NUTRIENT REMOVAL AND BIOMETHANATION POTENTIAL OF PROMINENT INVADING MACROPHYTES

THESIS SUBMITTED TO **AcSIR** FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGICAL SCIENCES

UNDER THE FACULTY OF SCIENCE

By

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July, 2018

Dedicated to

Myself

DECLARATION

*I hereby declare that the matter embodied in the thesis entitled: "**Nutrient removal and biomethanation potential of prominent invading macrophytes**" is the result of the work carried out by me at the Environmental Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (CSIR-NIIST), Thiruvananthapuram, under the supervision of Dr. Krishnakumar.B and the same has not been submitted elsewhere for any other degree.*

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CERTIFICATE

This is to certify that the work incorporated in this Ph. D. thesis entitled ***Nutrient removal and biomethanation potential of prominent invading macrophytes"*** *submitted by* ***Mrs. Priya. P.*** *to the Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Biological Sciences embodies original research work carried out by her under my supervision. I further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.*

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Acknowledgement

"No act of kindness, however small is ever wasted"-Aesop

First and foremost, I would like to acknowledge my thesis supervisor Dr. B. Krishnakumar for his constant guidance and support throughout the last six years. He has guided, criticized and corrected me throughout the period and has helped me to maintain the balance between my personal and professional life.

I am thankful to the present director of CSIR NIIST, Dr. A Ajayghosh and former director Dr. Suresh Das for the infrastructures and facilities provided for completing the doctoral works. I would also like to thank Dr. Mangalam Nair and Dr. Luxmi Varma for their timely advice in keeping up with AcSIR requirements to qualify for the doctoral degree. My gratitude to Dr. Elizabeth Jacob, HOD for her kind support and guidance during the period.

My heartfelt gratitude and respect to Dr. Ajit Haridas, Chief Scientist, who has guided and criticically reviewed my work whenever I contacted him. His approach and outlook towards the research are so motivating for a young researcher.

I would like to thank Dr. V B Manilal, Dr. J Ansari, Dr. Rugmini Sukumar, Dr. K.P.Prathish, Mr. Abdul Haleem, Mrs. Vijaya Prasad, Mr. Shajikumar V K, Mrs. Saharuba. P.M, Dr. Joshy George from the ETD Division, for their guidance during different studies during the doctoral work. I would like to specifically thank Dr. Savithri S for her insights and ideas for the kinetics study.

I am grateful to Dr. Anupama V N, Mr. Faisal M, Dr. Aneesh C, Dr. Anju S, Dr. Jayaprabha J S, Mrs Nisha and Mrs. Arya Bhasi for their love and support throughout the period. Special mentioning to Mr. Pradeesh P R and Mr. Rajendra Prasad for their lion's share in supporting the sample collection and reactor maintanence. I also want to express my gratitude to Dr Churchil Angel Antony Raj, Mr Amal and Mr Aneesh Kumar for their assistance at various points of doctoral work.

I want to express my sincere love to Anie Mariya, Jasmin, Suryalakshmi, Sanitha, Sayana, Cincy, Chinchu, Dyuthi, Reshma, Sajitha, Rothish, Ashik, Sakthi, Fahad, Kiran, Sanath, Ajay, Jiffin, Kashyap, Dayal, Dipin, Rahu, Adarsh, Anand, Chitrajith, and Arunkumar for the memorable days at NIIST.

It was my family's back up which helped me to finish the doctoral work and thesis and just words cannot express my love for them. I am forever indebted to my husband, Ani T Nair, for holding on to my dreams stronger than me and my little angel Shivangi for her understanding. God has always held me in his hands and these blessings are enough to be the evidence for it.

The list of gratitude is not complete and I want to apologize if I have missed anybody's name. Thank you all for your kindness and love.

Priya

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Abbreviations

WH	Water hyacinth
AV	Akkulam Veli Lake
UASB	Upflow Sludge Blanket Reactor
ALBR	Anaerobic Lead bed Reactor
FISH	Fluorescent In Situ Hybridization
qPCR	quantitative Polymerase Chain Reaction
HPLC	High Performance Liquid Choromatography
GC	Gas Chromatography
CW	Constructed Wetland
AD	Anaerobic Digestion
VS	Volatile Solids
TS	Total Solids
VFA	Volatile Fatty Acids
LR	Loading Rate
SRT	Sludge Retention Time
HRT	Hydraulic Retention Time
ppt	Parts per thousand

PREFACE

Eutrophication is primarily caused by the regular inputs of a range of nutrients into the water bodies. The subsequent luxuriant growth of invading macrophytes and associated ecological and socio-economic impacts are very high. The direct socio-economic impact ranges from loss in fisheries, tourism, water transport, massive vector (mosquito) breeding and associated epidemic outbreak, blockage of irrigation canals, etc. However, the higher nutrient uptake property of dominant macrophytes like water hyacinth (*Eichhornia* sp.) can be explored for nutrient stripping from eutrophic water bodies. In this approach controlled harvesting of the biomass followed by recovering (post-harvest treatment) value added products (biogas and manure) from the biomass can a sustainable way of managing eutrophication.

The present study focus on this aspect covering following objectives (1) evaluation of locally available macrophytes for application in nutrient removal activities, (2) to study the anaerobic digestion (biomethanation) of macrophyte biomass for recovering of value added products and (3) to study the microbial ecology of selected macrophyte rhizosphere and anaerobic digester for treating the macrophyte biomass.

An over view of eutrophication including the major factor responsible, its impacts and control measures etc are presented in Chapter Chapter 2 presents a detailed review of update literature covering biological nutrient removal studies, phytoremediation approaches, role of microorganisms in the nutrient uptake, anaerobic digestion of lignocellulosic biomass in general, anaerobic digestion of water hyacinth, co-digestion studies with water hyacinth, Ensilation of various crops materials for feeding purpose and biogas production, etc. are covered in this chapter.

Nutrient uptake efficacies of various common macrophytes such as *Eichhornia crassipes*, *Pistia stratiotes*, *Salvinia minima* and *Lemna minor* are compared in Chapter 3. The plants were tested for their phytoremediation potential to remove nitrate, phosphate and ammonia. The kinetics of nutrient removal, influence of

environmental factors on nutrient removal as well as the functional role of rhizospheric microflora in nutrient removal is also covered in Chapter 3. The water quality details of a local eutrophic brackish lake (Aakulam Veli Lake) covered with water hyacinth biomass is presented in Chapter 4. The nutrient load at different seasons and water quality parameters like pH, temperature, salinity and nutrient concentration were also covered here. The extend of macrophyte coverage during different seasons was done so that harvesting period of these plants can be decided. Moreover using geographical Information tools and field sample measurements, a quantitative assessment of WH biomass (Ton/Hec) were also done and the results are covered in Chapter 4.

Chapter 5 deals with the biomethanation of prominent macrophytes (*Pistia* and *Eichhornia*). Based on the preliminary observation, detailed biomethanation study was done with WH biomass. Different pre-treatment methods for WH biomass and the results of biomethanation in batch experimental systems are covered. Different approaches to improve the biogas yield such as improving the dry solid content, co-digestion with local waste substrates (food waste and MSW sludge) are included in this chapter. Additionally, to address the periodical availability of WH biomass in bulk, sample preservation method ensilation is also tested and the results are covered in this chapter. Observations on the qualitative analysis of the phylogenetic diversity of bacterial communities present in the biodigester are also encompassed in this chapter.

