rhodocyclaceae*, *Carnobacteriaceae* and *Moraxaellaceae* were detected. *Rhodocyclaceae* was leading family in 1st chamber of December (Fig. 4.1). Dominant/leading microbial families present along 1st chamber of large ABR during different months were, *Sulfurovaceae* in February, *Syntrophorhabdaceae* in April, *Sulfurovaceae* in June, *Burkholderiaceae* in August,

Xanthobacteraceae and *Pseudomonadaceae* in October and *Rhodocyclaceae* in December. Members of family *Veilonellaceae* (belong to Firmicutes phylum) are mostly carryout carbohydrates fermentation and nitrate reduction in anaerobic digesters. *Rhodocyclaceae* belongs to betaproteobacteria and is commonly present in wastewater treatment plants. *Carnobacteraceae* is shown to be affected significantly by operational parameters. *Moraxellaceae* detected in December at 14˚C, are psychrobacters and prevail at low temperature and ferment sugars. No archaeal family was detected along 1st chamber along the months. Hydrolytic microorganisms are detected along 1st chamber, as in 1st chamber the process of hydrolysis is being caried out.

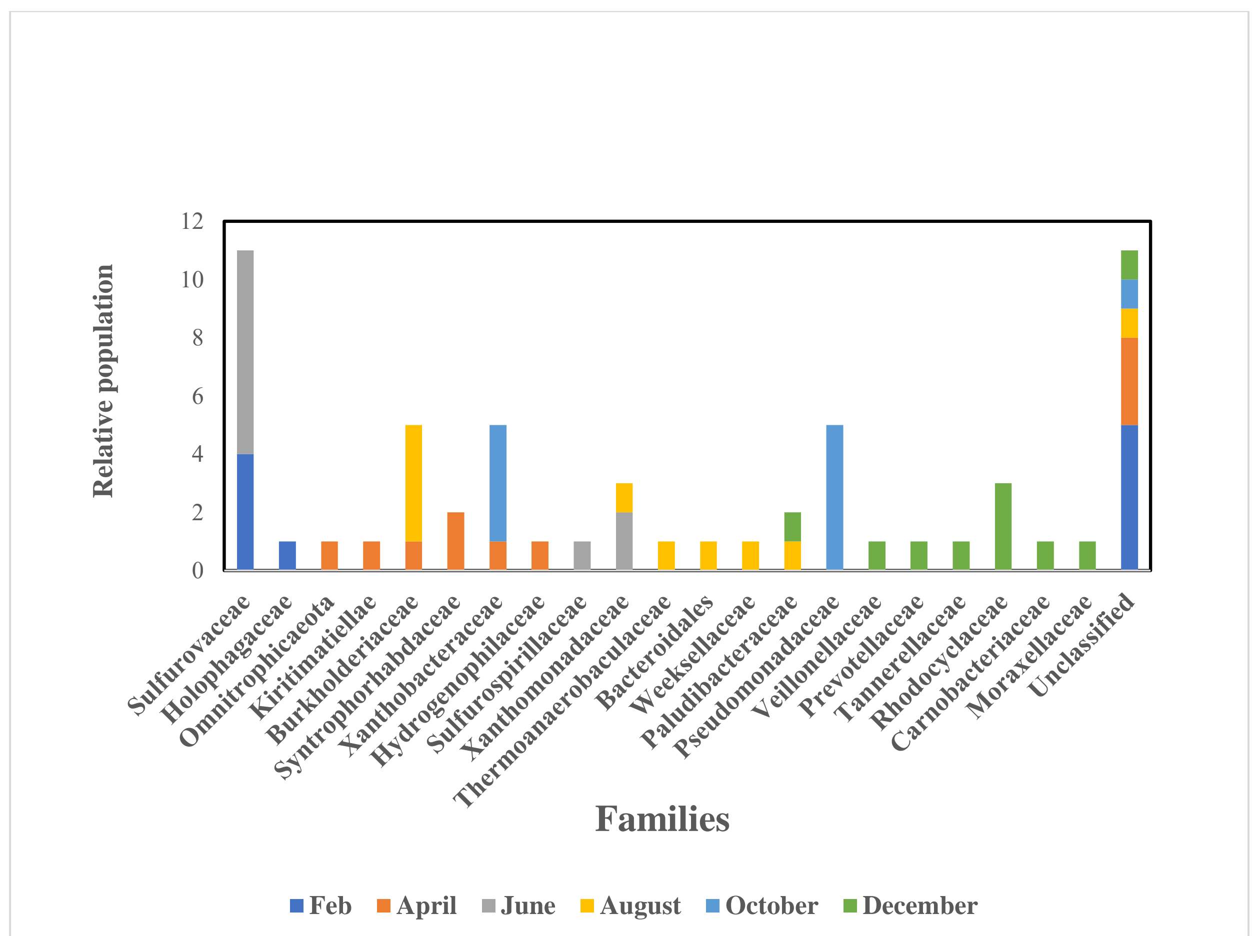


Figure 4.1 Relative bacterial population along 1st chamber of large scale ABR during six alternate months

4.5.1.2 Microbial structure along 4th chamber of ABR

Community shift was observed along 4th chamber of ABR during six alternate months. Diverse bacterial community structure was observed in 4th chamber during month of February and detected families were *Clostridiaceae*, *Rikenellaceae*, *Sphingomondaceae*, *Latescibacteria*, *Tannerelaceae*, *Omnitrophicaeota*, *Rhizobiales* and *Thiovulaceae* (Fig. 4.2). *Rikenellaceae* and *Tannerelaceae* were comparatively higher than other families.

In 4th chamber during April, bacterial diversity was lower as compared to February and only 3 families *Paenibacillaceae*, *Pseudomonadaceae* and *desulfomicrobiaceae* were detected, which were different from families detected in February (Fig. 4.2). *Pseudomonadaceae* was dominant family, *Paenibacillaceae* was 2nd dominant family and *desulfomicrobiaceae* was lowest in count.

In 4th chamber of June, microbial diversity was improved and six families *Clostridiaceae*, *Pedospharaceae*, Bacteroidales, *Deltaproteobacteria*, *Halomonadaceae* and *Hydrogenedensaceae* were detected which were completely different from families appeared in April (Fig. 4.2). While *Clostridiaceae* was shared family with samples of February in 4th chamber.

In 4th chamber of August *Rikenellaceae*, *Sphingomondaceae*, *Pedosphearaceae*, *Sulfurovaceae* and *Xanthomonadaceae* were detected (Fig. 4.2). *Sulfurovaceae* and *Xanthomonadaceae* were newly appeared families of August sample (4th chamber). *Rikenellaceae* and *Sphingomondaceae* are shared families of August with 4th chamber of February sample and *Pedosphearaceae* is shared with 4th chamber of June. Five families detected in 4th chamber of October *Pseudomonadaceae*, Bacteroidales, *Xanthomonadaceae, Burkholderiaceae* and gammproteobacteria. *Burkholderiaceae* and gammproteobacteria are different from 4th chamber of other months while *Rikenellaceae* is shared with February and August (4th chamber), *Pseudomonadaceae* is shared with April (4th chamber), bacteroidales is shared with June (4th chamber), *Xanthomonadaceae* is shared with August (4th chamber).

Families detected in 4th chamber for the month of December were *Sulfurovaceae*, *Xanthomonadaceae*, *Burkholderiaceae*, *Paludibacteraceae*, *Actinobacteraceae*,

Rhodocyclaceae and *Rhizobiacea* (Fig. 4.2). *Xanthomonadaceae*, *Burkholderiaceae* and *Rhodocyclaceae* had larger proportion. *Sulfurovaceae* was shared family of December and August (4th chamber), *Xanthomonadaceae* was shared family of 4th chamber for months December, October, and August, while *Burkholderiaceae* was shared family of December and October.

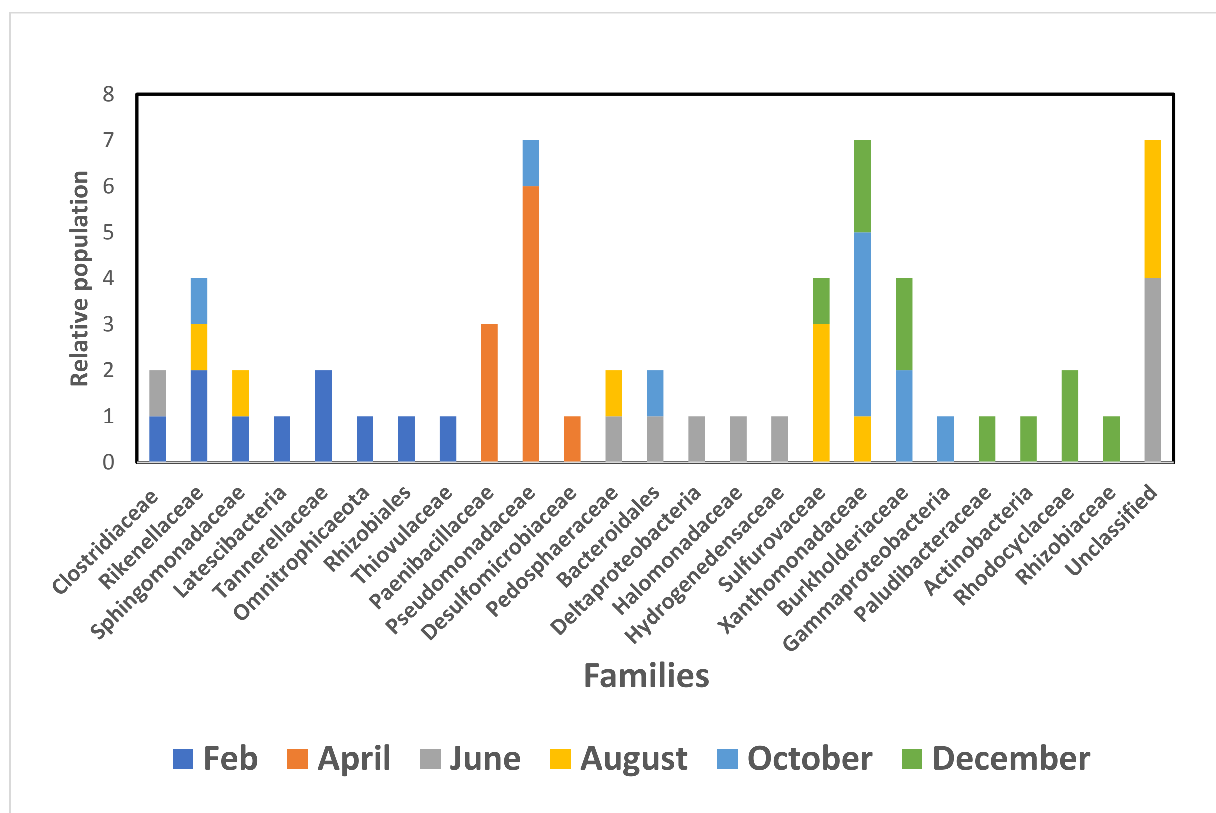


Figure 4.2 Relative bacterial populations along the 4th chamber of large scale ABR during six alternate months

4.3.2.2 Evaluation of variation in microbial community structure between 1st and 4th chamber

Microbial composition of 1st and 4th chamber was dissimilar overall each month. While overall bacterial diversity observed in both chambers was almost same. Comparatively more diversity was observed in 4th chamber (8 families) than 1st chamber (2 families) during the month of February. A completely different microbial composition appeared in 4th chamber in February. While during April 1st chamber

showed more microbial diversity (6 families) as compared to 4th chamber (3 families) and again community structure appeared was dissimilar to detected in 4th chamber. During month of June microbial diversity was increased in 4th chamber (6 families) compared to 1st (3 families) chamber, and completely new microbial flora was established in 4th chamber. In August not significant difference was observed in microbial diversity of both chambers as in 1st chamber (6 families) detected while in 4th chamber (5 families). All the families observed in 4th chamber were dissimilar to 1st chamber. Only 2 families detected in 1st chamber of October, while in 4th chamber 5 families were identified which were not like 1st chamber. In the month of December, both chambers (1st and 4th) had same microbial diversity, as 7 families detected in each chamber, but different microbial composition. Similar families identified along 1st and 4th chambers were *Tannerellaceae*, Omnitrophicaeota, *Pseudomonadaceae*, Bacteroidales, *Paludibacteraceae*, *Sulfurovaceae*, *Xanthomonadaceae*, *Burkholderiaceae* and *Rhodocyclaceae*.

4.3.2.3 Relative microbial populations of laboratory scale ABR

At low temperature

Relative microbial population was evaluated for 1st and 4th chamber of ABR working at low temperature. Bacterial families detected in 1st chamber were *Burkholderiaceae*, *Caulobacteraceae*, *Rhodocyclaceae*, *Chitinophagaceae*, Bacteroaidales and *Erysipelotrichaceae*. While families detected in 4th chamber were Betaproteobacteria, *Rhodocyclaceae*, *Methylococcaceae*, *Devosiaceae*, *Xanthomonadaceae*, *Schlesneriaceae* and *Sphingomonadaceae*. *Rhodocyclaceae* was only shared family of 1st and 4th chamber, all other families were different in both chambers. Dominant family in 1st chamber was *Rhodocyclaceae* and in 4th chamber no dominant family detected. One methanogenic family *Methylococcacea* was detected in 4th chamber (Fig. 4.3).

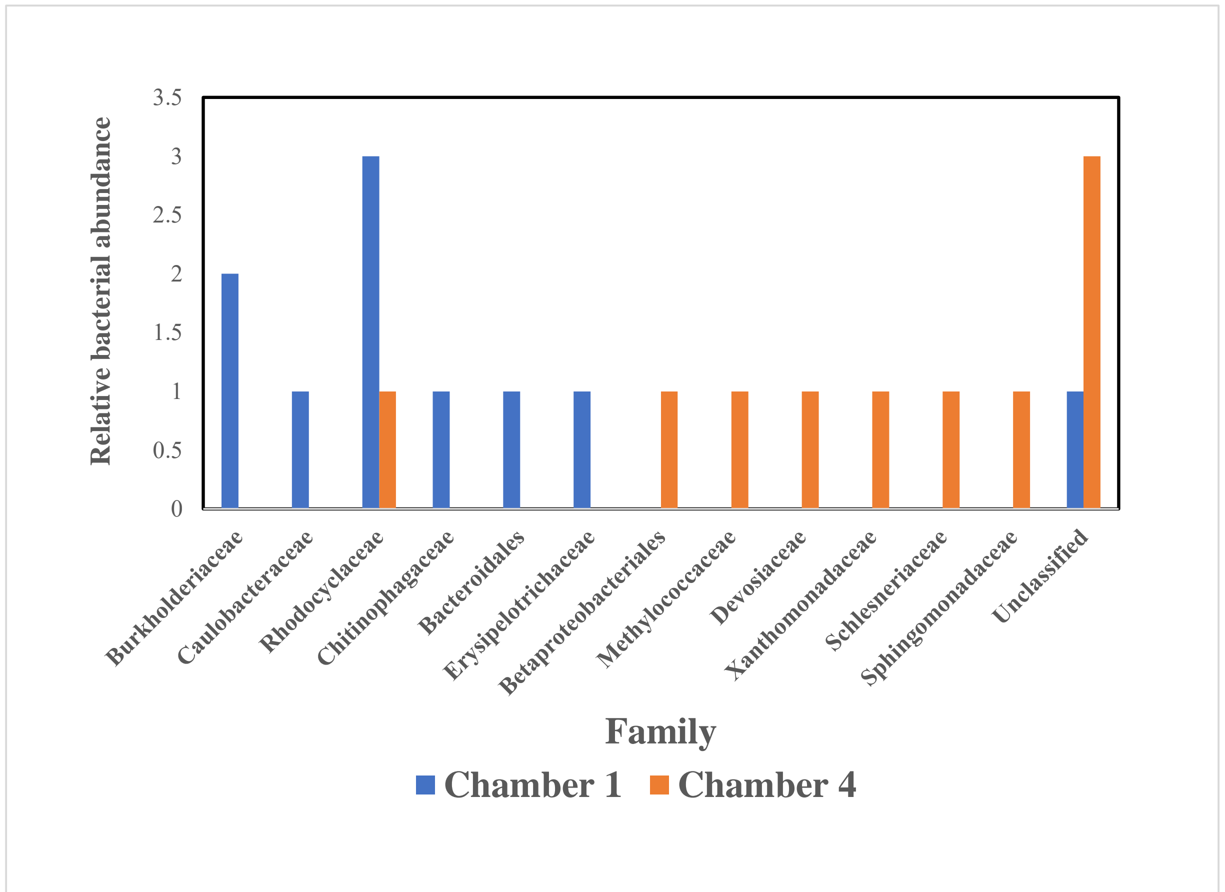


Figure 4.3 Relative abundance bacterial community in laboratory scale ABR working at low temperature

At high temperature

Different microbial community structures were identified in 1st and 4th chamber of laboratory scale ABR working at high temperature. In 1st chamber detected families were *Paludibacteraceae*, *Pseudomonadaceae*, *Rhodocyclaceae*, *Anaerolinaceae*, *Bacteroidetes*, *Xanthomonadaceae*, *Solibacteraceae* and *Pedosphaeraceae*. There was no dominant family in 1st chamber. In 4th chamber *Rhodocyclaceae*, *Bacteroidetes*, Planctomycetes, *Syntrophaceae* and Sphingobacteriales were detected. *Rhodocyclaceae* and Bacteroidetes were more in count in 4th chamber. Bacteriodetes and *Rhodocyclaceae* were common/shared families of both chambers (chamber 1st &4th), other families were different (Fig. 4.4).

Comparison of microbial community composition of high temperature ABR and low temperature ABR *Rhodocyclaceae* (chamber 1) and *Xanthomonadaceae* (in 1st chamber of high temperature and 4th chamber of low temperature).