A thorough investigation on the microbial ecology, more specifically the role of protozoa in an anaerobic digester for the biomethanation of water hyacinth biomass was done and the results are presented in Chapter 6. Experimental data about the dominant protozoa and their functional role in different stages of anaerobic digestion (VFA accumulation and biogas yield) are presented in detail. Furthermore, based on the data generated, a hypothetical ecological niche of protozoa during anaerobic digestion is also included in this chapter. A general Discussion is included as

Chapter 7 and the major findings are concluded in Chapter 8 as summary of the doctoral work.

Chapter 1
Introduction

1.1. Eutrophication

The exponential increase in population across the world had its significant impact on pollution. Some major problems that humanity is facing in the twenty-first century are related to water quantity and water quality issues (UNESCO, 1992). As water is a necessity for sustaining of life, the pollution of water bodies is a major concern on the global scale. "Eutrophication is an enrichment of water by nutrient salts that cause structural changes to the ecosystem such as increased production of algae and aquatic plants, depletion of fish species, general deterioration of water quality and other effects that reduce and preclude use." This is one of the first definitions given to the eutrophic process by the OECD (Organization for Economic Cooperation and Development) in the 70s (http://www.eniscuola.net). Human activities have altered the fluxes of the freshwater as well as marine ecosystems. Aquatic plants require two major nutrients for its growth, and they are Nitrogen (N) and Phosphorus (P). They receive these nutrients through a process known as natural eutrophication or aging of rivers, in which water bodies accumulate plant nutrients, typically from nutrient-rich land drainage which takes hundreds of years to complete (Smith, 2003). The natural deposition of these nutrients on water bodies will be on a limiting amount which helps in restricting the over plant growth which may cause the imbalance of the aquatic ecosystem.

Due to anthropogenic activities like overuse of fertilizers and dumping of untreated industrial effluents and organic wastes to the water bodies, the excessive accumulation of nutrients occurs, there will be an undesirable overgrowth of phytoplankton, and this forms a greenish matt layer over the surface of water bodies which further prevents the light penetration to it. The death and decay of these organic matters further demand the oxygen present in the water for putrefaction, and this creates a "no-oxygen" zone in the water body along with the increased turbidity and foul smell. There will be changes in species diversity, and there will be decreased dynamics in the species. Almost 60 % of the freshwater bodies and canals

have been suffering from eutrophication across the globe. It includes Lakes in China, Denmark, West Africa, and Asia including India, South and North America, etc. (Xia et al., 2016). The primary outcome of anthropogenic eutrophication is that there will be accelerated growth of invading macrophytes like Water hyacinth and *Pistia* sp., which forms mats over the surface of water bodies. It causes the loss in fishery wealth of the water system by affecting dissolved oxygen and causes hindrance in water transport. They also provide the breeding place for diseasing causing vectors like mosquitoes.

The major nutrients responsible for eutrophication are nitrogen and phosphorous.

a) Nitrogen

In water, nitrogen exists as inorganic and organic species. Inorganic nitrogen is present in the oxidized form (e.g., nitrite and nitrate) and reduced form (e.g., ammonia/ammonium and dinitrogen gas). Total nitrogen (TN) is the sum of all forms of nitrogen in the water sample. TKN (Total Kjeldahl Nitrogen) is the total concentration of organic nitrogen and ammonia. All the forms of nitrogen are interconvertible and they form the components of nitrogen cycle in water. Among these forms, organic nitrogen is the available nitrogen for the phytoplankton which is the organically bound nitrogen in the tri-negative oxidation state. Organic nitrogen and ammonia are the dominant forms found in water bodies. Organic nitrogen includes proteins, peptides, nucleic acids, urea and various synthetic organic materials that reach the runoff water from the organic wastes. However, before being used as a nutrient, the organic nitrogen must first be converted to ammonia. The organic nitrogen in the water body can range from micrograms to milligrams per liter depending upon the extent of pollution. Nitrite is the intermediate oxidation state of nitrogen both in oxidation of ammonia to nitrate and in the reduction of nitrate. The primary input source of nitrite to water bodies is from industries where it is used as a corrosion inhibitor. In acidic waters, nitrites can form nitrous acid which reacts with secondary amines to form nitrosamines which can act as carcinogens. Ammonia is present both on the surface as well as wastewater. They are produced mainly by the de-amination of organic nitrogen-containing compounds

and by hydrolysis of urea. In some water treatment plants, ammonia is added along with chlorine to form chlorine residuals. Natural levels of ammonia in groundwater are usually below 0.2 mg/L and inland surface water may contain free ammonia up to 5 mg/L. Ammonia limits in lake water is up to 1.2 mg/L according to CPCB standards. The presence of ammonia can range from 10 micrograms to 50 milligrams per liter in wastewaters (Westerhoff and Mash, 2002; Deborde and Von Gunten, 2008).

b) Phosphorus

Phosphorus is an essential element required for the growth of organisms because of its presence in nucleic acids like DNA and RNA. Phosphorus is the single most nutrient to manage for controlling accelerated eutrophication in freshwater lakes (USEPA, 1992). The common occurrence of Phosphorus is in the form of rocks in nature. The major sources of phosphorus in water bodies occur as i) atmospheric inputs like rain and dust ii) Point sources like effluents from sewage treatment plants and industries iii) Non point sources including stormwater, agricultural and land clearing off and iv) non point sources within water bodies like internal loading of re-suspended sediments (WEP, 2010). Phosphorus is the limiting nutrient of primary productivity in water. If present in excess it can promote the growth of micro and macro organisms in nuisance quantities. Phosphorus exists as inorganic orthophosphate (orthophosphate (PO_4^{3-}), and organic phosphate in wastewater and natural waters whereas particulate phosphorus is found in suspension or sediment. Total phosphorus (TP) is a measure of all forms of phosphorus found in water.

1.1.1. Impact of eutrophication in Fresh water and coastal water systems

The nutrient conditions of many natural water bodies are mostly oligotrophic in nature with limited primary and secondary productivity due to poor nutrient availability (Beeby, 1995). Once the nutrients are available in abundance, this will promote the diversity of organisms in the ecosystem which may cause dynamics in phytoplankton's as well as zooplanktons residing in the system. But the natural

aging will take thousands of years to happen and hence the fluxes in nutrients, as well as diversity, occur only slowly.

Anthropogenic eutrophication or cultural eutrophication happens due to regular inputs of a range of nutrients into the water bodies due to human activities. Phosphorus is the limiting nutrient that determines the extent of eutrophication in freshwater systems and the amount of nitrogen and phosphorus released into the water body depend upon the sources of pollution. This will result in the extensive growth of algae as well as aquatic flora which reduces the sunlight penetration to the water. The phytoplankton communities are the major altered biomass due to varying total nitrogen to total phosphorus ratio (Seip, 1994). The matted growth of phytoplanktons restricts the oxygen supply which will affect the growth of photosynthetic aquatic organisms. The decomposition of the dead algae or plants will be done by putrefying microorganisms which will use the available oxygen and release more phosphorus to the water body which will promote the growth of more algae. Consumption of oxygen by putrefying bacteria creates a "no life zone" which affects the life of aquatic organisms. This can change the economics as well as amenity value of the water body. As the sediment deposition increases, the depth of the river sink decreases which results in shallowing of the river body and eventually it will become swamps. Thus eutrophication can cause the death of a water body (Dorgham, 2014).

1.1.2. Indicators for Eutrophication

An approved indicator by environmental protection agencies or laws are always required for evaluating and tracking the trend in eutrophication, and this may lead to saving a water body from complete degradation. The symptoms of eutrophication can be divided to the primary sign or direct effect and secondary symptoms or indirect effect. Increased primary production will always lead to increase in chlorophyll 'a' along with increased macroalgal disturbances. This is considered as the primary symptoms of eutrophication. Loss of dissolved oxygen, losses of

submerged aquatic vegetation (SAV) and occurrence of toxic algal blooms are considered as secondary symptoms (Bricker et al., 1999, 2003, 2008, Xiao et al., 2007, Ansari and Khan, 2007). The selection of indicator should be relevant to the issue in both coastal and fresh water. The European Environment Agency – Environmental Monitoring and Assessment (EEA- EMMA) 'indicator comparison process" (y Royo et al., 2008) concluded the indicator choice as "nutrient concentrations when used jointly with Chl a are a closer step toward a eutrophication assessment". But the nutrient concentration assessment will not work in coastal waters. There mixing and residence time, and to underwater light, the climate has to be considered as susceptible factors (Fereira et al., 2010).

Schnitzler (1996) studied the response of aquatic macrophyte communities to levels of phosphorus and nitrogen in an old swamp on the upper Rhine plain in eastern France and worked out the utility of some aquatic macrophytes as bioindicators of eutrophication. *Vallisneria americana* is reported to be the efficient biomonitor of organic contamination and stressed aquatic ecosystems (Biernacki et al., 1996; Doust et al., 1994; Potter & Lovett-Doust, 2001). Some important bioindicators listed by Stojanovic et al. (1998) include *Wolffia arrhiza*, *Lemna gibba*, *L. minor*, *L. tris*ulca, *Spirodela polyrrhiza*, *Ceratophylum demersum*, *Elodea canadensis*, *Vallisneria spiralis*, *Stratiotes aloides*, *Nupher lutea*, *Bolboschoenus maritimus*, *Typha angustifolia*, *T. latifolia*, and *Phragmites communis*. These species were reported to be the best indicators of eutrophication caused by organic effluents and nutrients. The growth of *Spirodela polyrrhiza* was found to be directly related to the nutrient concentration of water (Khan and Ansari, 2005). The population and growth of *Lemna minor* and *Spirodela polyrrhiza* were studied as a measure of eutrophication caused by household detergents (Ansari et al., 2010).

1.1.3. Eutrophication in India

The discharge of untreated/poorly treated sewage is one of the major causes of nutrient overload and subsequent eutrophication in india. A number of studies have reported eutrophication of water bodies in India. Anthropogenic influences play a

major role in degradation of most of the lakes in the country to eutrophic or hyper eutrophic conditions. Garg et al. (2002) studied three lakes of Bhopal (Upper Lake, Lower Lake, and Mansarovar Lake) in India, to assess the potential fertility of lentic waters and to analyze the floral ecology. The highest level of eutrophication was found in Mansarovar Lake in their study and its increasing nutrient concentrations have found to eliminate the sensitive species of phytoplankton. Jha and Barat (2003) did a hydrobiological study of Lake Mirik in Himalayas and found that nutrient concentration was higher in certain pockets of lake. One of the major lakes of Bangalore which is Bellandur Lake is found to have converted to artificial reservoir of sewage and industrial effluents due to the urbanization of the city (Chandrasekhar et al., 2003). Lake Robertson which is located in Jabalpur has found to have low species density, fast shallowing, dominance of detritus food webs, and water unsuitable for human consumption (Singhal and Mahto, 2004). Dixit et al (2005) studied the hydrobiology of Shahpura lake in Bhopal, Madhyapradesh and found it as highly eutrophic. The phosphate content of the lake water studied was found in the range of 6.05 to 9.21 ppm. The nitrate content of the water was found to be in the range 2.02 to 15.22 ppm. The major reason for increasing eutrophication in india is pointed as phosphorous than nitrogen. The estimated annual consumption of phosphate-containing laundry detergents for the current population in India is about 2.88 million tonnes and the total outflow of P is estimated to be 146 thousand tonnes per year (Kundu et al., 2015). An investigation on point and non point sources of Phosphorus on Upper Lake of Bhopal was done by Coumar et al. (2018). It was found that among the P fractions, bioavailable P fraction was highest from the domestic waste water.

Aquatic ecosystems of the Kerala are also affected by anthropogenic activities. Analysis of change in the area of Vembanad Lake during 2002-2014 revealed growing anthropogenic pressure, with a reduction of 465 ha of area. Fisheries of the lake have been seriously affected by continuing reclamation, pollution,

eutrophication and anthropogenic interventions which have caused serious damage to the wetland systems (Varkey et al., 2016).

1.2. Nutrient removal from water bodies

Nutrient removal from water bodies is tried using various methods like physical, chemical and biological methods. Physical methods include controlling external nutrient input to the water bodies like banning phosphorus-containing detergents, hypolimnetic aeration and hypolimnetic withdrawal, dredging and also by using ultrasound. Chemical processes include inactivation using chemicals like alum, but this method can be adopted only for shallow lakes. Biological removal includes removing nutrients using aquatic natural treatment systems and microorganisms (WEP, 2010). Aquatic natural treatment systems have engineered system in the form of wetlands and ponds for treating pollutants for lake restoration and treating wastewater. Ponds and lagoons are for managing stormwater and industrial wastewater respectively. Plants with good adsorption and tolerance to contaminants are used for the vegetation in the ponds. Floating plants like Duckweed (*Lemna minor*) or water hyacinth (*Eichhornia crassipes*) and submerged plants like waterweed or water milfoil are used for the treatment of pollutants. Constructed wetlands are another method of nutrient removal where the different type of vegetations is used in different basins. Free water surface constructed wetlands and subsurface flow wetland constructed wetlands are being used for nutrient removal and the vegetation once harvested is used for value-added product extraction (Daniel et al., 1994; Kivaisi, 2001; Abbas et al., 2009; Cai et al., 2013).

1.2.1. Nutrient Removal studies in India

The nutrient removal studies in India are very limited. Even though sewage disposal is identified as a major source of eutrophication in India, our treatment systems are not tuned for removing/recovering materials. Engineered biological system as well as natural treatment systems have been reported for nutrient removal studies in India. Sequening batch reactor (SBR) is specifically designed for removing nutrients

in addition to organics (Kalbar et al., 2013). Constructed wetlands are another method of removing nutrients from waste water. Use of CW for treatment of waste water to allow for safe river discharge was studied in the Ganga River basin at Haridwar (India) and has been recommended for conservation of river water quality (Rai et al., 2013). To evaluate the suitability of three aquatic macrophytes which are *Typha latifolia*, *Colocasia esculenta* and *Phragmites australis* for their nutrient and trace element removal potential is studied using horizontal sub-surface flow constructed wetland (HSSF) for conserving Gang River ecosystem (Rai et al., 2015).

Natural treatment systems (NTSs) are viewed as a cost-effective alternative for treating waste water. A waste stabilization pond was built around 10 years ago in Mathura by the local water board with a capacity of 14.5 ML/D and treats domestic wastewater in a series of anaerobic and aerobic ponds. Similarly a water hyacinth pond is located close to a rural community in Naruana near Bathinda, Punjab which receives 0.25 ML/D of domestic wastewater from the local households. The treatment system was extended by adding a pond with water hyacinths and an oxidation pond but the quality of discarded water is not checked before releasing (Starkl et al., 2013).