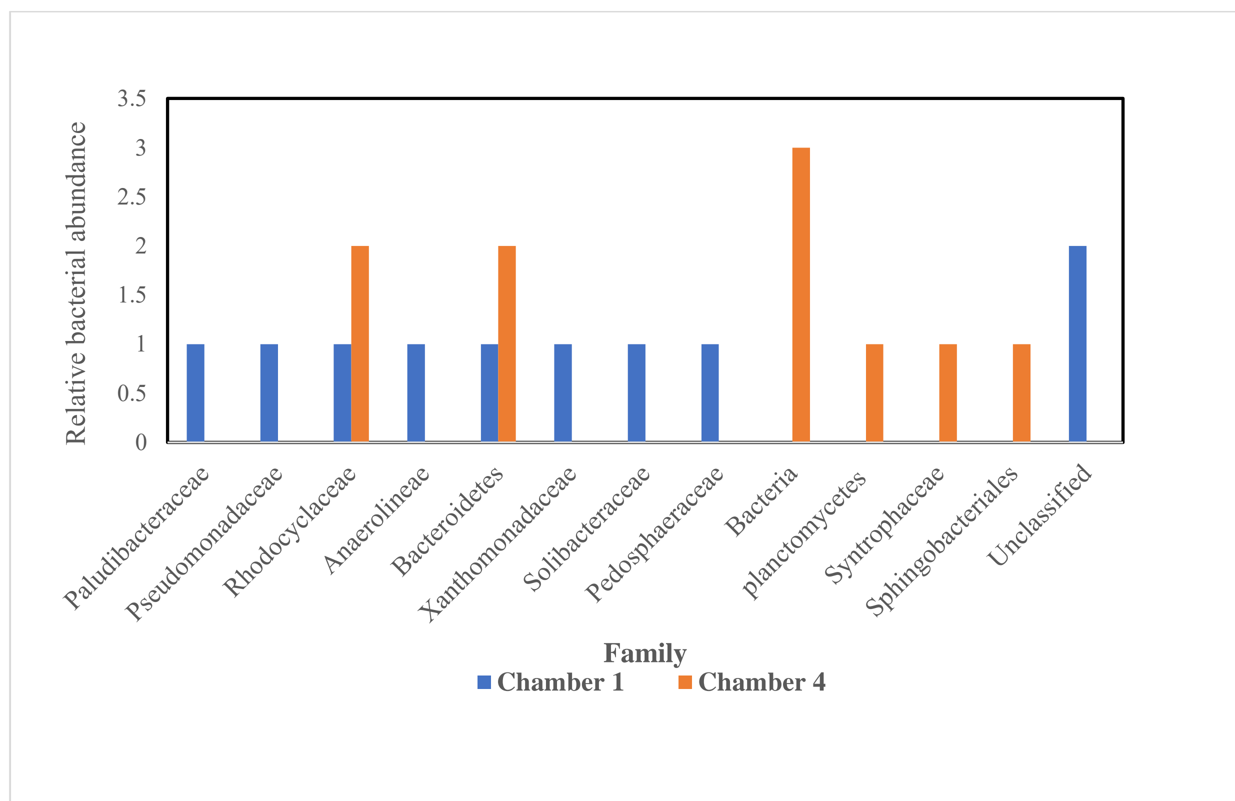


Figure 4.4 Relative abundance of bacterial community in laboratory scale ABR working at high temperature.

Detection of methanogenic microorganisms in ABRs

Wastewater samples were collected from 4th chamber for detection of methanogens in 4th chamber of field scale and laboratory scale ABR. For field scale ABR wastewater samples were collected for six months and for laboratory scale ABR one sample collected in last month of operation of the reactors.

Eight primer pairs were tested to describe entirely methanogenic population from ecosystem of 4th chamber of three ABRs. Selected primers were targeting different genes of wide range of methanogenic taxa. Primers used are: MCRB for target gene mcrB, MCRG1 target gene mcrG1, MTAB target gene mtaB and MTBA for target gene mtbA, MLf and MLr for target gene mcrA, MCRf & MCRr for target gene

mcrA, Met83F and Met1340R target 16S rRNA gene and 146f & 1324r target 16S rRNA gene of methanogens. No band was found with all these primers in any sample, except for ML primer pair. ML primer pair showed band for all the samples of 4th chamber except for month of February. A clear band was found for ML primer at annealing at temperature 50ºC and cycle 30x. PCR /topo cloning, and plasmid preparation was performed for ML primer band sequencing to confirm the detected band was for methanogen. The sequenced genome was identified as methanotroph, not of methanogen organism.

PCR was performed for primer sets MCRB, MCRG1, MTAB and MTBA with added PCR enhancer at temperature 55ºC with 39x cycles. Band (2000 bp) was found only with MTBA primer pair. After sequencing, the resulted genome was too large, not of methanogen.

4.3.2.4 Pathogens detection survey along chambers of ABRs

Detection of diarrheagenic bacterial pathogens

Wastewater samples collected from 1st and 4th chamber were tested for detection of diarrheagenic pathogens. PCR were operated to uncover diarrheagenic bacterial pathogens with specific primers against the community DNA extracted from 3 ABRs. Primers used were able to identify specific strains EAEC, EPEC, ETEC, EHEC, *Salmonella enterica*, *Shigella spp.,* *Vibrio cholerae*, *Yersinia enterocolitica*, *Aeromonas hydrophila* and *Campylobacter*. Specificity of primers used in present study to identify respective strains were tested in study conducted by Asa et. al., (2015). Primers used with their amplicon size are given as: pCVD432 for EAEC (194 bp), LT for ETEC (273 bp), STp for ETEC (166 bp), Eae for EPEC/EHEC (482 bp), Bfp for typical EPEC (300 bp), CadF for campylobacter (119 bp), IpaB for *Salmonella enterica* (314 bp), IpaH for *Shigella spp*. (423bp), Vc for *Vibrio cholerae* (192 bp), Aero for *Aeromonas hydrophila* (720 bp), Ail for *Yersinia enterocolitica* (354 bp).

Detection of human adenovirus (HAdV)

HDdV detection in wastewater samples was made using samples of 1st and 4th chamber. PCR reactions carried out for finding human adenovirus strains ABCDEF from community DNA extracted from 3ABRs. Specific primers for each strain were used with single forward primer HAdV-ABCDEF-hexon 25f and reverse primers HAdV-A-hexon 343r, HAdV-B-hexon308r, HAdV-C-hexon358r, HAdV-D-hexon288r, HAdV-E-hexon373r, HAdV-F-hexon265r. Reactions were performed with Taq polymerase and high-fidelity DNA polymerase at different annealing temperatures 50ºC, 52ºC, 54ºC, 58ºC for both Taq polymerase and DNA polymerase. No band was found with taq polymerase. With DNA polymerase B serotype gave band at annealing temperature 54ºC. Others gave results with DNA polymerase in the form of scattered multiple bands but did not gave any corresponding result.

Results of the microbial communities of anaerobic baffled rectors with dominant families in the samples of two chambers throughout the years in large scale ABR showed *Holophagaceae* (phylum acidobacteria) which belongs to strict anaerobes indicating the existence of anaerobic environment in the digester during February. *Holophagaceae* detected in 1st chamber during February indicates that anaerobic conditions were developed soon in the ABR, as members of holophagaceae are observed be involved in process of denitrification, which occurs in anaerobic conditions (Liesack et al., 1994). Establishment of anaerobic environment in the ABR is positive indicator for the process of anaerobic digestion. Detection of Kiritimatiellae in 1st chamber in April also indicates establishment of anaerobic environment in 1st chamber. Identification of family *Burkholderiaceae* in 1st chamber during April and August is indication that hydrolysis is going on in 1st chamber as this family belongs to common degraders and involved in hydrolysis (phenol, diesel) (Bell et al., 2013). *Burkholderiaceae* was also detected in 4th chamber of field scale ABR during end months. Another indication of good start of anaerobic digestion is presence of family *Syntrophorhabdaceae* as a dominant family in April. Wastewater treatment plants having dominant population *Syntrophorhabdacea* in initial stages were observed to have improved startup with better performance of digesters (Leven et al., 2007). *Syntrophorhabdaceae* have positive effect on anaerobic degradation process of organics, and they are seemed to be involved in aromatic compounds

utilization (Chen et al., 2008; Wu et al., 2001). A pathogenic family *Weeksellacea* detected in 1st chamber during (August) disappeared in 4th chamber which indicated that field scale ABR had efficiency to remove pathogens (Gioacchini et al., 2018). Members of *Paludibacteracea* were detected in both chambers, are common fermenters and produce different fermentation products including propionate from sugars (Nelson et al., 2011). It is mostly detected at mesophilic temperature in August as it mostly identified at mesophilic temperature (Ueki et al., 2006), but in the present study it is also detected at low temperature at 14˚C temperature (in lab scale reactor). These are member of bacteriodetes. They are involved in complex substrates degradation and effectively perform it (Garcia et al., 2011).

Members of family *Veilonellaceae* detected in 1st chamber of December sample, (belong to Firmicutes phylum) mostly carryout carbohydrates fermentation and nitrate reduction in anaerobic digesters. Metabolic end products of fermentation are propionic acid, acetic acid and lactic acid. Members of family *Prevotellaceae* were isolated from feces of human (He et al., 2010). *Rhodocyclaceae* in 1st and 4th chamber during December and belongs to betaproteobacteria and is commonly present in wastewater treatment plants and have degrading ability under anaerobic and denitrification conditions (Foss et al., 1998; Yuanyuan et al., 2016). *Carnobacteraceae* in 1st chamber of December was shown to be affected significantly by operational parameters and positively associated with COD and ammonium concentration and may be involved in COD and nitrite removal (Chongjun et al., 2016). *Moraxellaceae* detected in December 1st chamber at 14˚C, are Psychrobacters and prevailed at low temperature. They ferment sugars. They are mostly parasites (of human and other animals) (Rossau et al., 1991). Members of bacteriodetes belonged to hydrolytic microorganisms (Zhang et al., 2019). Hydrolytic microorganisms were detected along 1st chamber, as in 1st chamber the process of hydrolysis is being caried out.

Clostridiaceae, *Sphingomonadaceae*, *Latescibacteria* observed along 4th chamber during month of February were characterized having strict anaerobic nature performing different activities in water ecosystem. As *Clostridiaceae* consists of fermenters which are strictly anaerobes (Zamanzadeh et al., 2016) and are observed to involve in different stages of anaerobic digestion performing their role of carryout

hydrolysis and final stage of anaerobic digestion it is seemed in having close syntrophic relationship with methanogens. Their activity (competitive/syntrophic) in process may alter depending on community composition and conditions (environmental factors) (Ziganshin et al., 2013). Members of family *Sphingomonadaceae* are present commonly in water ecosystem, adapted in low substrate conditions (Steven et al., 2008). Latescibacteria are found in different ecosystems including wastewater treatment plants (Schabereiter et al., 2004). They are strictly fermentative organisms found at varying temperature having anaerobioc characteristics (Ibrahim et al., 2017).

Desulphomicrobiaceae family (4th chamber during April) and *Deltaproteobacteria* (4th chamber of June) carryout sulphate reduction in anaerobic digesters (Sarti et al., 2010: Tetsuro et al., 2009). *Desulphomicrobiaceae* are strictly anaerobic, members of this family can grow at wide temperature range (Alex et al., 2009). Population shift was observed in last months in 4th chamber, which was positively related to anaerobic digestion. Presence of actinobacteria in 4th chamber of December shows stability of the reactor and steady state condition as actinobacteria prevail at steady state conditions of the process (Akira et al., 2003).

In lab-scale ABRs, only *Rhodocyclaceae* was shared family in both ABRs detected in both chambers in both ABRs, but its dominance varied in different chambers. In low temperature ABR, *Rhodocyclaceae* was dominant in 1st chamber while in high temperature ABR *Rhodocyclaceae* was dominant in 4th chamber. Presence of Rhodocyclacea at both temperatures may be due to their adaptation to all temperature conditions (Rusmus et al., 2007). *Paludibacteraceae* detected in high temperature ABR, but not in low temperature. These are member of bacteriodetes. *Paludibacteraceae* are mostly detected at mesophilic temperature and their primary fermentation products are propionate and acetate (Ueki et al., 2006). They are involved in complex matter degradation and effectively perform it (Garcia et al., 2011). Members of family Anaerolineae detected in high temperature ABR are involved in hydrolysis as they are involved in biodegradation of carbohydrates (Zamanzadeh et al., 2016).

4.3 Investigation of microbial community structure of ABRs by NGS Illumina sequencing technique

Microbial community composition and richness was calculated for three anaerobic baffled reactors (ABRs), each ABR working at different temperature conditions. One full-scale ABR working at ambient temperature and two lab-scale ABRs placed at controlled temperature, one at low temperature and other at high temperature. Field-scale ABR sampled for all the four chambers for five alternate months according to change in environmental temperature, lab-scale ABR at low temperature sampled from chamber 1 and 4 and for lab-scale ABR at high temperature sampling done from samples chamber 1, 3 and 4. All the samples were evaluated for their microbial community composition, two domains of the ecosystem in ABRs were evaluated; bacteria and archaea. Microbial communities in the system were explored using Next Generation Sequencing (NGS) technique 'Illumina –MiSeq' sequencing'. V3 and V4 regions of 16S were amplified. Sequence analysis was performed using QIIME2-2019.4 platform. Output was delivered in form of taxa bar plot to visualize taxonomy profile of all the samples, alpha diversity to find diversity within group/chamber using different metrics and rarefaction curve to evaluate sample group sequencing depth and beta diversity to find distance among groups/chambers in terms of species composition. Taxonomic composition of micro biomes (microbial communities) was predicted based on DNA sequences of 16S rRNA gene.

4.3.1 Microbial community structure of field scale ABR

4.3.1.1 Alpha diversity analysis

Richness and diversity indices were calculated for bacterial population in three ABRs. After removal of low quality and short reads, a total of 3351759 effective sequences of specific gene16S-rRNA were generated from 25 samples, collected from three ABRs. Species richness in each microbial community was analyzed by16S rRNA phylogenetic index which is conventional for bacterial or universal primers (Dabert, 2001).

Sequences were demultiplexed by source bioscience per DADA2 routine to denoise them. Sequences shorter than 240 bp were discarded as they are considered shorter

length than needed. Demultiplexed count summary explained that maximum sequences generated were 195279 (from April chamber 1), minimum sequences were 20138 (from sample October chamber 1), while mean 134070.36 and median 144124. Sequence length summary is that each sequence lie between 240-300 bases (range = 60) with mean length 291.96. Sequence count (number of feature/spp.) and standard deviation were 2233 and 1.25 respectively. Frequency/repetition of feature per sample includes minimum frequency 837, maximum frequency 25410. Frequency per feature for minimum was 2 and maximum was 5113. Resulting sequences (high quality reads) aligned against Silva reference database and then clustered in OTUs (operational taxonomy unit), that was recognized as species taxonomic level. OTUs then were assigned to most likely taxa. We can find out core microorganisms of each chamber with time and temperature and can compare microbial communities and dominant populations in ABR.

4.3.2 Rarefaction analysis

Rarefaction analysis was performed to explore alpha diversity as a function of sampling depth. Alpha diversity at initial level was presented by rarefaction curves and its calculations were made for each sample using different alpha diversity indices/metrics as observed OTUs for simple count of unique species (OTUs) detected in the sample, Chao1 for species richness and Shannon for diversity estimation, Faith pd is phylogenetic metric used to relate OTUs to phylogenetic tree, showing sufficient sequencing depth for all samples coverage, which takes into account microbial abundance and richness in the samples. Shannon indices were applied as function of the number of sequences that were retrieved from QIIME2 module and analyzed under rarefaction analysis. Sequencing depth achieved was adequate for all microbial consortia, as Shannon indices observed to plateau. Lower Simpson index and higher Shannon index shows more community diversity. Data clearly depicts higher community diversity and richness for almost all samples (Fig 4.5).

Operational taxonomy unit (OUT) was used to characterize bacteria on the basis of similarity of the sequence. Sample metadata columns are, groups (plot for each sample), time in chamber (plot showing comparison of 4 chambers) and treatment at ambient temperature (plot at different range of temperature). Richness was estimated

against temperature and chambers. All the samples (25) were rarefied under equal depth for all amplicons. Clusters having closeness together have relatively more similarity in their microbial community composition. Alpha rarefaction curves showed complete species saturation and richness in all samples except two samples for the month of October chamber 1 and chamber 2. Richness is measurement of a number of different microorganisms which are present in a sample.

Rarefaction curves for OUT based analysis vs sampling depth was generated in accordance with each set of conditions (metadata: sample/Group, Time in chamber and ambient recorded temperature). A plot of observed OTUs against sequencing depth of samples in case of groups, time in chamber and ambient recorded temperature showed that number of OTUs increased maximum up to 1500 sequencing depth. While increasing sequencing depth beyond 1500, OUT remain constant. It means OTUs richness was achieved as it was achieved at 1000. To find diversity, maximum OTUs diversity achieved in group was 180. OTUs in different chambers are as follows: across chamber 1 was 140, chamber 2 was 110, chamber 3 was 140, for chamber 4 was 70. Lower OTUs diversity was found across the chamber 4. OTUs diversity with temperature variation was as follows: at 13-14°C, 140; 15-17°C, 80; 24-25°C, 130; 27-30°C, 140 and at 32-35°C was 100 (Fig 4.5).

In case of time in chamber and ambient recorded temperature observed OTUs were reached to maximum at 140 and sequencing depth 1500. Maximum OTUs were found in sample April chamber 1 and April chamber 3, while minimum OTUs were found in October chamber 1 and October chamber 2. In case of plot drawn for chambers, chamber 3 of all the samples have higher OUT comparatively and chamber 4 for all the samples have lower OTUs. In case of OTUs variation with temperature, at temperature range 13-14°C and 27-30°C maximum OTUs were shown. At temperature 15-17°C observed OTUs were lower. Total H value for OUT group significance is 7.78. Total p-value is 0.22.

Shannon and Faith pd values were also observed against sampling depth. In case of group, minimum Shannon diversity indices obtained was 0.5 and maximum Shannon indices obtained was 6.5. Shannon indices for time in chamber, across chamber 1 was 6.5, for chamber 2 was 6, for chamber 3 was 6.8 and for chamber 4 was 5.5. Shannon indices variation for different set of temperatures showed that 13-14°C had 6.5, 15-

17°C had 5.5, 24-25°C had 6.5, 27-30°C had 6.5, while 32-35°C had 5.8. Minimum Simpson indices was 0.3 and maximum Simpson diversity was about to 1.0. Similarly, richness of samples was calculated using Chao1 (4.6).