1.3. Water Hyacinth , the predominant macrophyte in eutrophic waterbodies

The water hyacinth (WH), *Eichhornia crassipes* is a tropical species belonging to the pickerelweed family (*Ponte deriaceae*). A native of Brazil and possibly other central South American countries, now it occurs in lakes, slowly moving rivers and swamps in most countries of the world including India, South Africa and the USA. It is a free floating aquatic plant, well known for its production abilities and removal of pollutants from water. It can quickly grow to very high densities (over 60 kg/m^2); thereby completely clogging water bodies, which in turn may have negative effects on the environment, human health and economic development (Epstein, 1998). Water hyacinth grows over a wide variety of wetland types and prefers nutrient-

enriched water. However, it can tolerate considerable variation in nutrients, temperature and pH levels. The optimum pH for growth of water hyacinth is 6–8. It can grow in a wide range of temperature from 1 to 40 °C (optimum growth at 25–27.5 °C) but it is thought to be cold-sensitive (Wilson et al., 2005). Growth rates increase with the increase in water nitrogen amounts (Heard and Winterton, 2000). Salinity is a major constraint on water hyacinth growth in coastal regions as salinity levels at 6.0 and 8.0% are lethal (Olivares and Colonnello, 2000; Muramoto et al., 1991). Water hyacinth normally occurs in the form of dense mats and as a result blocks light penetration for the submerged plants and also reduces dissolved oxygen levels. By releasing allelochemicals it antagonizes the growth of other organisms and reduces the biological diversity (Brendonck et al., 2003).

Water hyacinth is listed as one of the most productive plants on earth and is considered one of the world's worst aquatic plants. It can double its size in 6.2 days and a mat of medium sized plants may contain 2 million plants per hectare that weigh 270 to 400T (Cornwell et al., 1977). These dense mats interfere with navigation, recreation, irrigation, and power generation (Shanab et al., 2010). Many large hydropower schemes have to devote significant time and money in clearing the weed in order to prevent it from entering the turbine and causing damage and power interruptions. The blockage of canals and rivers can even cause dangerous flooding (Mailu, 2000). On the other hand, increased evapo-transpiration due to water hyacinth can have serious implications where water is already scarce.

Water hyacinth has apparently become a problem in different parts of the world due to its uncontrolled and rapid growth. Water hyacinth can present many problems for the fisherman such as decreased fish population, difficult access to the fishing sites and loss of fishing equipment, resulting in reduction in catch and subsequent loss of livelihood. Water hyacinth is stated as the reason for the reduction of biodiversity as well (Masifwa et al., 2001). These mats competitively exclude native submerged and floating-leaved plants and its associated fauna, thereby causing an imbalance in the aquatic micro-ecosystem. Diversity of fish

stocks is also affected. Low oxygen conditions beneath the mats create good breeding conditions for mosquito vectors of malaria, encephalitis and filariasis. Therefore, there is a need to manage its spread.

1.3.1. Water hyacinth coverage on Indian Lakes

Varthur Lake, situated in the south of Bangalore, was built to store water for drinking and irrigation purposes and there is substantial algal blooms, Dissolved Oxygen (DO) depletion and malodour generation, and an extensive growth of water hyacinth that covers about 70–80% of the lake (74 hectare) in the dry season. Other than *Eichhornia*, major macrophytes found in the lake are *Typha augustifolia, Colocasia essculanta, Cyperus haspans, Alternanthera phyloxiriodes, Lemna gibba, Lemna minor* and *Pistia stratiotes* (Mahapatra et al., 2011). The Bhomra wetlands in West Bengal were found to be heavily infested by Water hyacinth whereas in Akaipur wetland, it was moderate infestation with increased nutrient concentration (Maitra et al., 2014). The wetland avain diversity of Kurukshethra, Haryana was found to be reduced due to the wetland infestation by water hyacinth. It has rapidly covered the water surface in village ponds and crocodile sanctuary reducing the feeding areas for water birds (Kumar and Gupta, 2009). Due to longer period of drying and influx of nutrients, an entire area of the Lake Santhragacchi, West Bengal was infested with water hyacinth and it resulted in reduction of capture fishery of about 4,000 MT. within 10 years period (Khan, 2010).

1.4. Anaerobic digestion

Anaerobic digestion (AD) is a technology widely used for treatment of organic waste for biogas production. AD that utilizes manure for biogas production is one of the most promising uses of biomass wastes because it provides a source of energy while simultaneously resolving ecological and agrochemical issues (Budiyano *et al*, 2010). The anaerobic fermentation of manure for biogas production does not reduce its value as a fertilizer supplement, as available nitrogen and other substances remain in the treated sludge (Alvarez et al., 2008). The principal product

of anaerobic digestion is biogas containing about 65% of methane gas, 35% of carbon dioxide and traces of ammonia, hydrogen sulphide and hydrogen (Table 1.1). This 'biogas' is a convenient and clean fuel and can either be used directly with or without the removal of carbon dioxide or can be converted into electricity with the help of suitable generators. AD is the consequence of a series of metabolic interactions among various groups of microorganisms. It occurs in three stages hydrolysis/liquefaction, acetogenesis, acidogenesis and methanogenesis (Figure 1.1). The first group of microorganism secretes enzymes, which hydrolyses polymeric materials to monomers such as glucose and amino acids. These are subsequently converted by second group i.e. acetogenic bacteria to higher volatile fatty acids, H_2 and acetic acid. Finally, the third group of bacteria, methanogenic, convert H_2, CO_2, and acetate, to CH_4. The AD is carried out in large digesters that are maintained at temperatures ranging from 30°C -65°C (Appels et al., 2008; Demirel and Scherer, 2008).

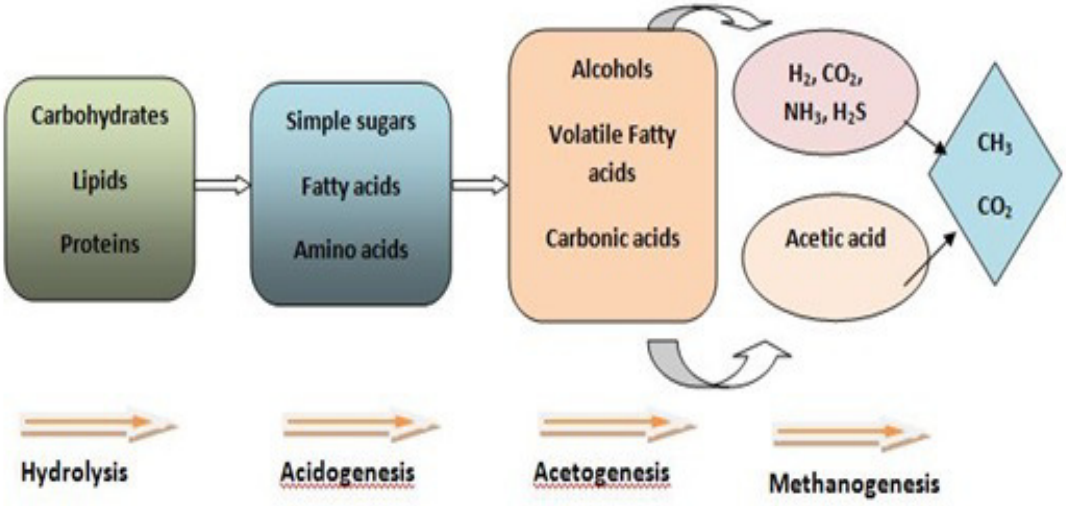

Figure 1.1: Anaerobic digestion

Three physiological groups of bacteria are involved in the anaerobic conversion of organic materials the first group of hydrolyzing and fermenting bacteria convert complex organic materials such as carbohydrates, proteins and lipids to fatty acids, alcohols, carbon dioxide, ammonia and hydrogen. The complex polymeric matter is hydrolyzed to monomer, e.g., cellulose to sugars or alcohols and proteins to peptides or amino acids, by hydrolytic enzymes, (lipases, proteases, cellulases, amylases, etc.) secreted by microbes. The hydrolytic activity is of significant importance in high organic waste and may become rate limiting. The second group producing acetogenic bacteria convert the product of the first group into hydrogen, carbon dioxide and acetic acid. They are also known as acid formers which convert the products of the first phase to simple organic acids, carbon dioxide and hydrogen. The principal acids produced are acetic acid (CH_3COOH), propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$), and ethanol (C_2H_5OH). The products formed during acetogenesis are due to a number of different microbes, e.g., *Syntrophobacter wolinii*, a propionate decomposer and *Sytrophomonos wolfei*, a butyrate decomposer (Molino et al., 2013).