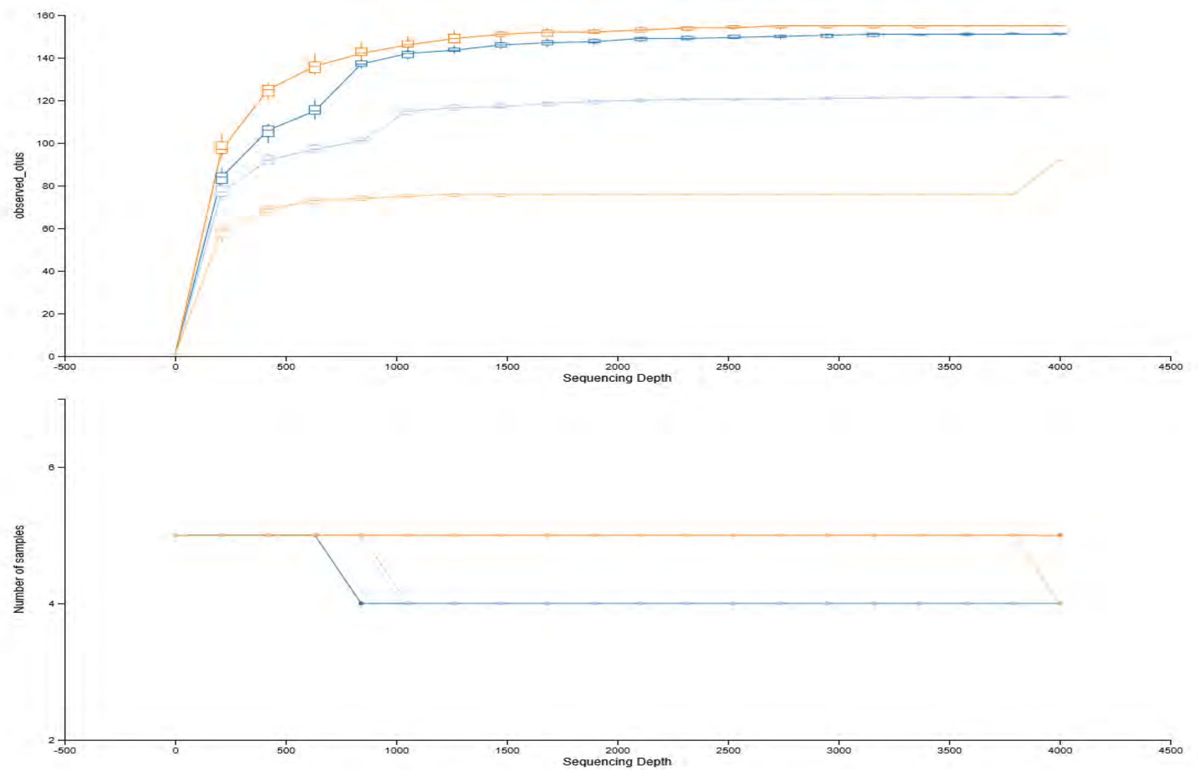


Figure 4.5 Comparison of Alpha diversity with rarefaction curve in terms of OTUs along the chambers of field scale ABR; *Chamber 1: dark blue, Chamber 2: light blue, Chamber 3: dark orange, Chamber 4: Light orange*

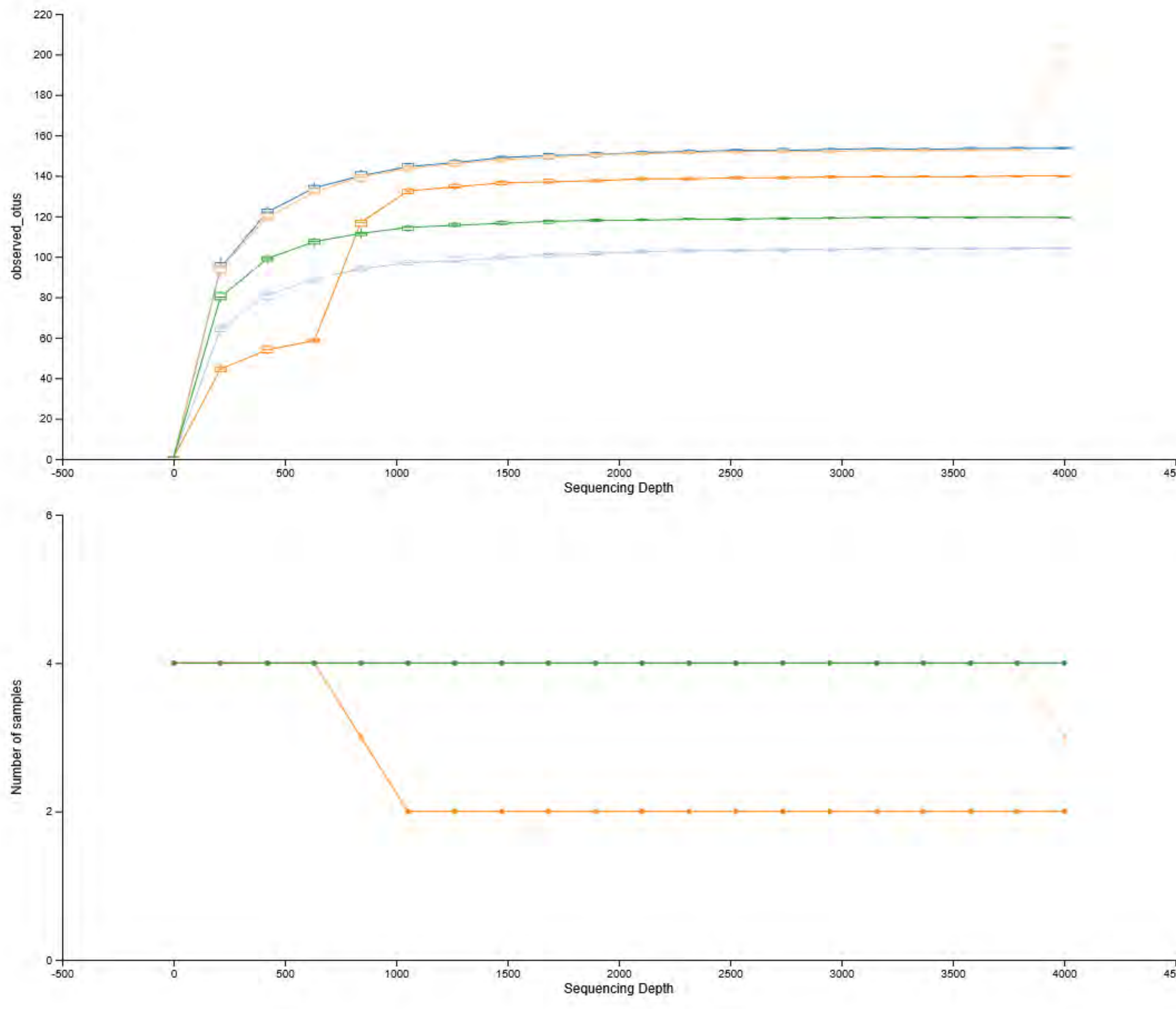


Figure 4.6 Comparison of Alpha diversity with OTUs rarefaction curve with temperature variation 13-14°C*: Dark blue, 15-17°C: light blue, 24-25°C: dark orange, 27-30°C: light orange, 32-35°C: green*

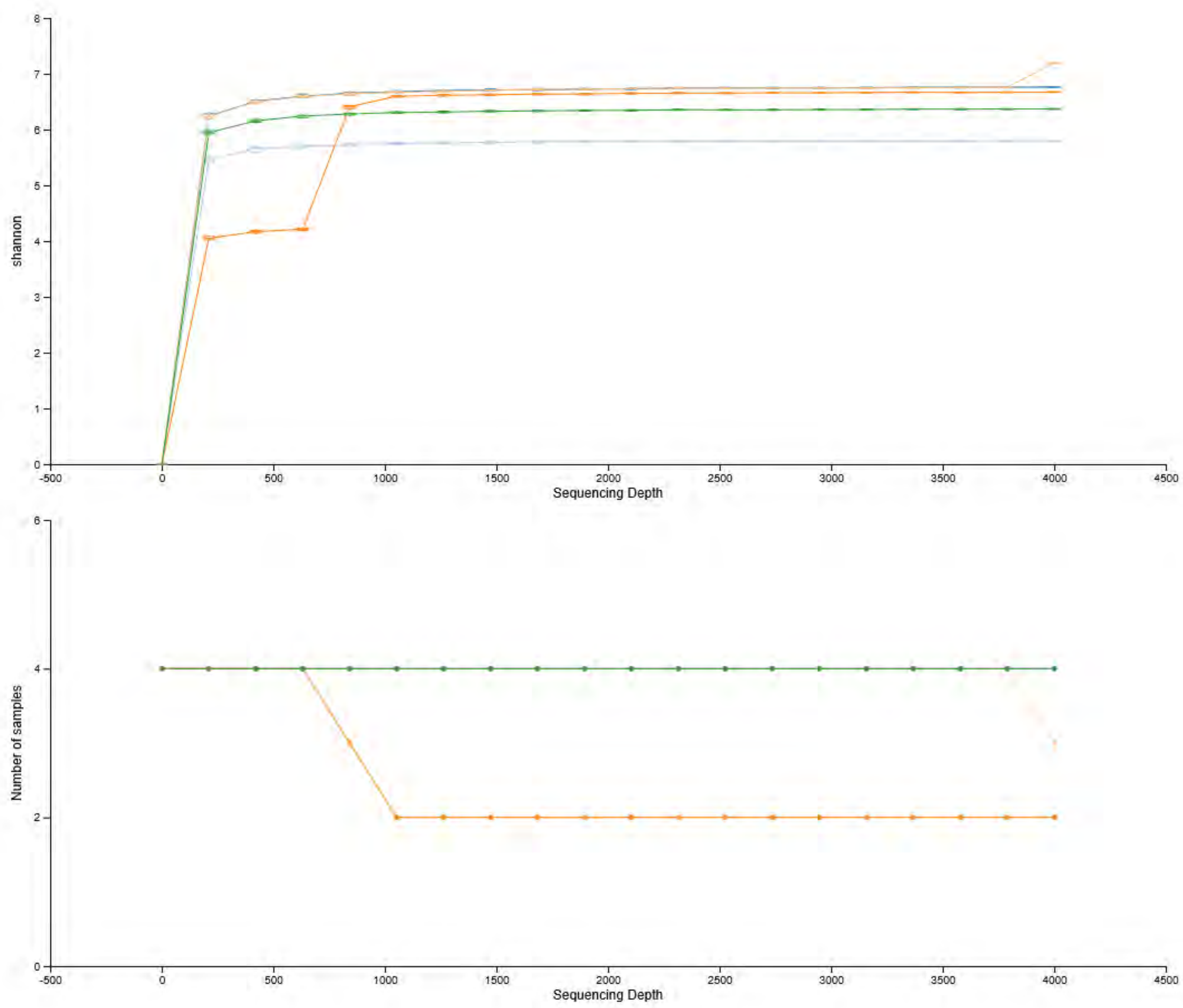


Figure 4.7 Comparison of Alpha diversity with Shannon rarefaction curve with temperature variation of environment 13-14 °C*: Dark blue, 15-17 °C: Light blue, 24-25 °C: Dark orange, 27-30 °C: Light orange, 32-35°C: Green*

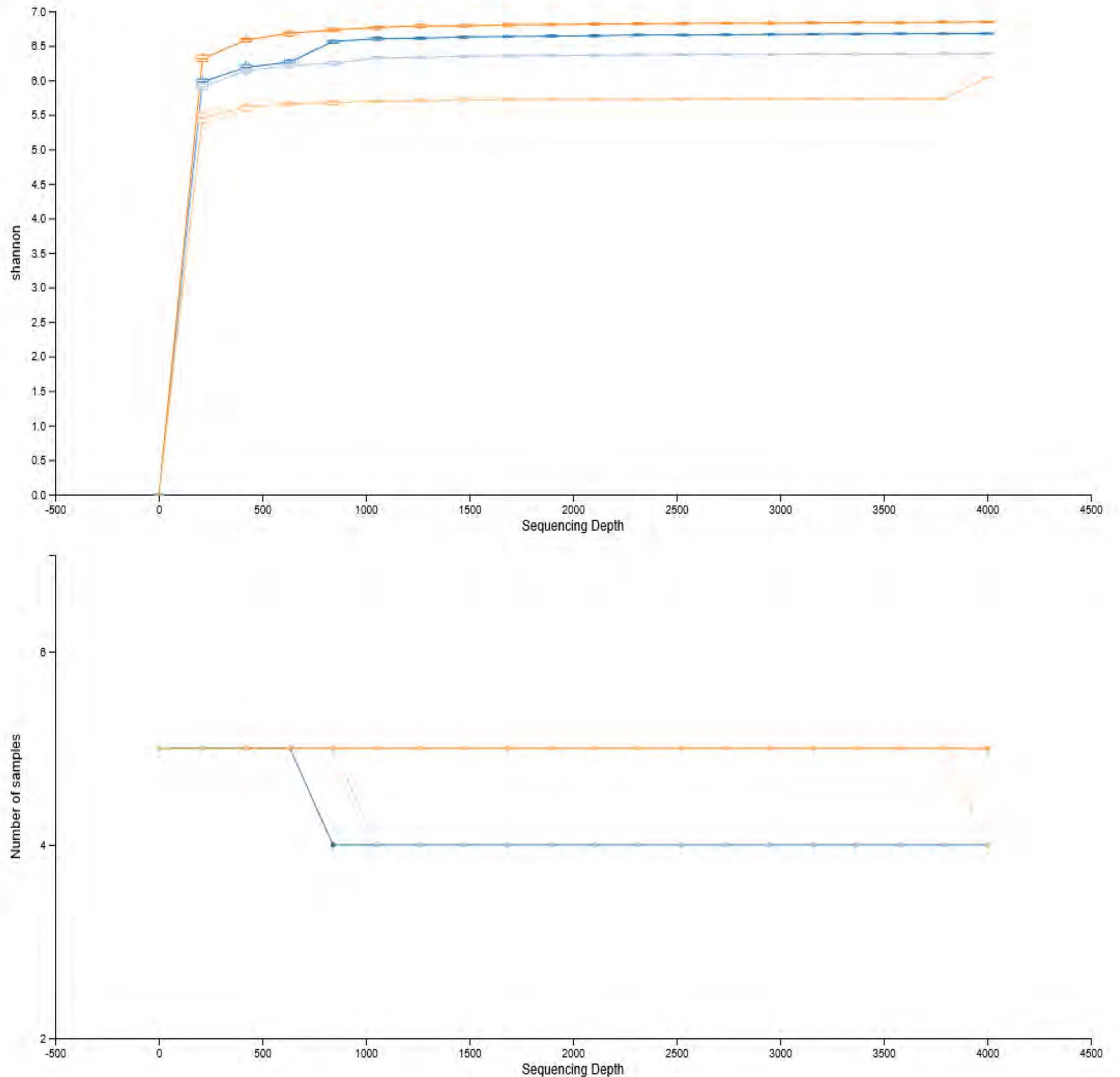
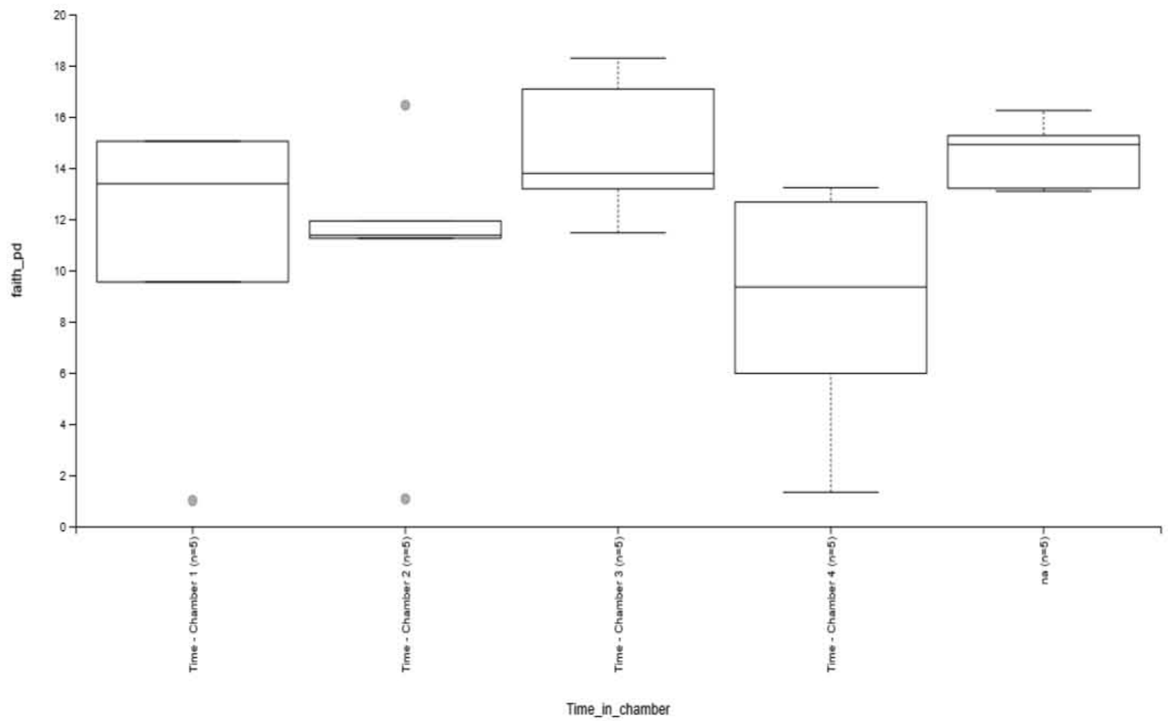


Figure 4.8 Comparison of Alpha diversity with Shannon rarefaction curve with temperature variation of. *Chamber 1: dark blue, Chamber 2: light blue, Chamber 3: dark orange, Chamber 4: light orange*

4.3.3 Faith pd group significance

Group significance was analyzed using Kruskal Wallis test. In Faith pd (phylogenetic diversity) group significance, *P*-value was varied among the samples. Good Overall sample evenness was achieved. Overall *p* value for time in chamber metadata was

0.12 and H value 7.3. No significant difference was observed between different pairs of chambers except between 3rd chamber and 4th chamber (*p* value 0.04) showed significant difference in their population. For ambient recorded temperature metadata *p*-value was 0.49 and H-value was 4.44. No statistically significant difference was shown at ambient recorded temperature (4.9).



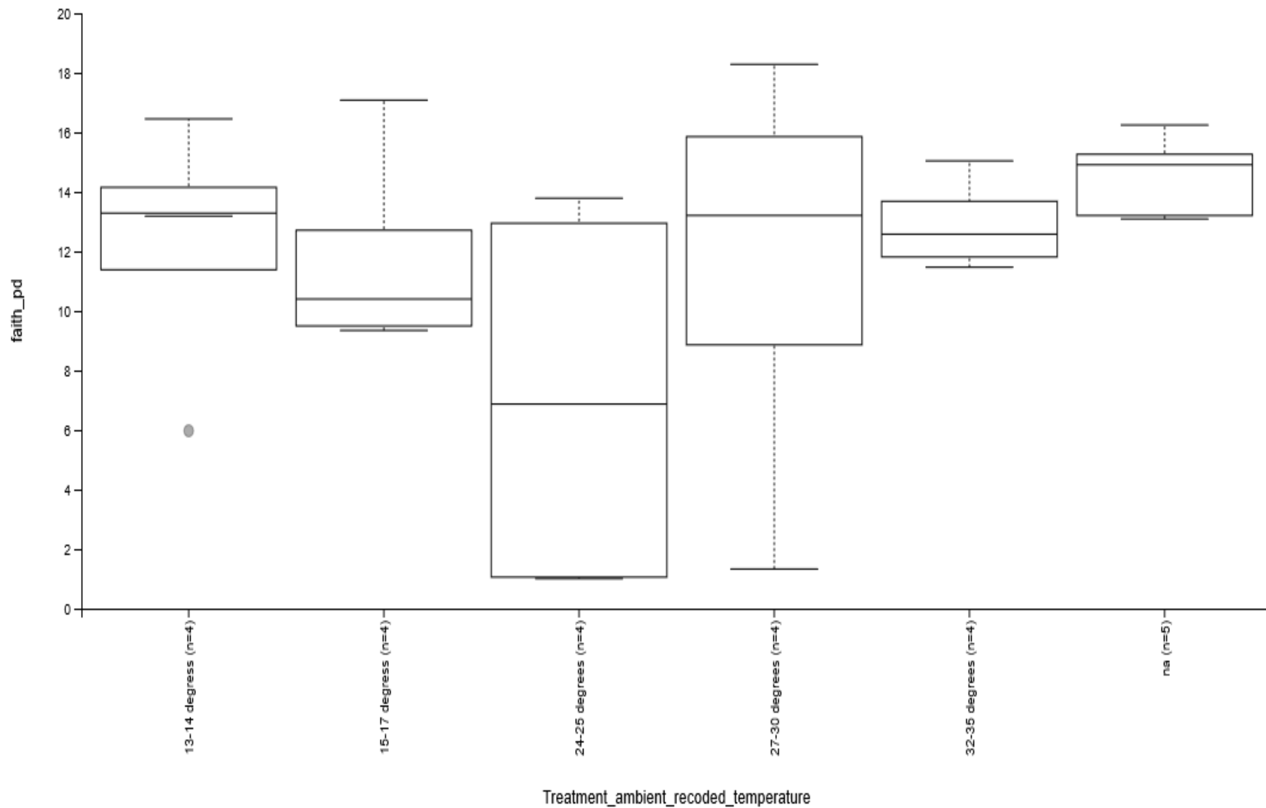


Figure 4.9 Faith pd group significance with shift in chamber and change in temperature

4.3.4 OTUs group significance

In case of time in chamber, overall *p* value was 0.22, showing insignificant group difference. Chamber 3 and chamber 4 showed statistically significant difference in species composition as shown by *p* value 0.028. For treatment ambient temperature, *p* value was 0.77 showing no significant group difference in composition based on species with temperature variation (4.10).