The third step or acetogenesis is a critical step where acetate is produced which is the immediate precursor of methane. Some acetate is produced through mixed acid fermentation while most of the acetate is produced through secondary fermentation. On this step, produced VFAs are converted to acids. Two groups of acetogens are involved in the process which is obligate hydrogen producing acetogens (OHPAs) and homoacetogens. The OHPAs are more dominant and produces acetate from fatty acids and the latter produces acetate through anaerobic respiration. Finally, in the fourth stage methane is produced by bacteria called methane formers (also known as methanogens) in two ways: either by means of cleavage of acetic acid molecules to generate carbon dioxide and methane, or by reduction of carbon dioxide with hydrogen. Methane production is higher from reduction of carbon dioxide but limited hydrogen concentration in digesters results in that the acetate reaction is the primary producer of methane (Omstead *et al.*, 1980). The methanogenic bacteria include *Methanobacterium, Methanobacillus, Methanococcus and Methanosarcina*.

Methanogens can also be divided into two groups: acetate and H_2/CO_2 consumers. *Methanosarcina* spp. and *Methanothrix* spp. (also, *Methanosaeta*) are considered to be important in AD both as acetate and H_2/CO_2 consumers (Schmidt and Ahring, 1996).

Table 1.1: Composition of normal biogas from anaerobic digester (Sitorus and Panjaitan, 2013)

Component	Composition levels
Methane	55-70% by vol
Carbon dioxide	30-45% by vol
Hydrogen sulphide	200-4000 ppm by vol
Energy content of AD gas product	20-25MJ/standard m3

The major benefit of anaerobic digestion as a waste stabilisation process is its lower energy requirement. The methane, soil conditioner and liquid fertilizer (digestate) produced as a by-products of the process provides potential sources of revenue. It reduces the green house gas production by reducing the demand for fossil fuels. Biomass acclimatisation allows most organic compounds to be transformed into biogas even in small volume reactors. There is rapid response to substrate addition after long periods without feeding. It provides a beneficial alternative method for reducing odour and sanitation issues caused by dumping of organic wastes in the land. However some disadvantages are also there with the process. It needs longer start-up time to develop necessary biomass inventory and sometimes it may require further treatment with an aerobic treatment process to meet discharge requirements. Biological nitrogen and phosphorus removal is not possible with the process.

1.5. Gap Areas

The nutrient accumulation in the water bodies increase the biological activity of the water body and causes dense growth of macrophytes like *Eichhornia* and *Pistia* over

water surface. Removal of these plants plays a significant role in improving the water quality as the decomposition of these plants on the water bodies increases the internal concentration of nutrients on the lake. Utilization of floating or submerged plants as phytoremediation agents is a promising method of removing nutrients and heavy metals from the waste water and industrial effluents. Once used for removal of contaminants, these plants can be harvested and can be used for valorization purposes like biomethanation. Biomethanation of lignocellulosic biomass, as a source of renewable energy faces lots of challenges like lower biogas yield and seasonal availability of substrate. To ensure the running of large scale digesters on field, these issues have to be addressed. Moreover, microbial ecology of such digesters, specifically the role of higher trophic organisms like protozoa is a least explored area. Population dynamics of these organisms influences the quality of the digestion and can be used as biological indicator of digestion.

This thesis covers the studies addressing these issues.

1.6. Research objectives

The research objectives addressed in this study are

(1) Evaluation of locally available macrophytes for application in nutrient removal activities,

(2) To study the anaerobic digestion (biomethanation) of macrophyte biomass for recovering of value added products and

(3) To study the microbial ecology of selected macrophyte rhizosphere and anaerobic digester for treating the macrophyte biomass.

Chapter 2

Review of Literature

2.1. Nutrient Removal approaches for waterbodies

Waterbody restoration is a need for the time, and the nutrient criteria are planned to depend upon the type of water body to be restored. Depending upon the sites, the water bodies will be prioritized in accordance with the nutrient removal regulations to be developed (Water Quality Assessment Division, 2006). For an effective regulation criterion to be developed, a nutrient database has to be created to analyze the seasonal changes on essential parameters like nutrients, Secchi depth, DO, turbidity (NTU), bacterial count and TDS over a period. The results obtained from the analysis are compared with the standards, a preliminary statistics is developed which will allow in developing and implementing a control measure for the water body restoration. The restoration can be done by treating the effect or the cause of eutrophication. Control of nutrient inputs is a successful method of regulating eutrophication. Physical, chemical and biological methods are being implemented for lake restoration. The method to be adopted depends upon the type of water body to be restored, its ecoregion and the nutrient input it receives.

2.1.1. Physical methods

Physical methods of nutrient regulation are done by either controlling the nutrient input or by removing the pollutants from the waterbed. The effluents released from the industrial wastes are supposed to be having pollutants only in the trace amount. But due to monetary constraints, the direct release of untreated effluent is a major concern in the state of remediation. Nutrient prevention is one of the management activities that are practiced to prevent aging of water bodies. Phosphorus is the primary limiting nutrient for eutrophication in most of the cases. The major sources of P input are excreta and detergents. Phosphorus in the form of phosphates is a major component in cleaning solutions because of its cleaning effect. But the removal of this phosphate from the effluents is expensive. Henceforth they are directly released to the water body where nutrient built up happens. So the discussions on the banning of detergents containing phosphates started from the

1970s and are implemented on several European Union countries by 2010 to reduce the phosphate level. In India, the estimated annual consumption of phosphate-containing laundry detergents for the current population in India is about 2.88 million tonnes, and the total outflow of P is expected to be 146 thousand tons per year (Kundu et al., 2014). But unfortunately, none of the synthetic detergents used in India are not phosphate free due to lack of mandatory regulations. The regulations in controlling the point sources of P were found to be useful in regulating the nutrient input to the river basins. In hypolimnetic aeration, oxygen is pumped to hypolimnion layer which is oxygen depleted. During anoxic conditions, critical nutrients and heavy metals are released into the water column from the sediment which increases nutrient load and so also, plant and algae growth. Surface spray, paddle wheels or diffusers are used for aeration. But the disadvantage of this method is that it is costly and there is difficulty in operating it without destratification of layers. This method has been successfully implemented in more than 350 states in the European countries and is most suitable for deep lakes. The success of implementation depends upon the degree of stratification and the air flow rate.