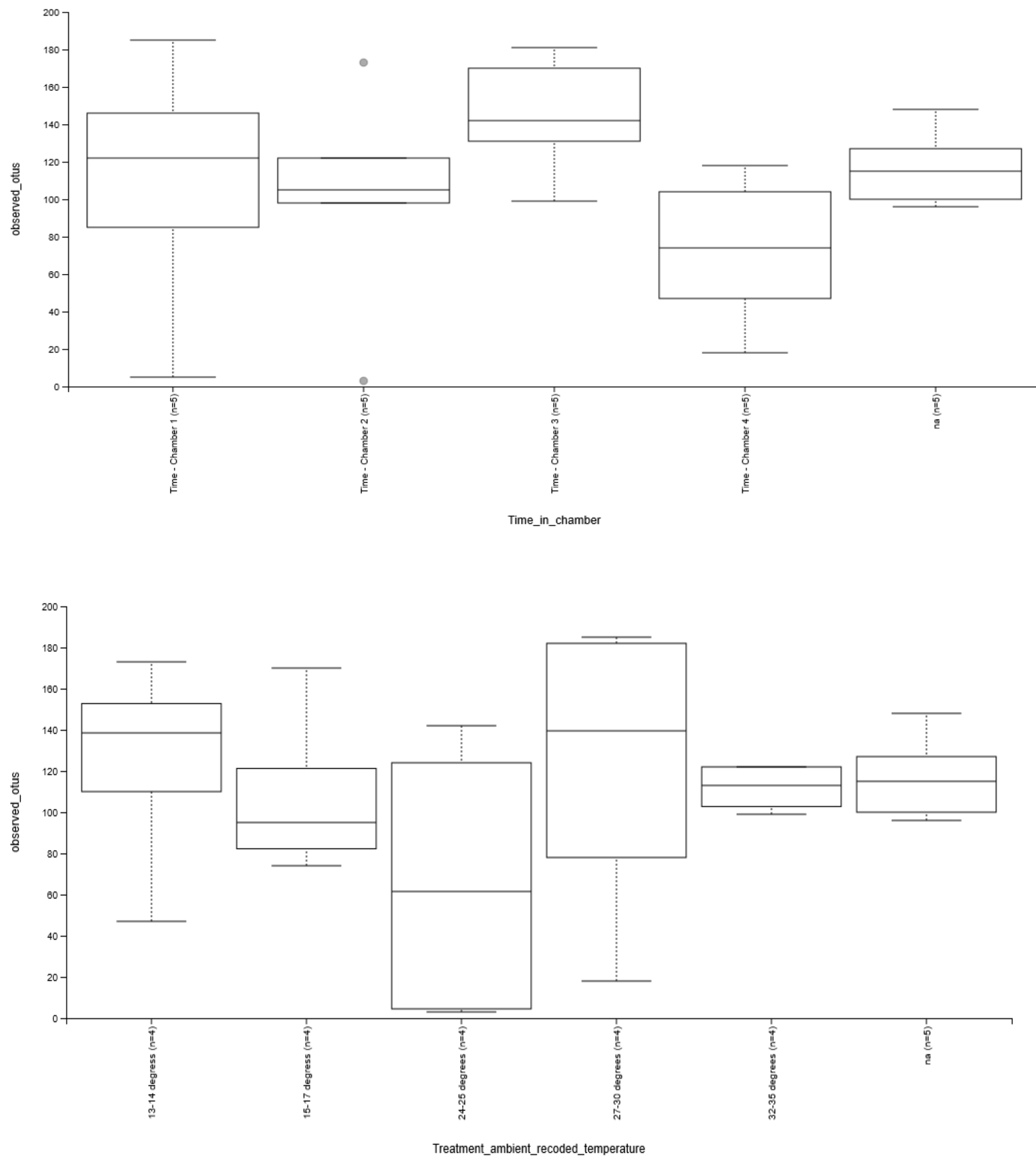


Figure 4.10 OTUs group significance with shift in chamber and change in temperature

4.3.5 Relative abundance of large scale ABR

Relative abundance and frequency in each sample was analyzed by taxa bar plot. We can group OTUs at different taxonomic levels as kingdom, phylum, class, order, family and species, the process is dependent on assigning taxonomic information to OTUs. In case of field scale ABR diverse microbial communities were observed in each of the four chambers. Significant difference was observed in composition of microbial community with variation in temperature, while community organization

along the chamber was less variable. It was observed that some taxa were consistently predominant more than one sample.

In taxa bar plot, at level 1 (kingdom level) of classification all the samples were categorized into 3 main groups: bacteria, archaea and unassigned 16S sequences. Dominant portion in all the samples was covered by bacteria. In some samples 100 % bacterial population was identified e.g. for the month of April chamber 4, December chamber 4 and October chamber 2. Archaea were present in most samples but comparatively in very low proportion than bacteria. Higher proportion of archaea among all was (4.31%) detected in April chamber 2 and lowest was (0.28%) in April chamber 3. Archaea were absent in April chamber 4, December chamber 4 and in October chamber 1&2. While unassigned sequences are present in very minute portion, and found only in four samples, February chamber 2 (0.095%), June chamber 2 (0.98%), June chamber 3 (0.292%) and June chamber 4 only 10 sequences (4.11).

Bacterial community of ABR exhibited higher diversity at upper taxonomic level (e.g., phylum). 19 different phyla of bacteria and two archaeal phyla were identified in 20 different samples taken from four chambers of ABR. At phylum level classification, overall examination of taxa bar plot depicted that *Proteobacteria* was found as an abundant phylum among other bacterial phyla across the samples. Abundance pattern for major bacterial phyla in all the samples independent of sample identity and conditions is as follows: *Proteobacteria* > *Bacteroidetes* > *Firmicutes. Proteobacteria* is a diverse group of microbes involving aerobes and anaerobes, able to degrade a wide range of organic matter and help to remove phosphorus and nitrogen. The second largest group *Bacteroidetes* are involved in fermentation system and break down of larger components of protein, starch etc. while the 3rd largest phylum *Firmicutes* mostly prevail in anaerobic environment and are reported as to produce extracellular enzymes as lipases and proteases for breakdown of organic matter.

Other less common bacterial phyla detected in four chambers of ABR are as follows: *chloroflexi, actinobacteria, planctomycetes, crenarchaeota, acidobacteria, armatimonadetes, cyanobacteria, NC10, OP9, verrucomicrobia, lentisphaerae, nitrospirae, elusimicrobia, fusobacteria, lentisphaerae, chlamydia, caldiserica and synergistetes.* Archeal phyla detected in ABR are *euryarchaeota* and *crenarchaeota.*

Higher bacterial diversity was observed in all the months at lower taxonomic level e.g. at family level. The striking aspect about the results is rapid apparent change in microbial community composition and dominance along the chambers of ABR within month and between the months (Fig. 4.11).

For the month of February, variable microbial diversity was observed in each chamber of ABR. 3rd chamber showed maximum microbial diversity while 1st chamber showed minimum diversity. Microbial community composition of the entire chamber was close to each other but not constant and percentage of each family was different in each chamber. Some bacterial families present in all the four chambers during the February were *Helicobacteraceae*, *Commamonadaceae* and *Desulphomicrobiaceae*.

Helicobacteraceae was dominant family of 1st and 2nd chambers 59.08% and 26.79% respectively, during in February sample *Comamonadaceae* (3.39 %) was less common. Other families less than 2 % are *Desulfomicrobiaceae* (1.367%) *Sphingomondaceae* 0.491%, *Anaerolinaceae* 0.66%, *Xanthomonadaceae* 0.71% and *Holophagaceae* 0.82%.

Less common families of February 2nd chamber were *Holophigaceae* (2.375%) and other families which were less than 2% include *Porphyromonadaceae* (1.611%), *Syntrophaceae* (1.011%), *Costridiaceae* (1.535%) and *Lachnospiraceae* (1.07 %). Families which were less than 1 % are *Commamonadaceae*, *Desulfomicrobiaceae*, *Sphingomonadaceae*, *Caulobacteraceae*, *Anaerolinaceae*, *Xanthomonadaceae*, *Peptostreptococcaceae*, *Rhodocyclaceae*, *Veillonelaceae* and *Bacillaceae*. Archaeal families in 2nd chamber were *Methanoregullaceae* (1.068 %), *Methanobacteraceae* (0.316%) and *Methanomicrobiales* (1.415%).

In case of 3rd and 4th chambers there was no dominant family. *Helicobacteraceae* was reduced in 3rd (2.481%) and 4th (3.448%) chamber during February.

In 3rd chamber *Porphyromonadaceae*, *Syntrophaceae*, *Helicobacteraceae* and *Desulfomicrobiaceae* were comparatively more among other families and each of these families constitute 2 to 4%. While other families which are less than 2 % were *Commamonadaceae*, *Sphingomonadaceae*, *Caulobacteraceae*, *Anaerolinaceae*,

Clostridiaceae, *Peptostreptococcaceae*, *Rhodocyclaceae*, *Erythrobacteraceae*, *Verucomicrobiaceae*, *Flavobacteraceae*, *Alcaliginaceae*, *Veillonellaceae*, Marinilabioaceae, *Acetobacteraceae*, *Desulphobacteraceae*, *Polyangiaceae* and *Ruminococcaceae*. Archaeal families in 3rd chamber are *Methanoregulaceae* (1.624%), *Methanobacteraceae* (0.411%), *Methylophillaceae* (1.013%) and *Methanosprillaceae* (0.382%).

In 4th chamber of ABR common families were *Desulfomicrobiaceae* 5.841%, *Commamonadaceae* 4.041%, *Helicobacteraceae* 3.44 %, *Caulobacteraceae* 3.14 %, *Syntrophaceae* 3.191%, *Porphyromonadaceae* 2.85 %, *Clostridiaceae* 2.41 % and *Xanthomonadaceae* 2.136 %. Families less than 2 % were *Peptostreptococcaceae* and *Pseudomonadaceae*. Archaeal families were *Methanoregulaceae* (1.248%), *Methanobacteraceae* (1.222%) and *Methylophillaceae* (1.763%).

Community shift from 1st to 4th chamber during month of February was observed as each chamber had different community composition with some families which were appeared in one chamber and absent in another (previous) chamber and by variation in their percentage. Methanogens were not detected in 1st chamber, but appeared in 2nd, 3rd and 4th chambers. Maximum archaeal families were detected in 3rd chamber, while 4th chamber had same archaeal families which were present in 3rd chamber. All the methanogenic families were less than 2% in three chambers. Significant difference was observed in community composition between 1st and 4th chamber showing complete shift in community composition. Diversity trend went on increasing from 1st chamber to onward and then dropped in 4th chamber.

During April microbial diversity observed was significantly more than that observed in the month of February in all the chambers of ABR except 4th chamber as only single family was detected in 4th chamber in April. Among those, 3rd chamber showed maximum diversity, 3rd chamber had more diversity as compared to all the months and all the chambers. Archaeal families were present in all the chambers except 4th chamber. Each chamber had different archaeal community composition. *Commamonadaceae* was in increased volume during April as compared to February and some new families were also appeared, which were absent during Feb.

Core microbial flora common in three chambers during April was *Helicobacteraceae*, *Commamonadaceae*, *Porphyromonadaceae*, *Sphingomonadaceae*, *Clostridiaceae*, *Methanoregulaceae*, *Methanobacteraceae*, *Weeksellaceae* and *Methylobacteraceae*.

In 1st chamber, 19 different families were detected in the month of April. *Commamonadaceae* (8.620%) and *Verrucomicrobiaceae* (7.447%) were main families of 1st chamber while *Helicobacteraceae* 3.346%, *Porphyromonadaceae* 2.661%, *Desulfomicrobiaceae* 2.674%, *Holophagaceae* (2.588%) and *Sphingomonadaceae* 2.270% were common families. The other were less common families in this chamber which were less than 2% including *Caulobacteraceae*, *Syntrophaceae*, *Clostridiaceae*, *Verrucomicrobiaceae*, *Rhodospiralaceae*, *Flavobacteraceae*, *Desulfobacteraceae*, *Weeksellaceae*, *Polyangiaceae* and *Rhizobiaceae*. Archaeal families in this chamber were *Methylobacteraceae* (0.106%), *Methanobacteraceae* (1.141%) and *Methanoregulaceae* (0.835%). Microbial community of April 1st chamber was almost completely changed than February 1st chamber.

In 2nd chamber during April common families were *Helicobacteraceae* (11.203%), *Commamonadaceae* (7.330 %), *Sphingomonadaceae* (3.006 %) and *Pseudomonadacea* (3.749%). Other families which constitute (1-3%) were *Porphyromonadaceae*, *Desulphomicrobiaceae*, *Syntrophaceae*, *Anaerolinaceae*, *Clostridiaceae*, *Erythrobacteraceae* and *Rhodobacteraceae*. Families which are less than 1% were *Caulobacteraceae*, *Enterobacteraceae*, *Peptostreptococcaceae*, *Alcaliginaceae*, *Veilonellaceae*, *Weeksellacea* and *Oxalobacteraceae*. Archaeal families in April 2nd chamber were *Methanoregullaceae* (1.194%), *Methanobacteraceae* (1.937%), *Methylophillaceae* (0.734%) and *Methanosaetaceae* (1.185%). Appearance of new families and change in proportion was observed in April 2nd chamber as compared to February 2nd chamber.

Highest diversity among all the months and chambers was observed in 3rd chamber during the month of April. Almost 37 families were observed in this chamber. Prevailing families were *Commamonadaceae* (7.202%), *Caulobacteraceae* (7.111%), *Sphingomonadaceae* (6.572%), *Xanthomonadaceae* (5.006%) and *Erythrobacteraceae* (3.888%). Other bacterial families were less than 1% including *Helicobacteraceae*, *Clostridiaceae*, *Porphyromonadaceae*, etc. While families which

constitute (1-3%) were *Verrucomicrobiaceae*, *Rhodospirillaceae*, *Flavobacteraceae*, *Acetobacteraceae*, *Bradyrhizobiaceae*, *Sphingobacteraceae*, *Microbacteraceae*, *Legionellaceae* and *Aurantimonadaceae*. 2nd and 3rd chamber in this month samples showed comparatively close community composition. Some new families were appeared in this chamber which were absent in February 3rd chamber. Archaeal families in April 3rd chamber were *Methylobacteraceae* (0.559 %), *Methyloregulaceae* (0.083 %), *Methylococcaceae* (0.417 %) and *Methylophillacea* (0.795 %). Community composition was changed in each chamber. In 4th chamber of April only single family *Paenibacillaceae* (1.451%) was identified. No methanogenic microorganisms were found.

Microbial diversity was observed in all the four chambers of ABR during the month of June. 2nd chamber had comparatively more diversity among all the chambers. Microbial community composition w shared some families along the chambers. Common families along the four chambers of ABR during June were *Helicobacteraceae*, *Comamonadaceae*, *Syntrophaceae*, *Anaeroliniaceae*, *Clostridiaceae*, *Holophagaceae*, *Methanoregulaceae* and *Peptostreptococcaceae*. All the chambers in this month have relatively close community composition.

Helicobacteraceae was predominant family in 1st, 2nd and 3rd chamber and significantly reduced in 4th chamber. In 1st chamber during June *Helicobacteraceae* (32.958%) was leading family. *Porphyromonadaceae* was less common family with 3.390% its total content. Minor families include *Commamonadaceae* 0.89 4%, *Desulfomicrobiaceae* 1.592 %, *Caulobacteraceae* 0.271 %, *Syntrophaceae* 1.792, *Anaerolinaceae* 0.948%, *Clostridiaceae* 0.552 %, *Holophagaceae* 0.531%, *Peptostreptococcaceae* 0.623 %, *Veilonellaceae* 0.390 %, *Desulfobacteraceae* 0.677 %, *Ruminococcaceae* 0.482%, *Lachnospirraceae* 0.309%. Two archeal families detected in this chamber were *Methanoregulaceae* 1.235% and *Methanobacteraceae* 0.574%.

In June chamber 2 *Helicobacteraceae* 32.029% was the dominant family. The other common families were *Commamonadaceae* 6.17 %, *Porphyromonadaceae* 2.574% and *Caulobacteraceae* 2.151%. minor families include *Sphingomonadaceae* 1.212 %, *Syntrophaceae* 1.545%, *Anaerolinaceae* 0.973%, *Xanthomonadaceae* 0.850%, *Clostridiaceae* 0.812%, *Holophagaceae* 1.340%, *Peptostreptococcaceae* 0.517%,

Erythrobacteraceae 0.873%, *Rhodospirillaceae* 0.217%, *Veillonellaceae* 0.778%, *Rhodobacteraceae* 1.895%, *Acetobacteraceae* 0.328%.

In June chamber 3 *Helicobacteraceae* 23.761% was dominant family but lower in proportion than in June chamber 2. Other common families include *Pseudomanadaceae* 5.308%, *Desulfomicrobiaceae* 3.297 %, *Syntrophaceae* 3.080% and *Holophagaceae* 3.514%. minor families include *Porphyromonadaceae* 1.953%, *Caulobacteraceae* 1.060%, *Anaerolinaceae* 0.517%, *Xanthomonadaceae* 1.210%, *Clostridiaceae* 1.093%, *Peptostreptococcaceae* 0.626%, *Verrucomicrobiaceae* 0.142%, *Flavobacteraceae* 0.751%, *Rhodobacteraceae* 0.901%. Archaeal families include *Methanoregulaceae* 0.951%, *Methylophillaceae* 0.951%, *Methylococcaceae* 1.761%.

For June 4th chamber, some common families were found including *Syntrophaceae* 5.796 %, *Desulfomicrobiaceae* 3.768 %, *Sphingomonadaceae* 3.549 % and *Holophagaceae* 3.245 %. Minor families of this sample were *Helicobacteraceae* 1.193%, *Caulobacteraceae* 1.934 %, *Anaerolinaceae* 0.515 %, *Clostridiaceae* 1.248 %, *Peptostreptococcaceae* 0.640 %, *Verrucomicrobiaceae* 0.398 %, *Flavobacteraceae* 0.952%, *Chitinophagaceae* 0.507% and *Desulphobacteraceae* 0.195 %. Archaeal families detected in this chamber were *Methylococcaceae* 0.437%, *Methanoregulaceae* 1.053% and *Methylophilaceae* 0.484%.

For the month of October 1st chamber of ABR was least characterized up-to family level and no family was detected in this chamber. It contained 100% population of gammaproteobacteria. In 2nd chamber single family *Caulobacteraceae* 11.853% was detected. The result for these two chambers during October was quite unexpected. While diversity in microbial community composition was observed in 3rd and 4th chamber, 3rd chamber had more microbial families than 4th chamber.

In 3rd chamber of ABR for the month of October *Commamonadaceae* 12.738 % was leading family. *Helicobacteraceae* accounts for 2.812 %, and *Enterobacteraceae* 3.901%. Other minor families were *Porphyromonadaceae* 1.956 %, *Desulphomicrobiaceae* 0.884 %, *Sphingomonadaceae* 0.778%, *Caulobacteraceae* 1.250 % etc, while among archaeal families in this only *Methanobacteraceae* (0.784 %) were detected.

Common families in 4th Chamber of October month sample were *Comamonadaceae* 7.312 %, *Porphyromonadaceae* 5.069 %, *Enterobacteraceae* 4.232 %, *Helicobacteraceae* 2.645 % and *Desulphomicrobiaceae* 2.282 %. *Syntrophaceae* 1.113 %, *Clostridiaceae* 1.492 %, and *Peptostreptocococaceae* 1.603 %. Other including *Caulobacteraceae, Anaerolinaceae*, *Pseudomonadaceae*, *Syntrophobacteraceae, Ruminococcaceae* and *Syntrophorhabdaceae* were less than 1 %. Archaeal families include *Methanoregulaceae* 0.340 %, *Methanobacteraceae* 0.150%, and *Methylophilaceae* 0.561%. All the families of 4th chamber of October were like 3rd chamber of ABR in October except *Anaerolinaceae*, *Methanoregulaceae* and *Methylophillaceae*.

Only few families *Porphyromonadaceae*, *Desulphomicrobiaceae*, *Caulobacteraceae* and *Weeksellaceae* were common among the four chambers of ABR during December. 2nd Chamber of ABR showed highest diversity while 4th chamber had lowest diversity during December. Community composition of all the chambers to some extent had similar families, but chamber 2 and chamber 3 were comparatively close to each other. Difference was observed in chamber 1 and chamber 4. Many families of chamber 1 disappeared in chamber 4. Fifty percent community composition of chamber 4 was like chamber 1.

Comamonadaceae was leading family of 1st chamber of December and constitutes 14.97 % of the total population. Other families including *Porphyromonadaceae* 3.929 %, *Desulfomicrobiaceae* 1.743 %, *Caulobacteraceae* 0.658 %, *Xanthomonadaceae* 0.323%, *Clostridiaceae* 1.462 %, *Enterobacteraceae* 2.517 %, *Peptostreptococcaceae* 0.6 %, *Erythrobacteraceae* 0.344 %, *Rhodospirallaceae* 0.244 %, *Veilonellaceae* 0.919 %, *Bradyrhizobiaceae* 0.642 %, *Marinilabiaceae* 0.874 %, *Weeksellaceae* 0.629 %, *Lachnospirraceae* 1.006 %, TTA-B6 0.244 %, *Oxalobacteraceae* 0.691%, *Aerococcaceae* 0.290 % and *Hyphomicrobiaceae* 1.006 %. Archaeal community include *Methanobacteraceae* 0.857 % and *Methanosarcinales* 0.099 %.

Hyphomicrobiaceae, *Oxalobacteraceae*, *Methanosarsinales* and *Bradyrhizobium* were unique families of chamber 1, which were absent in other 3 chambers during the month of December.

In December 2nd chamber *Comamonadaceae* was comparatively higher in proportion and constitute about 11.918%. other common families were *Porphyromonadaceae* 4.849 % and *Xanthomonadaceae* 2.273 %. Minor families include *Desulfomicrobiaceae*, *Sphingomonadaceae* *Caulobacteracea*, *Syntrophaceae*, *Anaerolinaceae*, *Peptostreptococcaceae,* etc. Archaeal community include *Methanobacteraceae* 1.047 %, *Methylophillaceae* 0.456 % and Methanosetaceae 0.237 %.

Chitinophagaceae was unique family of 2nd chamber which was absent in other chambers during December. In 3rd chamber for December *Comamonadaceae* was present in 10.881%, while *Porphyromonadaceae* and *Desulfomicrobiaceae* were present in 5.661% and 3.774% respectively. Minor families include Helicobacteraceae 1.045 %, *Caulobacteracea* 1.328 %, *Syntrophaceae* 1.593 %, *Anaerolinaceae* 0.972%, *Xanthomonadaceae* 1.621%, *Clostridiaceae* 1.0621%, *Peptostreptococcaceae* 1.006 %, *Veillonellaceae* 0.75 %, *Marinilabiaceae* 0.904%, *Weeksellaceae* 1.175%, *Lacnospiraceae* 0.915%, TTA-B6 0.305 %, *Aerococcaceae* 0.505%, and *Erysipelotrichaceae* 0.203%. Archaeal community includes *Methanoregulaceae* 0.944 %, *Methanobacteraceae* 1.887 %. *Erysipelotrichaceae* was new family of chamber 3 while absent in others.

Common families in December chamber 4 include *Rhodocyclaceae* 7.828 %, Helicobacteraceae 6.044 %, *Desulphomicrobiaceae* 5.289 %, *Porphyromonadaceae* 3.631 % and *Syntrophaceae* 2.560 %. Minor families were *Pseudomonadaceae* 0.609 %, *Verrucomicrobiaceae* 0.483%, *Alcaligenaceae* 0.315 %, *Weeksellaceae* 0.420 %, *Ruminococcaceae* 1.196 %. Member of archaea were absent in chamber 4 of December. *Alcaligenaceae* was new family of 4th chamber of December, absent in other chambers.

Overall bacterial diversity variation and abundance along the 4th chamber of field scale ABR was lower than the diversity along the 1st chamber of ABR. Many bacterial families were disappeared which were present in 1st chamber (Fig 4.12).

Community dynamics along 1st chamber of ABR

Among two microbial domains along the 1st chamber archaeal community organization comparatively was less variable than the bacterial community organization. Four archaeal families (*Methanoregulaceae, Methanobacteraceae, Methylobacteraceae* and *Methanosarcinales*) were detected along the 1st chamber during whole period. These families were identified in 1st chamber during April (*Methanoregulaceae, Methanobacteraceae, Methylobacteraceae*), June (*Methanoregulaceae* and *Methanobacteraceae*) and December (Methanobacteraceae and *methanosarcinales*). Archaeal families were not identified in 1st chamber during February and October.

A wide diversity of bacterial families detected along 1st chamber; 31 bacterial families were identified. Bacterial community organization was changed along 1st chamber showing that bacterial community composition changed over time from February to December and new families appeared in each month. Core bacterial families *Helicobacteraceae* (absent in December), *Commamonadaceae* and *Desulfomicrobiaceae* were shared in almost all months. In April and December maximum change in community composition was observed. From February to December complete shift in bacterial community was seen. Microbial community was uneven along the 1st chamber. Each month has few shared and more unique families. Abundance distribution of bacterial families along the 1st chamber of ABR depicted that *Helicobacteraceae* was leading family but it was not stable as its percentage varied randomly from one month to another and *Commamonadaceae* was 2nd common family. Higher differentiation of bacterial community is found at family level along 1st chamber of ABR (Fig 4.12).

Community dynamics along 4th chamber of large scale ABR

Difference and similarity in microbial community composition along 4th chamber of ABR was analyzed. No predominant family exists in 4th chamber of ABR along the months. Instead *Helicobacteraceae* and *Commamonadaceae* were common bacterial families. 25 families were present along 4th chamber of ABR. Bacterial community composition along 4th chamber was less dynamic as compared to 1st chamber. Many bacterial families were disappeared which were present in 1st chamber. Core bacterial

families along 4th chamber are *Helicobacteraceae*, *Comamonadaceae* and *Desulfomicrobiaceae*. Some unique families were present in 4th chamber of each month along 4th chamber. Four archaeal families were present along 4th chamber. It was less variable and diverse, but abundance distribution of archaeal families was more than 1st chamber. Archaea were absent in April and December 4th chamber. Development of microbial community is dynamic process and was affected by various factors (Fig 4.12).

4.3.6 Diversity observed at species level

Total number of species, their abundance and diversity were dissimilar in each month and each chamber. At species level, *Lithotrophicum* was found as core biomarker in sample of 1st chamber for the month of February 58.96 %. It belongs to phylum *Proteobacteria* and family *Helicobacteraceae* which constitute major portion of these two samples. Minor species in 1st chamber of February which were below 2 % include fermentans, orale, palustris, foetida, ruminatium, beijingense, acidiphilum, succinatiumandens, mobilis. Unique species of this chamber were acidophilum and succinatimandens. In 2nd chamber for the month of February *Lithotrophicum* was found as highlighted species. No unique species was detected in this chamber. While in case of chamber 3rd of February no highlighted species and no unique species was found. *Lithotrophicum* was reduced up to minimum (0.509%) in 3rd chamber. No dominant species is detected in overall chamber 4 samples during February and lower species diversity was observed along the chamber 4th at species level. Detected species in this chamber were *Lithotrophicum* 3.448 % *Fermentans* 2.239 %, *Orale* 5.841 %, *Palustris* 1.248 %, *Ruminantium* 1.145% and *Versatilis* 1.763%. *Lithotrophicum* was major species in chamber 1 of the February and was reduced up to 3.448%, in chamber 4 and *Fermentans* slightly increased in from (1st chamber)1.061% to (4th chamber) 2.239 %. *Orale* increased in chamber 4 from 1.367 to 5.841%, *Ruminantium* showed almost 1% increase, *Palustris* remained almost same. While *Foetida*, *Beijingense*, *Acidiphilum*, *Succinatiumandens*, *Mobilis* disappeared in chamber 4. A new species *Versatilis* (absent in chamber 1) appeared in chamber 4 of Feb and constitute 1.763%.

The later reduction in *Lithotrophicum* was noticed as during April as compared to February. At species level in 1st chamber of April *Orale* and *Foetida* were 2.674 %

and 2.588 % respectively. Minor species below 2 % range were *Lithotrophicum*, *Fermentans* and *Sulfurophila*, *Palustris*, *Ruminatium*, *Versatilis*, *Beijingense*, Aciditrophicus, *Lacus*, *Spinosum*, *Conservatix*, *Mobilis*, *Selenitireducens* and *Naphthalenivorans*. Unique species of this sample which were absent in other samples along the chamber 4 include *Versatilis*, *Aciditrophicus*, *Lacus*, *Spinosum*, *Selenitireducens* and *Naphthalenivorans*.

Lithotrophicum sp. in chamber 2nd of ABR for the month of April was 10.284 % and disappeared in 3rd chamber of April. *Pohangensis* was a unique species of this chamber, not present in others. While in case of 3rd chamber of April, a wide diversity with unique bacterial species was detected and most of them were found absent/rare in other chambers species which include: *Spinosum*, *mathuresis*, *massiliensis*, *Ansorpii* etc. For month of April chamber 4th, no species was identified.

Lithotrophicum was also found as core biomarker in sample of 1st chamber for the month of June 30.911%. Minor species in chamber 1 of June constitute below 2 % were fermentans, orales, palustris and sulfuriphila foetida, ruminantium, nitrogenifigens, beijingense, conservatix orale, xylanivorans and vannielii. Only single species *Nitrogenfigenes* belonging to this sample was unique among chamber 1 of ABR. In June chamber 2 contained *Lithotrophicum* (30.22 %) was highlighted species. Unique species of this chamber was *Xylanolytica* which was present in minor proportion. *Lithotrophicum* was reduced up to 21.35 in chamber 3rd of June. No unique species was appeared in this chamber. In June chamber 4th species of *Orale*, *Foetida* and *Acidiphilum* constitute 3.768 %, 3.245 % and 3.549 % respectively while Palustris and *Aciditrophicus* constitute lower proportion and are 1.053% and 1.240% respectively. Some minor species which are below 1% are *Ruminantium*, *Versatilis*, *Tardivitalis* and *Spinosum*. In June chamber 1 *Lithotrophicum* was dominant species and disappeared in chamber 4 of June. In chamber 4 of June no dominant species is identified. *Fermentans*, *Sulfuriphila*, *Nitrogenifigens*, *Beigingense* and *Conservatix* which were present in 1st chamber, also disappeared in 4th chamber. New species which appeared in 4th chamber for month of June were *Versatilis*, *Acidiphilum*, *Tradivitalis* and *Spinosum*.

Sample of October chamber 1 contained 100 % *gammaproteobacteria*. In October 2nd chamber, single unidentified species was detected. While in 3rd chamber of October

several species appeared having some unique species *Pohangensis* and *Thermalis* (1.184%). No dominant species was detected in this chamber. While in 3rd chamber of October several unique species appeared, *Pohangensis* and *Thermalis* (1.184%). No dominant species was detected in this chamber. October 4th chamber contain *Fermentans* 3.335%, *Orale* 2.82%, *Ruminants* 1.098% and *Aciditrophicus* 1.113%. Other species which account less than 1 %, were *Sulfuriphila*, *Versatilis*, *Pohangensis*, *Acidipica* and *Aromaticivorans*.

For December chamber 1 *Fermentans* were 3.370%. Other species which constitute from 1-2% species were *Orale*, *Xylanivorans* and *Vannielii*. Species which account lower than 1% were *Lithotrophicum*, *Sulfuriphila*, *Xylanolytica*, *Beijingense* and *Mobilis*. Unique species were *Xylanolytica*, *Xylanivorans* and *Vannielii*. No highlighted species were detected in 1st chamber for month of April and December. During December, in 2nd chamber good diversity of species was observed with some common/shared and unique species. Unique species include *Lysobacter*, *Thermalis*, *Kawasakiensis*, *Indigens*, *Sticklandii* and *Lapsinanis*. These were present as minor species. While chamber 3rd had less species diversity than 2nd chamber of December. Two species were unique in this chamber *Xylanovorans* and *Furcosa*. In December 4th chamber *Succinamendans* 7.824 %, *Lithotrophicum* 4.806 %, *Orale* 5.289 %, *Fermentans* 3.631% and *Selenitireducens* 2.078 % were comparatively higher. *Pohangensis* constitute lower than 1% of total population. *Lithotrophicum* and *Orale* increased in December chamber 4 up to 4.806 % and 5.289 % respectively. *Fermentans* remained almost same as in chamber 1 (3.631%). Three new species appeared which were absent in chamber 1, *Succinatimandens* 7.824%, *Selenitireducens* 2.078% and *Pohangensis* lower than 1%.

Lithotrophicum was found as core biomarker in sample of 1st chamber for the month of February and June. The later reduction in *Lithotrophicum* was noticed as during April. No highlighted species were detected for month of April, October and December. Some species are seen to fluctuate simultaneously. No dominant species is detected in overall along 4th chamber samples. Lower species diversity was observed along the 4th chamber as compared to 1st chamber.

Microbial community structure analysis shows that ABRs also share common microbial prevalence with other anaerobic reactors with some unique microorganisms

in each chamber and temperature set as helicobacteraceae and comamonadaceae. Overall dominance was shown by proteobacteria followed by bacteriodetes and firmicutes. The results agree with bacterial diversity of 14 wastewater treatment plants from China from varying geological locations (Wang et. al., 2012). Proteobacteria is dominant phylum generally consistent with fact that it is common shared phylum along other functional species in wastewater treatment (He et al., 2015). Abundance of chloroflexi in ABR may be due to its metabolic flexibility which make it able to grow and prevail in any conditions during wastewater treatment plants (Breuker et al., 2013). Decreasing pathogens count observed later shows their deactivation due to predation or naturally die off (Andersson et al., 2008).

a)

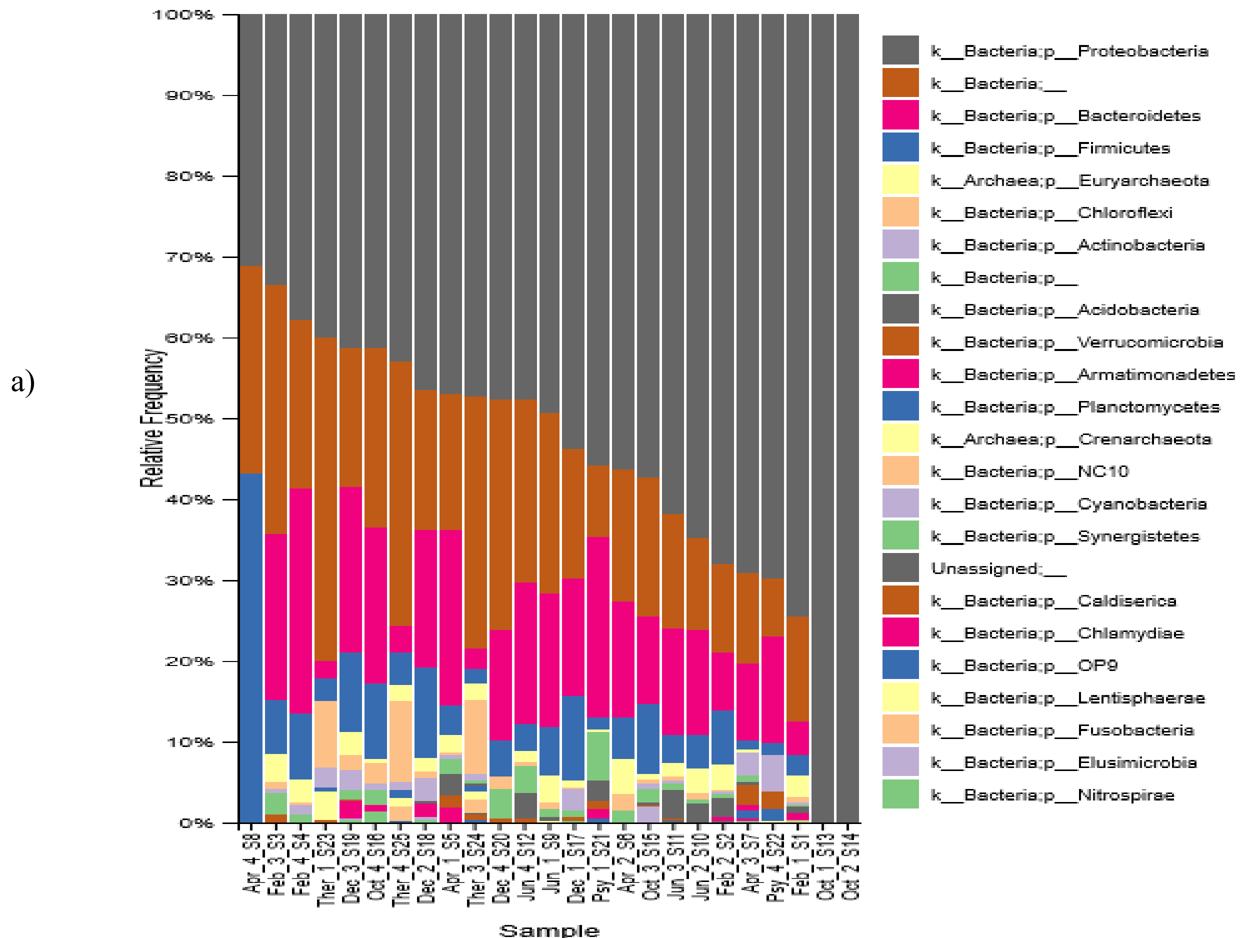


Figure 4.11 Stacked barplot depicting percentage relative abundance of 16S DNA community structure of 3 individual ABRs at phylum level