Hypolimnetic withdrawal is an inlake restoration technique where nutrient-rich water from the hypolimnion layer is siphoned to remove the internal nutrient accumulation. It is an active low-cost restoration technique and has the potential to reverse the process of eutrophication if repeatedly done over years. Hypolimnetic withdrawal along with aeration was applied to hoard the river from dying, and there was built up in dissolved oxygen across the layers with improved habitat for flora and fauna (Kumar Arun, 2008). A study conducted by comparing the water quality variables before and after this technique in stratified lakes of European and North American lakes was found to be successful as there was a massive removal of average phosphorus accumulation in sediments. But there will be temperature variation in the hypolimnic region because of water siphoning. This can lead to thermal instability in the water layers and may affect the aquatic habitat there within (Nurnberg, 2007). In Italy, for restoring Lake Varese (Surface area of 14.52 km^2),

hypolimnetic withdrawal in the deepest section (maximum depth: 26 m) and oxygenation in the shallower section, during summer stratification were done and succeeded (Premazzi et al., 2003). In India, Lake Nainital of 46 hectare, which is one among the national lake situated in Himalaya, was found to be suffering from anthropogenic activities.

Environmental dredging is the removal of nutrient-rich sediments from water bodies. This requires heavy equipment or specialized hydraulic dredges. Dredges remove sediments along with water from water bodies. The deposit is dewatered at the shore and the water before releasing back will be subjected to treatment to prevent resuspension of pollutants. This can control rooted vegetation and deepens the lake by increasing lake volume. Sediment dredging was found to be useful in dams and estuaries. A study to observe the effect of dredging on Phosphorus cycle was done by Jing et al., (2015) on Dongqian Lake, China. It was found that dredging cannot be adequate unless external loading of P is blocked. It was also found to be affecting the Iron cycling in the lake. Decrease of invertebrate species due to sediment change, increase of oxygen demand due to re-suspension of sediments that also affects lighting intensity, and increase of turbidity levels caused by plumes, can be triggered by dragging, scooping and dumping acts while dredging (Balchand and Rasheed, 2000; Crowe et al., 2010; de Leeuw, 2010). The drawback of this technique is expensive as well as requires permission from the governing bodies as the unscientific dredging can affect the whole ecosystem in and around the lake.

2.1.2. Chemical methods

Nutrient inactivation is the method in which aluminum, ferrous or calcium salts are used for the precipitation of nutrients especially soluble reactive Phosphorus. Phosphorus reacts with aluminum sulfate (Alum) to form aluminum phosphate or aluminum hydroxide which forms insoluble precipitate as flocs. For target concentrations above 2 mg/L, a dose of 1.0 mole of aluminium per mole of phosphorus is sufficient and the resultant precipitate in the form of wet sludge

makes up to 5% in the total quantity of processed water (Kluczka et al., 2017). This can be removed from the water column. This method can successfully reduce the level of phosphorus and particulates which subsequently control the algal growth. Due to its cost-effectiveness and efficiency, it has been applied across the globe including Mansi Lake in India (Shaha and Ghujjare, 2008). It can be added to ponds, lakes or reservoirs as single dose proportional to the stormwater reaching the water body. This technique is mostly applied in shallow lakes and can last for 8 or more years. Other than nutrients, alum impregnated alumina can be used for the removal of fluoride as well (Maheswari, 2006).

2.1.3. Biological removal

Over physical or chemical methods of lake restoration, biological processes are more preferred due to its cost-effectiveness and eco-friendly nature. Biological removal mainly consists of removal of nutrients by plants or microorganisms. Enhanced biological removal by plants and microorganisms are being used in different remediation aspects like storm water and industrial effluent treatments (Hu et al., 2003). Biological Nutrient Removal (BNR) uses engineered systems with anaerobic, aerobic or facultative anaerobic microorganisms for treating various wastewaters according to its quality and amount. The microorganisms utilize in the engineered system or in the natural treatment can biologically assimilate the nutrients and used in increasing their biomass (Nancharaiah et al., 2016).

Aquatic Natural Treatment Systems are engineered system in the form of wetlands and ponds for treating pollutants for lake restoration and treating wastewater (Rezania et al., 2016). Ponds and lagoons are for treating stormwater and industrial wastewater respectively. Plants with good adsorption and tolerance to pollutants are used for the vegetation in the lakes. The more it is engineered, the more predictable it is to treat its capacity of treatment. Floating plants like Duckweed (*Lemna minor*) or water hyacinth (*Eichhornia crassipes*) and submerged plants like waterweed or water milfoil are used for the treatment of pollutants (Robles-Pliego et al., 2015;

Rezania et al., 2015). Constructed wetlands are another method of nutrient removal where the different type of vegetations is used in different basins. Free water surface constructed wetlands and subsurface flow wetland constructed wetlands are being used for nutrient removal, and the vegetation is used for anaerobic digestion post harvesting.

Aquatic macrophytes are effective indicators of water quality. It will enhance the ability to absorb loads of nutrients because of these properties is well used in wastewater treatment. The water hyacinth, *Eichhornia crassipes* is a tropical species belonging to the pickerelweed family (Pontederiaceae). It is a native of Brazil and possibly other Central South American countries, now it occurs in lakes, slow-moving rivers and swamps in most countries of the world including India, South Africa and the USA.

Water hyacinth can tolerate considerable variation in nutrients, temperature and pH levels. The optimum pH for growth of the plant is 6-8. It can grow in a wide range of temperature from 10 to 40°C, but it is thought to be cold sensitive and optimum growth at 25°C to 27.5°C. Growth rates increases with the increase in water nutrient level especially nitrogen amount (Heard and Winterton, 2000). *Pistia stratiotes* (L.) is a floating perennial commonly called water lettuce belonging to the family Araceae. It floats on the surface of the water, and its roots hanging submerged beneath floating leaves. Plants are known to accumulate large quantities of nutrients during the period of rapid growth (Gupta et al., 2012).

2.2. Role of Microorganisms in nutrient assimilation

Biological assimilation done by plants results in biomass addition and microorganisms in the root microflora play a significant role in this. Microbial removal of nutrients is adopted in various forms of engineered systems depending upon the need and demand to be satisfied. Microbial removal of nitrogen forms is by ammonification, ammonia assimilation, denitrification and by nitrification. Ammonia is the most preferred form by bacteria due to its-3 oxidation state which is

identical as in biomass. Ammonification is converting organic reduced nitrogen forms to ammonia by heterotrophic bacteria. The primary determining factor of this process is the carbon to nitrogen ration in the medium (Grady et al., 1999). In the absence of NH_4^+-N, bacteria prefers more reduced forms like nitrate or nitrate, but a significant energy spending has to be done to reduce it to the -3 oxidation state (Rittmann and McCarty, 2001). Denitrification is another important step in nitrogen cycle where nitrate or nitrite is biologically reduced to N_2 gas where Nitrification is the biological oxidation of ammonia nitrogen to nitrite nitrogen and then to nitrate nitrogen. Ammonia-oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) are together called nitrifiers. Most of them are autotrophic, but heterotrophic populations such as *Bacillus* and *Pseudomonas* were also reported. Most of the bacterial communities in nitrifiers are aerobic but can survive in anaerobic conditions as well. In the absence of oxygen, some microorganisms can use ammonia as the inorganic electron donor and nitrate as electron acceptor which produces as N_2 gas and water as the by-product. This process is called anaerobic ammonia oxidation or ANAMMOX process (Egli, 2001; Egli et al., 2003).

Like Nitrogen, Phosphorus is another essential macronutrient required for the growth of organisms. Phosphorus is majorly solubilized by Phosphate solubilizing microorganisms (PSMs). The major amount of phosphorus available in the soil is in insoluble form. They are solubilized by PSMs and made available to plants. PSMs isolated are majorly from the species *Pseudomonas* and *Bacillus* (Illmer and Schinner, 1992) and *Aspergillus* and *Penicillium* from the fungal species (Wakelin et al., 2004). Other than bacteria and fungi, actinomycetes and algae are also reported to show phosphate solubilizing activity. PSMs can solubilize Phosphorus by releasing organic acids, or by releasing extracellular enzymes or by substrate degradation (McGill and Cole, 1981).

The significance of identifying the role of microorganisms in nutrient removal holds an important aspect called bioaugmentation. Bioaugmentation has been implemented in reducing the nutrient concentration from sludge by hydrolysis and is

found to be useful in North China (Ma et al., 2011). Phragmites constructed as wetland microcosm when inoculated with various denitrifying bacteria was found to be effective in nutrient removal from polluted lake water (Hong Bo et al., 2010). Studies on floating bed systems of perennial grass inoculated with effective microorganisms are gaining its significance in in-situ treatment of polluted water bodies. The major plants and microorganisms which are significant in nutrient removal are listed in Table 2.1

Table 2.1: Plants and Microbes used for nitrogen and Phosphorus removal

Plant	Type	References
Water Hyacinth (*Eichhornia crassipes*), *Pistia stratiotes*, *Lemna minor*, *Salvinia minima*	Free floating aquatic vascular plants with high reproduction rate	Boyd, 1970; Reddy and De Busk, 1985; Jayaweera and Kasturiarachchi, 2004
Pennywort (*Hydrocotyle umbelleta*), Water lily (*Nymphoides indica*), Aquatic vines *(Ipomoea* spp.)	Rooted floating macrophytes	Sooknak and Wilie (2004); Greenway and Wooley (1999)
Reed canary grass (Phalaris arundinacea), cattails (Typha spp.), *Juncus effusus*, *Scirpus lacustris,*	Emergent plants widespread in Europe and Asia	Guntenspergen et. al., 1989; Vymazal, 2013
Microorganisms	Type	References
(*Neochloris oleoabundans*, *Chlorella vulgaris* and *Scenedesmus obliquus*)	Microalgae with 99 % of Nitrogen and Phosphorus removal	Franchino et al., 2013
Acinetobacter genus	Phosphate accumulating organisms (PAOs)	Van Loosdrecht et al., 1997
Microlunatus phosphovorus	Phosphate accumulating organisms (PAOs)	Nakamura 1991,1995
Accumulibacter phosphatis	Phosphate accumulating organisms (PAOs)	Dabert 2001, Lee 2001
Nitrococcus mobilis	Ammonia oxidizer	Juretschko 1998
Nitrobacter sp.,	Nitrate oxidizer	Henze et al., 1997
Alkaligens, Pseudomonas, Methylobacterium, Bacilus, Paracoccus, Hyphomicrobium	Denitrifying bacteria	Wagner et al., 2002

2.3. Anaerobic digestion

Anaerobic Digestion (AD) is a complex microbial process which requires strict anaerobic conditions (oxidation reduction potential (ORP $\leq$ 200 mV) to proceed, and depends on the coordinated activity of a diverse microbial communities to transform organic material into mostly methane (CH_4) and carbon dioxide (CO_2). The major factors affecting the process are pH, temperature, alkalinity, volatile fatty acids to alkalinity ratio, solid and hydraulic retention time (HRT). The inhibitors commonly present in anaerobic digesters include ammonia, sulphide, light metal ions, heavy metals etc. Co-digestion with other waste, adaptation of microorganisms to inhibitory substances, or adding methods to remove the toxicants can massively improve the efficacy of the waste treatment (Chen et al., 2008).

2.3.1. Anaerobic digestion of aquatic macrophytes

Anaerobic digestion of different aquatic macrophytes like Water hyacinth, *Cabomba*, and *Salvinia* were reported in the past. Aquatic weeds including submerged macrophyte like *Ceratophyllum demersum*, *Egeria densa*, *Elodea nuttallii*, *Potamogeton maackianus* and *Potamogeton malaianus* were studied for their biomethanation potential (Koyama et al., 2014). It was found that *C. demersum, El. nuttallii* and *P. malaianus* are feasible for anaerobic digestion due to the high methane recovery, and the rate of methane recovery was found regulated by the lignin content. The results of the pilot-scale batch digestion study reported digestion of both Water hyacinth and Cabomba yielding 267 L biogas/kg VS and 221 L biogas/kg VS, respectively, with a methane content of 50%. In the same study, *Salvinia* produced only 155 L biogas/kg VS with 50% methane (Shah et al., 2015). Patil et al., (2014) have reported that dried water hyacinth (DWH) produced slightly more biogas as compared to digester with cow manure (CM). This indicates the fact that substrates for methanogenic bacteria are readily available in water hyacinth. However, the period for attaining the maximum production rate is more extended (45 – 55 days) for water hyacinth as compared to cow manure (35-45

days). This is because bacteria needed for biogas production in the case of water hyacinth takes a longer period to grow whereas in ruminants waste such as cow manure pathogens are already present and bacterial growth takes a little time for biogas production.

a) Anaerobic digestion of water hyacinth biomass

Water hyacinth has been suggested as a strong candidate for the production of methane because of high biomass yield potential (Ghosh and Klass, 1977). They have reported that WH under conventional digestion conditions exhibited higher methane yields and energy recovery efficiencies when grown in sewage-fed lagoons as compared to the corresponding values obtained with WH grown in a fresh-water pond. Mesophilic digestion provided the highest feed energy recovered in the product gas like methane, while thermophilic digestion, when operated at sufficiently high loading rates and reduced detention times, gave the highest specific methane production rates which are supported by recent studies. Both batch and semi-continuous digestion experiments were performed. The highest apparent biogas yields reported were obtained in the batch mode of operation over long detention times (Wolverton and McDonald, 1981).

A comparative study on the effect of different pre-treatment methods on the biogas yield from WH was carried out by Patil et al., (2012). WH was pre-treated as chopped, dried and ground, treated with NaOH, ground WH combined with poultry waste and ground WH combined with primary sludge. The results of the study showed the highest cumulative biogas yield was from ground WH combined with poultry waste. The biogas yield of the fresh WH was negligible. The composition of biogas from WH and poultry waste and primary sludge showed methane 65 % methane and 35 % CO_2 whereas fresh water hyacinth contained methane 60 % methane and 39.94% CO_2. NaOH treated WH yielded biogas with 71% methane and 29 % CO_2. In an early study, Itodo et al., (1992) have proposed that several steps such as the introduction of bacteria having the cellulolytic capacity, preheating the

media material, milling the media material, chemical treatments with NaOH, and drying have been shown to improve biogas yield.

Chanakya et al. (1992), by coupling a solid-phase acidogenic system to an up-flow anaerobic packed-bed methanogenic digester. The leachate from the acidogenic reactor was fed to the methanogenic reactor for methane production. A two-stage rumen-derived anaerobic digestion process for the conversion of WH shoots with cow dung into biogas. Under conditions similar to those of the rumen and loading rates (LR) in the range of 11.6–19.3 g volatile solids (VS) /L.day, the degradation efficiencies were 38% for the shoots and 43% for the mixture. Furthermore by applying a loading rate of 154 gVS/day, an SRT of 90 hr, and connecting it to a methanogenic reactor of the UASB type, 100% conversion efficiency of the VFA into biogas with the methane content of 80% was achieved. The average methane gas yield was 0.44lg/VS (Kivaisi and Mtila, 2007).