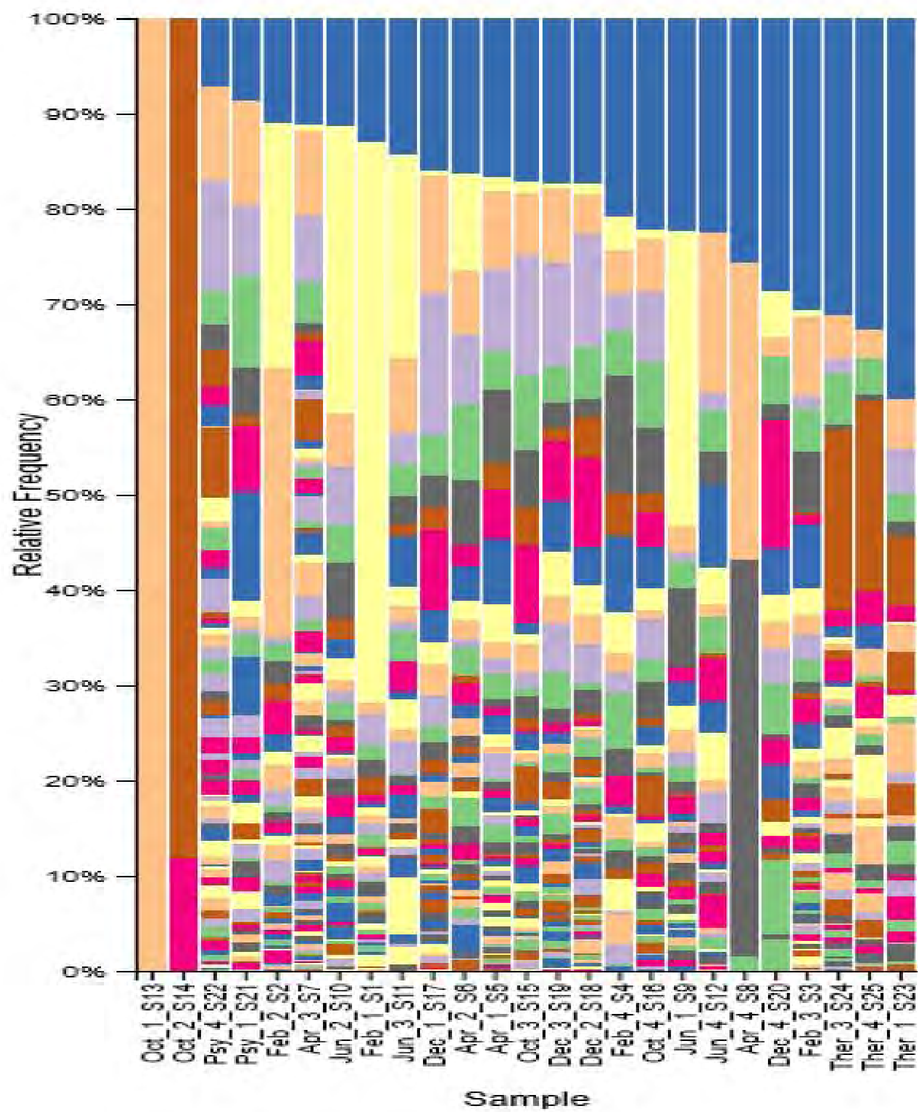


Figure 4.12 Stacked barplot depicting percentage relative abundance of 16S DNA community structure of 3 individual ABRs at fsmily level

Beta diversity refers to species differences between the communities of different samples. Diversity associated to relationship of two or more samples is called beta diversity. It showed that bacterial and archaeal community composition was varied within the reactor. It calculates similarity and dissimilarity/distance between the samples. Beta diversity between samples measured using Bray Curtis, weighted Unifrac and unweighted Unifrac to measure distance between samples based on relatedness and abundance of shared and unshared OTUs. Analysis was performed to measure distance between samples in terms of species composition in following ways: ambient recorded temperature and time in chamber. PCoA (Principal coordinates analysis) was widely used for conceptualization of distance metrics. PCoA used to examine samples dissimilarity using Bray Curtis distance. Emperor (a local visualization tool which enables to visualize scattered plot) was used for visualization of scattered plot and distance metrics. It generated 3D plots. Each dot in a plot represents one sample plotted according to composition of OTUs and abundance showing correlation between two dots (samples), their similarity and distance. Visualization allows efficient understanding of information. Pairwise distance metric is represented by PCoA. With increase of sequencing depth, some samples are lost. In this case 1 or 2 samples were lost.

Bacterial diversity between different samples was calculated by using weighted & unweighted Unifrac distances to measure diversity between the samples. The method is phylogenetically sensitive as distance between the sample is measured based on relatedness and abundance of shared and unshared OTUs. Principle coordinate analysis (PCoA) was used to represent pair wise distance matrix.

Emperor plots showed how the grouping/group composition of the species change with change in condition. As species vary in groups when conditions are changed, it

affects whole group composition. Bray Curtis was used to analyze either group composition is different or same with change in condition. It showed how the group composition changed with change in condition, as cluster formation varies in species composition by changing condition.

Species which were closer to each other in the plot, they have more similarity in features/ characteristics. It does not give result in terms of significant or non-significant or in terms of number of species; it gives the way in which they disperse while changing the condition. Dispersion depends on condition. It gives us a picture of species grouping. If more the distance, more will be the difference in species characters.

Metagenomic analysis based on taxonomy depicts that microbial communities were dominated by bacteria, as bacteria form major portion of all the ABR samples. Proportion of archaea was very less in all the samples. All these metrics used for beta diversity measurement refer to distance metrics, which measured difference between community compositions of different samples. All these 4 metrics are same except they use different method. As how the samples vary in terms of species abundance and difference created in the groups when condition was changed. Some of the samples (balls) overlap as those samples shared some communities in them/ have same species. Three different approaches were used to evaluate beta diversity measures. Beta diversity explored additional informative aspects about variation of communities with time, temperature and chamber, as they are not captured by modeling alpha diversity only. Pair wise measures are helpful to identify time or situation at which community shifts are observed. Effect of temperature and chamber number of ABR on microbial communities' studies showed variation in results. Pairwise dissimilarities in microbial communities of different samples were compared. Comparisons display not much variability; some communities were different in some samples. Each sample presented by a ball with a different color. Communities within a sample also show some variation but that variation is lower than that of variation between the samples. PCoA analysis is used to evaluate relationship between samples along orthogonal axes of variance.

Through PCoA (beta diversity) divergences of bacterial communities were revealed in different chambers of ABR and at different temperature. Development rate of

archaeal community was at slower as compared to bacterial community. It relates to community organization between the samples. It investigates differences in two or more than two communities.

Bray Curtis distance

It measures compositional dissimilarity between samples. At temperature 13-14°C all four groups of samples had different but closely related species composition as these four samples showed composition different from each other having distinct clusters. All the four samples move in relatively same direction. December chamber 1, 2 & 3 have less difference in species composition while those had more distance in composition from chamber 4. Bacterial communities at temperature 15-17°C showed variation in their composition two groups of samples (Feb. chamber 3rd and 4th) at this temperature move towards one side and other two groups (Feb. 1 & 2) move in other direction. It showed these four groups at one temperature range have very different composition in pairs (Figure 4.13).

At temperature 24-25°C two samples (October, chamber 3 and 4) move in same direction and have closer community composition. While the other two samples (October, chamber 1st & 2nd) move in the same direction and also have close relation in community composition. At temperature 27-30°C, three samples (April chamber 1st, 2nd and 3rd) have divergent species composition. It clearly separates all the samples for their composition as expected from their environmental conditions. It shows that temperature has effect on bacterial community composition. Each sample form different cluster within given environment. At temperature 32-35°C, three samples June 1st, 2nd & 3rd chambers have related species composition (Figure 4.16). Samples of chamber 1st & 2nd had closer species composition than chamber 3. The community was comparatively stable in 3 samples and developed slowly. While sample 4 (June, chamber 4th) have very far composition of species from other three samples. Temperature has greater effect on microbial community composition as some samples are located on opposite sides under same condition.

In chamber 1 of ABR all the samples had distinct community composition in terms of species. Two samples February, chamber 1st and June chamber 1st were relatively close in species composition. In case of chamber 2, divergence of composition was

observed but two samples (Feb 2nd chamber and June 2nd chamber) demonstrated closely related species composition. In chamber 3 of December and October comparatively a close composition was observed while other samples were dispersed, and June chamber 3rd had very far species composition. Greatest distance was calculated between February 1st chamber and December chamber 1. Also, a greatest distance was measured of December chamber 2nd sample from that of February chamber 2nd and June chamber 2nd. While June chamber 2nd and February chamber 2nd had close community composition. In chamber 4 of December and February there was related species while others had dispersed species composition (Fig. 4.13).

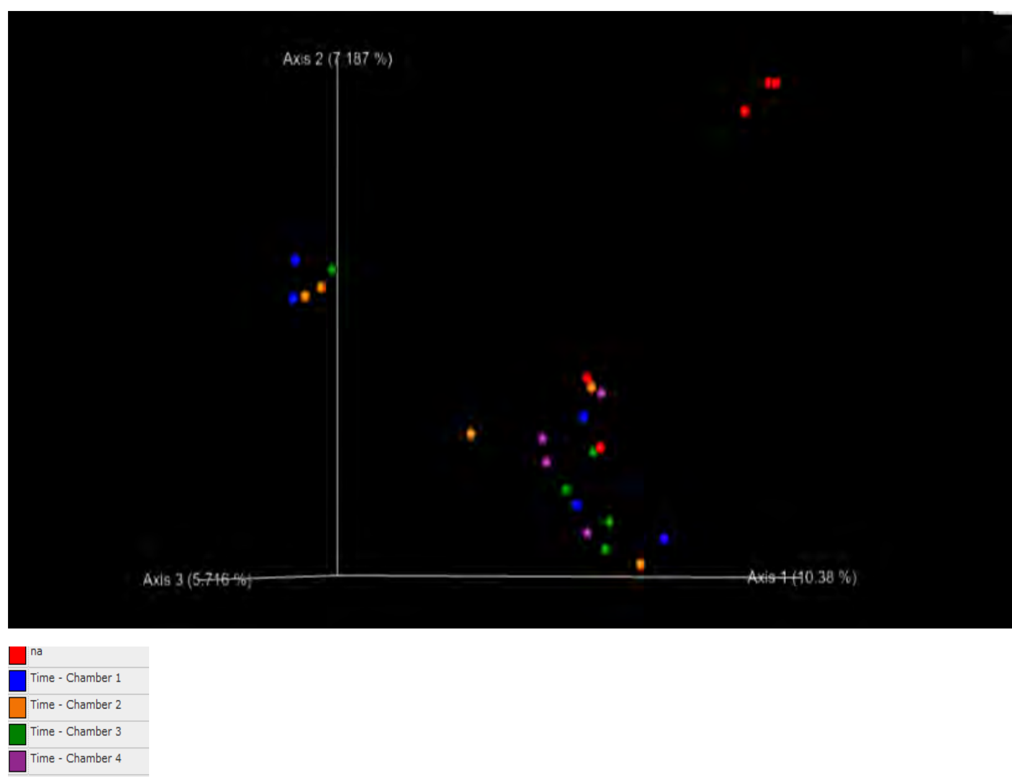


Figure 4.13 Multidimensional scaling of the bacterial and archaeal communities in 3 ABRs samples derived from Bray Curtis distance with respect to time in chamber.

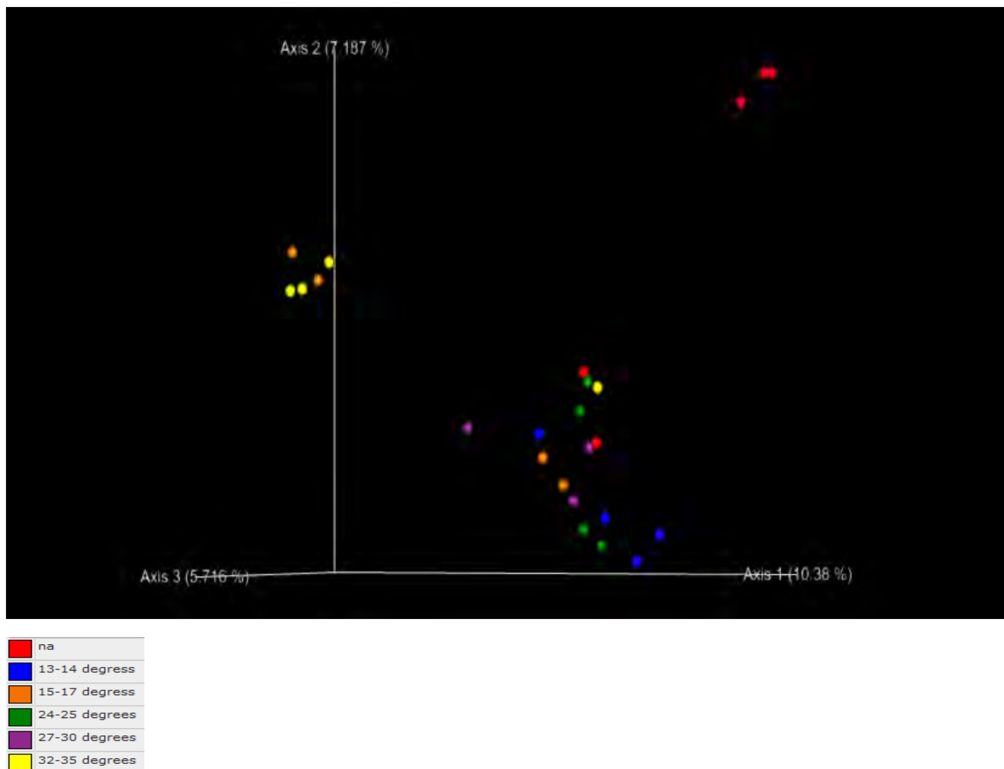


Figure 4.14 Multidimensional scaling of the bacterial and archaeal communities in 3 ABRs samples derived from Bray Curtis distance with respect to treatment temperature.

Weighted Unifrac distance

Unifrac method expanse to unweighted and weighted Unifrac distance to identify a broad range of biologically relevant shift. This discrepancy happens because unifrac method takes into account phylogenetic distance of taxa (as OTUs) while calculating distance among samples. Significant difference in community composition was observed overall at ambient temperature treatment. Significant positions differences were occurred between the samples from different compartments and months. At temperature 13-14°C composition was closer. At temperature 15-17°C sample of February chamber 3rd and 4th overlap indicating that they shared community composition. Sample of October chamber 4 at temperature 24-25°C and April chamber 1st at temperature 27-30°C also showed very close composition. In group significance comparative analysis for each set of chambers (e.g. five samples of chamber 1, five samples of chamber 2) and temperature was performed in terms of significant or insignificant difference (Figure 4.15). In case of time in chamber group

significance, no significant difference is observed, as *p*-value was greater than 0.05 for all the 2 groups. For temperature group significance, a significance difference in community composition was seen between the community composition of samples at temperature 13-14°C and 15-17°C having *p*-value 0.028, between samples at temperature range 13-14°C and temperature 32-35°C having *p*-value 0.023 and between samples at temperature 24-25°C and 32-35°C having *p*-value 0.022. While other comparisons do not show a significant difference in community composition (Figure 4.16).

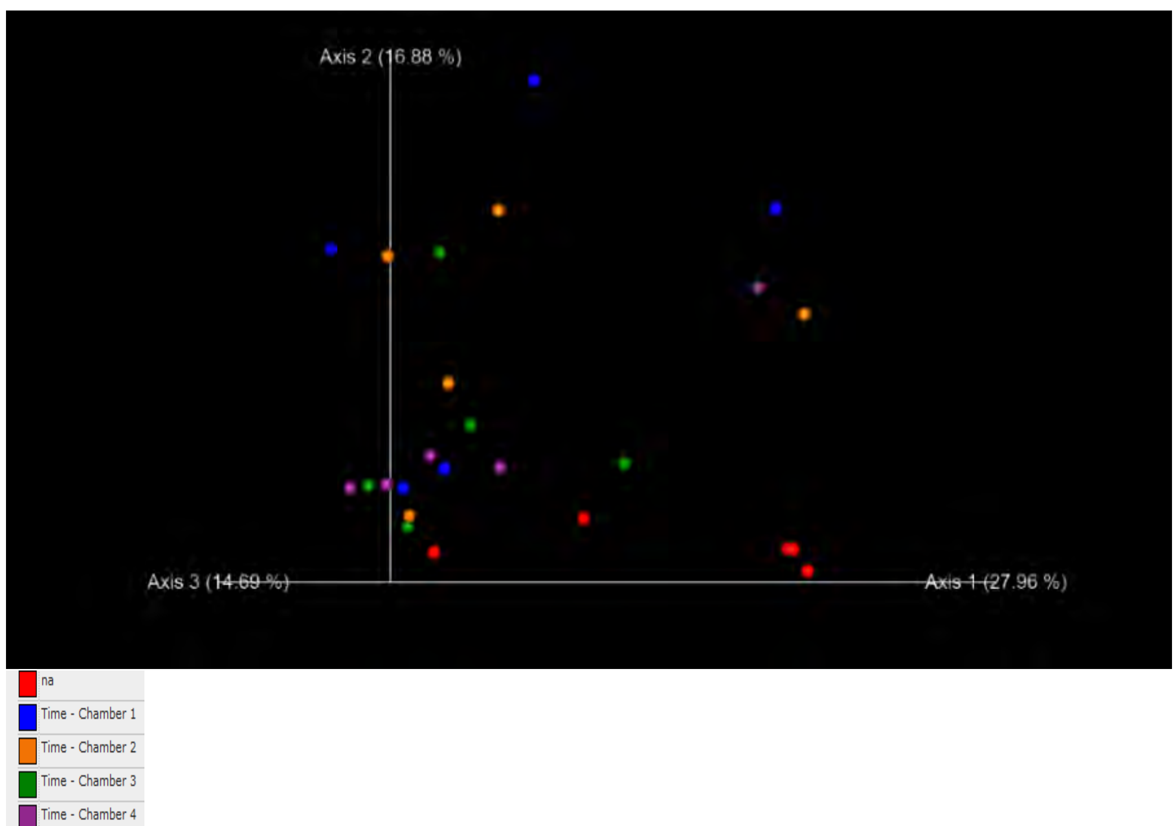


Figure 4.15 Multidimensional scaling of the bacterial and archaeal communities in 3 ABRs samples derived from Weighted UniFrac distance with respect to time in chamber.

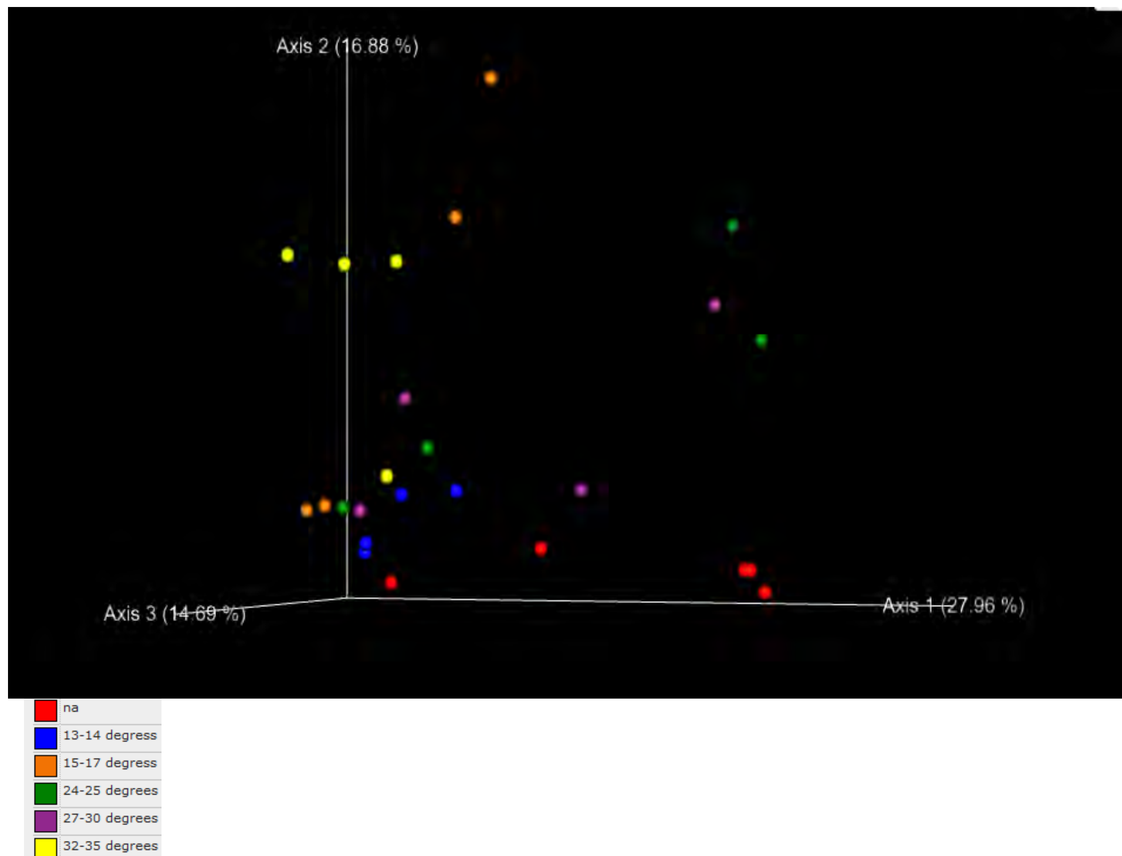


Figure 4.16 Multidimentional scaling of the bacterial and archaeal communities in samples of 3 ABRs derived from Weighted UniFrac distance with respect to temperature.

Unweighted Unifrac distance

With variation of temperature and chamber for unweighted Unifrac all the samples showed very close community composition in terms of quality except some samples. At temperature 24-25°C October chamber 1st & 2nd and at temperature 27-30°C April chamber 4th was located at another pole. In case of chamber October chamber 1st & 2nd and April chamber 4th were located on the other pole having greater difference in their community composition. For pairwise difference among groups based on chamber number was not significant and overall *p*-value for this was 0.66 (Figure 4.17). While in case of group significance with temperature significant difference in group composition at different temperature were shown significant between samples at temperature 13-14°C and 15-15°C with *p*-value 0.023, between 13-14°C and 32-35°C with *p*-value 0.034, between 15-17°C and 24-25°C with *p*-value 0.037, 24-25°C and 32-35°C with *p*-value 0.053. Other groups have insignificant difference in terms

of community composition. Overall p-value for difference in groups at different temperature was significant having p-value 0.002 (Figure 4.18).

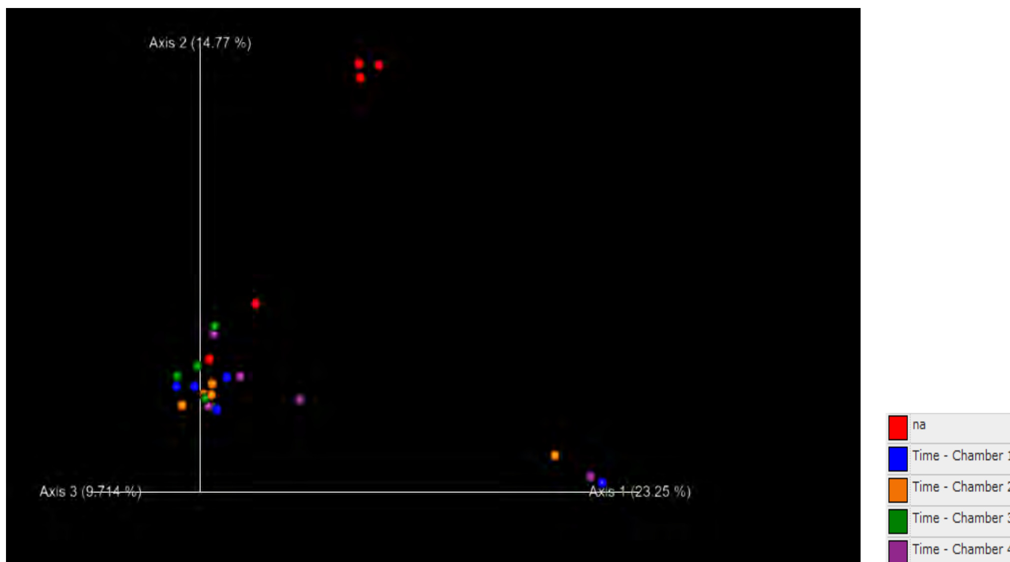


Figure 4.17 Multidimentional scaling of the bacterial and archaeal communities in 3 ABRs samples derived from Unweighted UniFrac distance with respect to chamber.

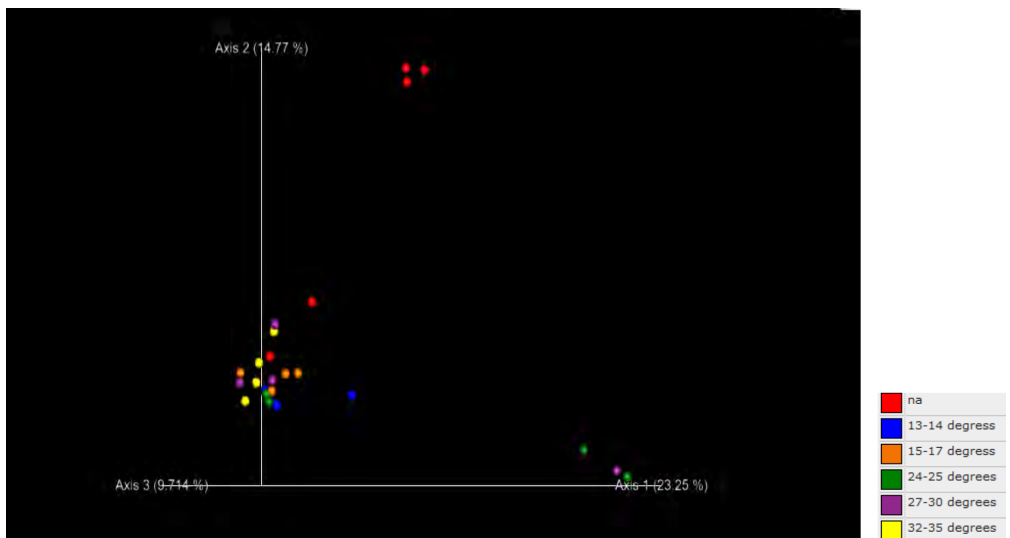


Figure 4.18 Multidimensional scaling of the bacterial and archaeal communities in 3 ABRs samples derived from Unweighted UniFrac distance with respect to temperature

4.3.7 Microbial analysis of Lab scale ABR

At low temperature

For ABR working at low temperature sampled from 2 chambers two chambers (1st chamber and 4th chamber) was assessed for their microbial ecosystem. For laboratory scale ABR working at low temperature, great bacterial diversity was observed. At phylum level, 1st chamber showed more diversity as compared to 4th chamber. 8 bacterial phyla detected with abundant *proteobacteria* 55.851%. Other phyla are *bacteriodetes, firmicutes, acidobacteria, verrucomicrobia, armatinonadetes, planctomycetes* and one archaeal phylum euryarchaeota were detected in 1st chamber. Six bacterial phyla detected in 4th chamber include *proteobacteria, bacteriodetes, firmicutes, actinobacteria, verucomicrobia* and *planctomycetes*. At family level both chambers (1st and 4th chamber) had significant diversity, but 4th chamber showed comparatively higher diversity than 1st chamber.

Significant diversity was observed in laboratory scale ABR working at low temperature. If we compare microbial diversity in 1st and 4th chamber of low temperature ABR, it was observed that at phylum level greater diversity was observed in 1st chamber (*proteobacteria, Bacteroidetes, firmicutes, euryarchaeota, acidobacteria, verrucomicrobia, armatimonadetes, planctomycetes*) than in 4th chamber (*proteobacteria, bacteriodetes, firmicutes, actinobacteria, verrucomicrobia, planctomycetes*). But dominance of phyla was consistent in both chambers. As in both chambers *proteobacteria* was the most abundant phylum and *bacteriodetes* was 2nd most abundant phylum. Proteobacteria was observed to increase in 4th chamber as compared to 1st chamber, and *bacteriodetes* instead decreased in 4th chamber as compared to 1st chamber. While at family level 4th chamber had more diversity than 1st chamber. No archaeal phylum was found in 4th chamber while one archaeal phylum (*euryarchaeota*) was detected in 1st chamber.

At family level, in 1st chamber no dominant family was observed instead *Comamonadaceae* was comparatively abundant. Other bacterial families detected were *Helicobacteraceae*, *Desulfomicrobiaceae*, *Sphingomonadaceae*, *Caulobacteraceae*, *Holophagaceae*, *Pseudomonadaceae*, *Rhodocyclaceae*, *Verrucomicrobiaceae*, *Rhodospirillaceae*, *Chitinophagaceae*, *Veilonellaceae*,

Acetobacteraceae, *Marinilabiaceae*, *Desulfobacteraceae*, *Weeksellaceae*, *Pirrellulaceae* and *Nitrosomonadaceae*. While methanogenic families identified in this chamber are *Methanobacteraceae* (0.45 %) and *Methylophillaceae* (2.106 %).

In 4th chamber more bacterial diversity was observed at family level. *Comamonadaceae* was comparatively more than other bacterial families. Other families detected were, *Sphingomonadaceae*, *Caulobacteraceae*, *Pseudomonadaceae*, *Rhodocyclaceae*, *Verrucomicrobiaceae*, *Rhodospirillaceae*, *Chitinophagaceae*, *Veilonellaceae*, *Acetobacteraceae*, *Marinilabiaceae*, *Desulfobacteraceae*, *Weeksellaceae*, *Pirrellulaceae* and *Nitrosomonadaceae*, *Xanthomonadaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, *Erythrobacteraceae*, *Flavobacteraceae*, *Alcaliginaceae*, *Rhodobacteraceae*, *Sphingobacteraceae*, *Legionellaceae*, *Cyclobacteraceae* and *Lactobacillaceae*. Methanogenic families detected in 4th chamber were *Methylophillaceae*, *Methylococcaceae* and *Methylobacteraceae*.

At high temperature

For ABR working at high temperature three chambers (1st chamber, 3rd chamber and 4th chamber) were evaluated for their microbial ecology. At phylum level classification good diversity was observed in all the three chambers with proteobacteria as dominant phylum in all chambers. In 1st chamber *bacteriodetes* was second abundant phylum while in other two chambers *bacteriodetes* was present in small number. The other common phyla in three chambers were *firmicutes, chloroflexi, planctomycetes, crenarchaeota*. While *caldiserica* was present in 1st and 3rd chamber and absent in 4th chamber. Other phyla of 3rd chamber were *actinobacteria, acidobacteria, crenarchaeota, NC10, OP9* and in 4th chamber were *actinobacteria, crenarchaeota, NC10 and OP9*. Only one archaeal phylum *euryarchaeota* was detected in 3rd and 4th chamber while no archaeal phylum was detected in 1st chamber.

At family level, 3rd chamber had comparatively more bacterial diversity as compared to other two chambers. No dominant family was observed in three chambers. Common families of three chambers were *Anaeroliniaceae*, *Cystobacteraceae*, *Pirellulaceae*, *Polyangiaceae* and *Desulfurococcaceae*. Other families of 1st chamber were *Comamonadaceae*, *Syntrophaceae*, *Rhodocyclaceae*, *Chitinophagaceae*,

Alcaliginaceae, *Acetobacteraceae*, *Bradyrhizobiaceae*, *Nocardiaceae*, *Caldosericaceae* and *Caldilineaceae*. No archaeal family detected in 1st chamber. Families of 3rd chamber were *Comamonadacea*, Sphingomonadaceae, *Syntrophaceae*, *Clostridiaceae*, *Holophagaceae*, *Peptostreptococcaceae*, *Rhodocyclaceae*, *Rhodospirillaceae*, *Chitinophagaceae*, *Alcaliginaceae*, *Bradirhizobiaceae*, *Syntrophobacteraceae*, *Caldisericaceae*, *Bacillaceae* and *Gemmalaceae*. Archaeal families of 3rd chamber were *Methanoregulaceae* and *Methanobacteraceae*. Families found in 4th chamber were *Desulphomicrobiaceae*, *Syntrophaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, *Rhodospirillaceae* and *Syntrophobacteraceae*. Archaeal family of 4th chamber was *Methanoregulaceae*.

Anaerobic digesters have multiple sets of microbial communities e.g. each of the four stages of AD has different types of microorganisms. Community composition and efficiency of these microorganisms depends on various factors including temperature. In the present study, microbial diversity (based on V4 and V5 regions) of three ABRs was evaluated. For field scale ABR each of the four chambers of ABR were analyzed for their microbiology with relation to temperature variation. And for lab scale ABRs, 2 chambers (1st and 4th) for low temperature and three chambers (1st, 3rd, 4th) for high temperature were studied. NGS sequencing analysis showed clear composition of microbial populations (bacterial and archaeal) present in different chambers of ABRs at different taxonomic levels. Diversity in microbial community of a digester indicates metabolic diversity of the microorganisms performing in the anaerobic digester. It is important to maintain active bacterial population within a digester to avoid its failure and overcome rate limiting steps for balanced process. And it is important to understand complex microbial/bacterial communities of AD to run properly, to improve the process efficiency and stability and operation strategies. Structure of microbial community performing in a digester is dependent on many factors, two of which are substrate characteristics and operational conditions. Type of compounds entering in a digester decides which microorganisms thrive in system (Miron et al., 2000). Microbial (bacterial and archaeal) diversity was varied in each chamber within a month and along the months. New families appeared in each month. Bacterial diversity of digesters varies depending upon different factors and rapid significant shift in composition of microbial species may occur (Zumstein et al., 2000).

A correlation was observed in microbial community composition among the four chambers of field scale ABR along the months at variable temperature. Structure of microbial community was dissimilar between months and chambers, degree of dissimilarity varied with time. This variation in microbial community structure may be due to change in temperature along the months. As microbial communities of anaerobic digesters are sensitive to temperature change and respond to every 1˚C variation in temperature. As a result, whole process of anaerobic digestion is altered (Pap et al., 2015; Risberg et al., 2013; Gou et al., 2014). Proteobacteria was detected as dominant phylum in both chambers (1st and 4th chamber) of low temperature ABR working at lab scale and 1st, 3^{rd,} and 4th chambers of lab scale ABR working at high temperature. Bacteriodetes was 2nd dominant phylum in both chambers of low temperature ABR but in three chambers of high temperature ABR bacteriodetes was in minor proportion 3%. Proportion of bacteriodetes was constant in all three cambers of high temperature ABR. Archaeal phyla were also detected in both lab scale ABRs. Archaeal phyla detected in 1st chamber but not in 4th chamber at in ABR at low temperature, but in high temperature lab scale ABR archaeal phyla detected in 1st, 3rd, and 4th chambers. The only difference between two reactors was temperature. It showed that high temperature lab scale ABR favored the growth of archaea. As temperature variation of reactor is an important factor that determines structure of microbial communities present in the digester. Microbial community composition of digester was varied by both increase and decrease in temperature (Labatut et al., 2014).

Bacterial community of ABR exhibited higher diversity at phylum level, 19 different phyla of bacteria and two phyla of archaea were identified in field scale ABR. Proteobacteria was dominant phylum during Febuaray 1st and 4th chamber, April 1st chamber, Firmicutes dominant phylum in April 4th chamber and proteobacteria second dominant phylum. Different studies demonstrate that phylum Proteobacteria (approx. 35%-65% of total sequences) (Meerbergen et. al., 2017), Bacteroidetes and Firmicutes domestic mostly predominate in municipal wastewater treatment plant (Gao et al., 2016). Proteobacteria are known to perform significant role in multiple environmental functions due to having metabolic diversity, as by regulating N, C, P and S cycles in anaerobic digesters (Friedrich et al., 2005), especially its class betaproteobacteria is most efficient in organic matter and nutrients removal in

municipal wastewater treatment plants (Nielsen et al., 2010). These three phyla are positively linked to process of anaerobic digestion and are involved in degradation of different types of organic material present in wastewater. Presence of these phyla as dominant, may be attributed to mesophilic temperature conditions of the ABR which make environment feasible for their survival and growth (Tang et al., 2005). Bacteroidetes, and proteobacteria are affected by temperature (Lauber et al., 2009).

Phylum Proteobacteria play crucial role in two stages of anaerobic digestion, hydrolysis and acetogenesis. Bacteria of this phylum are involved in removal of wide range of organic matter. Some bacteria belong to this group are sulphate reducers, denitrifiers (Crocetti et al., 2000) and nitrifiers (Jabari et al., 2016; Snaidr et al., 1997).

Members of Phylum Bacteroidetes and firmicutes are involved in hydrolysis and acidogenesis (fermentative bacteria) of anaerobic digestion (Ariunbaatar et al., 2014). Their presence indicates the occurrence of fermentation within the reactor as bacteria in these two groups has the property to hydrolyze polymers substrates (proteins, lipids and polysaccharides) which are not degraded in remediation stages of anaerobic digestion, into format, acetate, long chain fatty acids hydrogen and CO_2 (Godon et al., 1997).

Members of phylum Bacteroidetes are known to have proteolytic activity, involved in protein degradation in anaerobic digester with final products of volatile phenolic acids and ammonia. They degrade complex substances (Yi et al., 2014). Clostridia and Bacteroidetes were common fermenters present in anaerobic digesters. Change in wastewater composition with season and its organic load content can shift the archaeal and bacterial community of the anaerobic digester. But effect for clostridia and Bacteroidetes not major changes are induced due to these factors. On the other hand, phosphorus and VFAs concentration of the digester also affect the microbial community composition and may change it (Hori et al., 2006). Members of phylum proteobacteria, bacteriodetes, firmicutes and chloroflexi are typical hydrolytic (fermentative) bacterial phyla (Zhang et. al., 2019).

Phylum Firmicutes degrade wide range of organic substrates and is an abundant group of bacteria prevailing in most anaerobic reactors. It can grow at wide temperature

range i.e. at mesophilic and thermophilic temperature (Linda et al., 2016; Lui et. al., 2016). Firmicutes especially its class clostridia increase with increase in temperature. Firmicutes may have metabolic pathway with end-product volatile fatty acids, utilized by some other group of microorganisms present in ecosystem (De Verieze et al., 2015; Shnurer et al., 2016).

Phylum chloroflexi constitutes some part of the digestors microflora (Hug et al 2013; Riviere et al., 2009) and involved in hydrolysis as it is capable of degradation of complex substances and polymers, bacteria in these groups decompose exo-polysaccharaides (EPS) (having proteins and polysaccharides) and dead cells into simple molecules as lactate and ethanol (Zhang et al., 2014).

Actinobacteria belongs to polyphosphate accumulating bacteria and supports de-acidification process of the sludge and allows pH to become neutral by reducing VFAs (Lerm et al., 2012) and are extremotolerant to pH changes (Nascimento et al., 2018). Clostridia and actinobacteria play vital roles in hydrolysis and acidogenesis during anaerobic digestion (Ziganshina et al., 2016).

Acinetobactor are phosphorus accumulating organisms (PAOs) and belong to gammaproteobacteria (Xia et al., 2010). Acidobacteria, Bacteroidetes, and proteobacteria are affected by temperature (Lauber et al., 2009). Another study shows that acidobacteria are affected by pH also (Lauber et al 2009). Synergistese group of bacteria are involved in degradation of amino acids present in anaerobic digesters (Jumas et. al 2009).

pH control microbial community by regulating nutrients availability and enzymatic processes which are essential for microbial process (Madigan et al., 2016). Limited archaeal diversity is seen in digester. Presence of two methanogenic phyla euryarchaeota and crenarchaeota in ABR shows that anaerobic digestion is successfully being carried out in digester. Crenarchaeota are highly thermoacidophiles. (Godon et al., 1997). Euryarchaeota includes all members of methanogens involved in final step of anaerobic digestion (methanogenesis) (Stams et. al., 2012).

Minor phyla/families in each chamber of ABR varied greatly along the months and even microbiology of one chamber varied from other chamber in the same month. This was due to involvement of different groups of microorganisms which take part in food chain of anaerobic digestion. Products of one stage of AD are used as food source for microorganisms of other stage, which favors rapid change in microbial community in next chamber. Rapid succession due to very dynamic system of wastewater treatment is another factor contributing to this rapid change in microbiology of the digester (Shu et al., 2015). Microbial community shift observed in every month in each chamber has deep relation with temperature variation in each month. Microbial diversity in start (February) was comparatively lower because microbial consortia of digester take time to develop and flourish in the provided conditions of digester. Temperature had greater effect on microbial shift in this study. Microbial diversity of ABR observed to increase with increase in temperature. Increase in microbial adaptability towards increasing temperature was noticed. Different studies concluded that increasing microbial adaptability towards increasing temperature (Bouskova et al., 2005; Pap et al., 2015). Reactors working at mesophilic temperature may have rich microbial communities than reactors working at thermophilic communities (Lapara et.al., 2000).

Clostridia detected along 1st chamber of ABR belongs to strictly anaerobic group of fermentative microorganisms and contribute to acid formation during anaerobic digestion. Detection of this bacterial group indicates established anaerobic conditions within reactor (Jabari et al., 2016; Walter et al., 2012). Detection of clostridia along different chamber indicate that clostridia perform multiple functions in the anaerobic digesters as hydrolysis, acidogenesis, acetogenesis and also in association with methanogens. Species of clostridia hydrolyze proteins (Gannoun et al 2013) protein, starch, lipids and cellulose during 1st stage (hydrolysis) of anaerobic digestion (Niu et al., 2014). Their presence in domestic wastewater is expected as they belong to microbiota of human intestine (Lopetuso et al., 2013).

Results showed that *Helicobacteraceae* was present in almost in all the chambers while in some chambers it was dominant indicating presence of Sulphur in the reactor, as *Helicobacteraceae* belongs to sulfur oxidizing bacteria and regulate sulfur cycle (Meyer et al., 2016).Rapid variation observed in helicobacteraceae in different

chambers along the month may be due to high sensitivity of Sulphur oxidizing bacteria towards the environmental factors including wastewater shock loads, competition and predation which can cause abrupt fluctuation in taxa (Comas et al., 2008; Mohanakrishnan et al 2011). Another reason may be change in sulphur content during the AD process as reduction in sulphur can cause drop in *Helicobacteraceae*.

Lithotrophicum a member of *Helicobacteraceae* (proteobacteria) was found as core biomarker in some chambers. *Lithotrophicum* is sulphur oxidizing bacteria grow at wide range of mesophilic temperature (10-40°C) and is facultative anaerobe (Inagaki et al., 2004). The later reduction in *Lithotrophicum* number indicates the reduction in ammonium chloride in the reactor during the second month of start of the reactor. Reduction in number of species in 4th chamber indicates that ABR is established. Members of family bacteriodaceae detected along 1st chamber are well known fermentative bacteria and play important role in hydrolysis of anaerobic digestion. As a result, it produces hydrogen, CO_2 and organic acids (Traversi et al., 2012). Members of the family *Chitinophagaceae* and *Saprospiraceae* have ability of hydrolyzing some organic matter (Lim et al., 2009). *Saprospiraceae* family is protein degrader (Xia et. al., 2008). Clostridia were also detected along 2nd, 3rd and 4th chamber. Studies showed that clostridia are also associated with acidogenesis (acid forming stage (Asad et. al., 2014) forming acidogenesis products formate, acetate, butyrate and lactate (Li et al 2011) and also have role in acetogenesis (3rd stage of AD) (Ziganshin et al., 2013). Syntrophomonas and syntrophobacter belong to acetogens (Dhamosharan and Ajay 2014).

Actinomyces detected in ABR belong to acid producing bacteria, significant growth of acid producing bacteria accumulate VFAs in the digester, which may also deteriorate the AD process. VFAs accumulation induce proliferation of clostridia, clostridia oxidize fatty acids in association with hydrogenotrophic methanogens. Balance between hydrogenotrophic methanogens and clostridia decide process balance as with increase of clostridia acetoclastic methanogens does not shift to hydrogenotrophic and also hydrogenotrophic methanogensdecline in number. This mismatch in process metabolism may also affect process badly (Wirth et al., 2012).

Fluctuation of methanogens observed along the months may be with temperature as *Methanosarcinaceae* and *Methanomicrobiales* increase with increase in temperature

and decrease in abundance of methanomicrobiales with decrease in temperature shows effect of temperature variation on microbial community (Westerholm et al., 2018). An archaeal family *Methylophillaceae* was detected in ABR is common family identified in from domestic sewage plants (Eyice et al., 2015). *Comamonadaceae* was second dominant family along chambers of ABR. Species which belong to family *Comamonadaceae*, are denitrifying bacteria involved in nitrogen cycle during anaerobic digestion (Wang et al., 2016). Change in wastewater composition with season and its organic load content can shift the archaeal and bacterial community of the anaerobic digester. But effect for clostridia and Bacteroidetes not major changes are induced due to these factors. On the other hand, pH and VFAs concentration of the digester also affect the microbial community composition and may change it (Hori et al., 2006).

Rhizobiaceae and *comamonadaceae* were positively associated with ammonium and COD concentrations and both may be involved in nitrite and COD removal (Chongjun et al., 2016). *Comamonadaceae* can also degrade organic acids as acetate (Ensley et al., 1995). Change in microbial community structure was observed with passage of time along the months. Another study showed the shift in dominance, with change in COD (enhanced COD) addition changed the dominance from chloroflexi to proteobacteria which indicate COD as an important factor in regulating structure of bacterial community (Chongjun et al., 2016).

Caldilineaceae and *Anarolinaceae* are phosphate accumulating microbes in wastewater treatment system. They have ability of nitrification and phosphorus accumulation and have good removal rate of phosphorus and NH_4^+-N during wastewater treatment (Kindaichi et al., 2013). *Caldilineaceae* and *Anarolinaceae* are highly associated with domestic wastewater system. *Oxalobacteraceae* detected is strictly aerobe, reduces nitrate/nitrite to nitrogen gas. It prevails in anoxic environment due to presence of residual dissolved oxygen and nitrates which is suitable environment for its growth. Family holophagaceae also belongs to is anaerobic microorganisms related to denitrification. *Ruminococcaceae* and *Eubacteraceae* belonged to human associated microbiota (gut microbiota) (Li et al., 2011).

Paludibacter, succiniclasticum and acidaminococcus belong to anaerobes and produce propionate and other products by utilizing sugars, succinate and glutamate respectively (Nelson et.al., 2011). Core genera have crucial role in wastewater treatment systems irrespective of type of treatment system and location. Pseudomonas methylobacterium, bacillus, paracoccus isolated from wastewater treatment plants, are a part of denitrifying microbial flora (Vedenina & Govorukhina 1988). *Porphyromonadaceae* and *Lachnospiraceae* are detected in the environment with high ammonium concentration. Especially porphyromonadaceae was higher in the reactors with high ammonia concentration as compared to low ammonia concentration (Muller et. al., 2016). Marinilabiaceae was also detected in such environment as with high ammonia concentration (Ziganshina et. al., 2015).

It was noticed that reactors treating domestic wastewater show more bacterial diversity compared to others. Because domestic wastewater has larger portion of easily degradable organic matter as microbial diversity is reduced due to recalcitrant and toxic pollutants. And bacterial community composition of domestic sewer is more variable due to large fraction of readily degradable organic matter (Meerbergen et al., 2017). The values of pH also affects bacterial community composition even 1 pH unit change have considerable effect on bacterial community structure (Fierer et al., 2006). High pH values mostly decrease diversity of microbial communities (Farzadkia and Bazrafshan 2014). To know how the anaerobic digestion system works, studies of microbial community within the digester are of great importance to get full image of whole system. Present study added valuable knowledge and insight into the factors affecting process of anaerobic digestion and its key players. As information about species interaction and adaptation in particular circumstances like at specific temperature are helpful in optimization the way in which microbes handle conditions of environmental stress. Results indicated that respective microbial flora of each of the four stages was present in the respective chamber of ABR, as each chamber represents one stage of AD. Hydrolytic bacteria were isolated from 1st chamber, acidogenic bacteria in 2nd chamber, acetogenic bacteria in 3rd chamber and methanogenic bacteria (archaea) in 4th chamber of ABR. Microbial community shift was observed with temperature variation.

4.4 Conclusions

- Bacterial shift was observed along chambers of fields scale and lab scale ABRs indicating progress of the process.
- Miseq analysis exhibited that proteobacteria is the overall predominant phylum along the months but a change was observed in predominant phyla with temperature.
- Respective microbial flora of each of the four stages was present in the respective chamber of ABR.
- Significant change in microbial composition was observed with temperature shift indicating direct influence of temperature in microbial community determination and its performance.
- Change in microbial community structure was also observed along the chambers but not significant as compared to temperature.
- A good anaerobic microbial diversity was observed along the ABRs at all temperatures.
- ABR demonstrated efficient removal of pathogens, with more significant removal at comparatively higher temperature.
- Phase specific microorganisms were present at each stage as hydrolytic, acidogenic, acetogenic and methanogenic microorganisms were identified in the respective chamber. But their structure was varied with time and temperature.
- Microbial structure and efficiency showed reactor stability with efficient organic matter removal.
- Methanotrophs detection indicates methane production and development of anaerobic conditions from the very start period of rector operation.

5. CONCLUSIONS

When analyzing the efficiency of anaerobic baffled reactor in the current study it is concluded that ABR working as a primary treatment unit removes considerable organic material and pathogens from wastewater. Efficiency of ABR fluctuates with seasonal variations with comparatively better efficiency and stability in summer season. Performance of ABR was increased with increasing temperature and reactor was more stable at high temperature including pathogens removal.

Microbial diversity analyses showed continuous bacterial shift along chambers of ABRs indicating progress of the process, but the change was more prominent with temperature shift. Respective microbial flora of each of the four stages was present in the respective chamber of ABR. Complete microbial shift was observed during summer and winter seasons. Some unique families (absent in previous chamber) appeared in each chamber. For further studies temperature is among the significant parameters for control and operation of AD process.

FUTURE PROSPECTS

- Implementation of four chambered ABR as primary treatment system to treat wastewater in Pakistan.
- Treatment efficiency analysis of four chambered ABR to treat wastewater originating from resources other than domestic wastewater.
- Investigation of bioavailability of micronutrients and their impact on metabolic activity of microbial consortia of ABR.
- Further modification of ABR to develop hybridized anaerobic inclining baffled bioreactor which could be more suitable for high strength wastewater treatment.
- Modification of ABR configuration to eight compartments and study of its microbial community composition of chambers.
- Sampling of sludge, instead of wastewater for more deep insight into microbiology of ABR could be studied.

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Appendix 1: Key for Stacked bar plot Fig. 4.11

Appendix 2: Key for Stacked bar plot Fig. 4.12

k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae
k__Bacteria;p__Proteobacteria;__;__;__
k__Bacteria;p__Bacteroidetes;__;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;__;__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;__
k__Bacteria;p__Firmicutes;__;__;__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfovibrionales;f__Desulfomicrobiaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadace
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Caulobacterales;f__Caulobacteraceae
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Syntrophobacterales;f__Syntrophaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;__
k__Bacteria;p__;c__;o__;f__
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Anaerolineales;f__Anaerolinaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadace
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Clostridiaceae
k__Bacteria;p__Acidobacteria;c__Holophagae;o__Holophagales;f__Holophagaceae
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;__
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanomicrobiales;f__Methanoregulaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae
k__Archaea;p__Euryarchaeota;c__Methanobacteria;o__Methanobacteriales;f__Methanobacteriaceae
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadace

k__Bacteria;p__Chloroflexi;c__Anaerolineae;__;__

k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;f__Rhodocyclaceae

k__Bacteria;p__Armatimonadetes;c__[Fimbriimonadia];o__;f__

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraces

k__Bacteria;p__Verrucomicrobia;c__Verrucomicrobiae;o__Verrucomicrobiales;f__Verrucomicrobiacea

k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales;f__Methylophilaceae

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae

k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae

k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Chitinophagaceae

k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae

k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfuromonadales;__

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Acetobacteraceae

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae

k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Marinilabiaceae

k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Desulfobacterales;f__Desulfobacteraceae

k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Cystobacteraceae

k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae]

k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Syntrophobacterales;f__Syntrophobactera

k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae

k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;__

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;__

k__Bacteria;p__Actinobacteria;c__Actinobacteria;__;__

k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;__

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Methylococcales;f__Methylococcaceae

k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Polyangiaceae

k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae

k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae
k__Bacteria;p__NC10;c__12-24;o__;f__
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Lachnospiraceae
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;__;__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;__;__
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanomicrobiales;__
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Legionellaceae
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Nitrosomonadales;f__Nitrosomonadaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Rhizobiaceae
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__TTA_B6
k__Archaea;p__Crenarchaeota;c__Thermoprotei;o__Desulfurococcales;__
k__Archaea;p__Crenarchaeota;c__Thermoprotei;o__Desulfurococcales;f__Desulfurococcaceae
k__Bacteria;p__Verrucomicrobia;__;__;__
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Nocardiaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Xanthobacteraceae
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cyclobacteriaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Aurantimonadaceae
k__Bacteria;p__Cyanobacteria;c__Oscillatoriophycideae;o__Chroococcales;f__Xenococcaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Methylobacteriaceae
Unassigned;__;__;__;__
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;__
k__Bacteria;p__Caldiserica;c__Caldisericia;o__Caldisericales;f__Caldisericaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosaetaceae
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;__
k__Bacteria;p__Caldiserica;c__Caldisericia;o__Caldisericales;f__Caldisericaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosaetaceae
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;__
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Beijerinckiaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae
k__Bacteria;p__Cyanobacteria;__;__;__
k__Bacteria;p__Firmicutes;c__Bacilli;__;__
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Gemmatales;f__Isosphaeraceae
k__Bacteria;p__Chlamydiae;c__Chlamydiia;o__Chlamydiales;f__Parachlamydiaceae
k__Bacteria;p__OP9;c__OPB46;o__;f__
k__Bacteria;p__Chloroflexi;c__Anaerolineae;o__Caldilineales;f__Caldilineaceae
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanomicrobiales;f__Methanospirillaceae
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Syntrophobacterales;f__Syntrophorhabdace
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae
k__Bacteria;p__Firmicutes;c__Erysipelotrichi;o__Erysipelotrichales;f__Erysipelotrichaceae
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bacteriovoracaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae
k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Lactobacillaceae
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Gemmatales;f__Gemmataceae
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae
k__Bacteria;p__Elusimicrobia;c__Elusimicrobia;o__Elusimicrobiales;f__Elusimicrobiaceae