Patil et al., (2012) have conducted a series of experiments on biomethanation using fresh WH, dry WH, poultry litter, cow manure and primary sludge with 60 days retention time. The digester fed with poultry litter produced the highest biogas followed by the digester fed with primary sludge. The results also revealed that digester with dried WH produced slightly more biogas as compared to digester with cow manure. However, the period for attaining the maximum production rate is longer for WH as compared to cow manure. The use of enriched and pre-treated WH for biogas generation increases gas production, therefore, will be a good energy source for those residing in the coastal areas, which face the menace of clogging of waterways by the weed.

Momoh et al., (2011) have reported that the co-digestion of cow dung, WH and waste paper is feasible at room temperature. However, the effect of waste paper on the fixed amount of cow dung and WH was found to increase biogas production in a parabolic manner. It was observed that a waste paper concentration of 17.5 gm is the maximum amount of waste paper needed to combine with 5 gm of cow dung and 5

gm of WH for maximum production of biogas. Almoustapha et al., (2008) carried out a pilot project with discontinuous-type installation (batch reactors), that investigates the possibility of producing biogas from a mixture of WH and fresh rumen residue, and replacing fire-wood as a source of fuel. The study revealed improved gas production during the summer season which is approximately 1.8 times greater than it is during the winter season.

Singhal and Rai (2003) compared biogas production from water hyacinth (*Eichhornia crassipes*) and channel grass (*Vallisneria spiralis*) which were employed separately for phytoremediation of lignin and metal-rich pulp and paper mill and highly acidic distillery effluents. They found that biogas production from channel grass was relatively greater and quicker (maximum in 6-9 days) than that from water hyacinth (in 9-12 days) and such variation in biogas production by the two macrophytes was found to be correlated with the changes in C, N and C/N ratio of their slurry brought by phytoremediation. A study compared biomethanation of water hyacinth and *Salvinia* in Santhiniketan and found that the yield of biogas produced from water hyacinth and *Salvinia* were 552 L kg-1 volatile solids (VS) and 221 L kg-1 VS, respectively. The maximum methane content obtained in the current study was 62 and 63 % for water hyacinth and *Salvinia* (Mathew et al., 2015).

Jagadish H Patil et al., (2011) studied the effect of volatile fatty acid on biomethanation of WH. All biodigesters were fed with the fermentation slurry and were seeded with inoculum obtained from an anaerobic primary sludge digester. Acetic acid (lower volatile fatty acid) 10% by volume was added in different amounts to each of the biodigesters. A maximum cumulative biogas yield was produced by the digester which was fed with 0.4 ml of acetic acid. The overall results showed that the addition of acetic acid in an optimum quantity has a remarkable effect on the cumulative biogas production. However the addition of acetic acid more than the optimum quantity evolved very less quantity of biogas because of imbalance in the syntrophic interaction between acetogens and methanogens, which might have caused accumulation of volatile acids thus

increasing the pH of fermentation slurry. Effect of heavy metals on biomethanation of water hyacinth was studied by Patel et al., (1993). They have examined the effect of $FeCl_3$, $NiCl_2$, $CoCl_2$, $CuCl_2$, and $ZnCl_2$, on anaerobic digestion of water hyacinth-cattle dung and concluded that $FeCl_3$ caused a more than 60 % increase in biogas production with high biomethane content.

Other than as energy crop, water hyacinth is having another application as fish and ruminant feed. Studies have reported the usage of sun dried WH for feeding and has found to be superiorto rice hay in terms of crude protein and digestability (Abdelhamid and Gabr, 1991). For paper production from WH, it was sun-dried to a DM content of 160 to 200 g/kg and sprayed with molasses (Tham, 2016). Rezani et al., (2015) has suggested the usage of dried WH biomass to be fabricated as briquettes, which is suitable as co-firing agent in coal power plant. On studying the effect of microwave pre treatment of WH for biogas production, it was found that the unpretreated fresh and dried water hyacinth produced biogas of 37,56 and 33,56 mL/g TS, respectively (Sumardiono et al., 2015). Ganguly et al., (2016) have used 3 dys sundried water hyacinth biomass with different catalysts like rice beer cake, acetic acid and cows urine for biogas production and found that cows urine can act as catalyst for improved gas production from dried water hyacinth.

b) Co-digestion

Anaerobic digestion is one of the widely accepted solutions for managing different kinds of organic wastes. Until recently anaerobic digestion (AD) was a single purpose treatment of single substrate. For example manure was digested to produce energy. Today, linking the substrate characteristics, the limits and the possibilities of AD are better known and co-digestion has therefore become a feasible solution to overcome the drawbacks of mono-digestion. Common co-digestion substrates include sewage sludge and the organic fraction of the municipal solid waste. The merits of co digestion are improved nutrient balance for an optimal digestion and a good fertilizer quality, homogenisation of particulate, floating, or settling wastes

through mixing with animal manures or sewage sludge, increased, steady biogas production throughout the seasons, additional fertilizer (soil conditioner) and renewable biomass production for digestion ("Energy crop") as a potential new income of agriculture (Braun and Wellinger, 2003).

Studies have shown that co-digestion of several substrates, for example, banana and plantain peels, spent grains and rice husk, pig waste and cassava peels, sewage and brewery sludge, among many others, have resulted in improved methane yield by as much as 60% compared to that obtained from single substrates (Adeyanju, 2008; Babel et al., 2009). In a co-digestion study conducted by Ofoefule and Uzodinma (2009) using cassava peels, cow dung, poultry droppins and swine dung, it was found that among the different combinations tried, cassava peels and swine dung had the highest cumulative gas yield of 169.60 L/total mass of slurry whereas the cassava peel and cow dung experienced fastest onset of flammable gas production. In a study varying amounts of sawdust waste complimented with a fixed amount of cow dung and water hyacinth was anaerobically fermented in batch-fed digesters, it was found an increase in 8 to 9.5% of total solid content obtained by the addition of saw dust improved biogas production (Otaraku and Ogedengbe, 2013).

Anaerobic co-digestion of sheep waste which is rich in anaerobic bacteria along with water hyacinth on various ratios of substrate was done by Patil et al., (2014) and was found to have an yield of 346 ml/ gm of VS. The addition of waste paper in the co-digestion of cow dung and water hyacinth was suggested as a feasible means of improving biogas yield and also alternative means of recycling waste paper (Yusuf and Ify, 2011). Patil et al., (2011) has showed biogas production of 0.35L/ gm of VS with 69% methane content by co digesting alkali pre treated water hyacinth with primary sludge in a batch digester with a retention period of 60 days. Comparison of biomethanation of various organic wastes like water hyacinth, poultry litter, cow manure and primary sludge were studied and comparative analysis was done on biogas yield and it was found that water hyacinth and poultry litter yielded 1.08 l per gm VS on a 250 ml batch experiment on 60 days retention period. The addition of

waste paper to fixed amount of cow dung and water hyacinth was observed to improve biogas production. However, biogas yield was observed to decrease with increase in waste paper concentration (Yusuf and Ify, 2011). The effect of fish waste (FW), slaughter house wastewater and waste activated sludge(WAS) addition as co-substrates on the fruit and vegetable waste (FVW) anaerobic digestion performance was investigated under mesophilic by Bouallagui et al., (2009) for finding the better co-substrate for the enhanced performance of co-digestion. A C/N ratio between 22 and 25 seemed to be better for anaerobic co-digestion of FVW with its co-substrates. The most significant factor for enhanced FVW digestion performance was the improved organic nitrogen content provided by the additional wastes. The major areas that can be modified for the novelty of the anaerobic co-digestion are pre-treatment, microbial dynamics and modelling. Understanding the role of each factor improves the predictability as well as economic feasibility of the process.

c) Ensilation

Ensiling is a crop preservation method based on natural lactic acid fermentation under anaerobic conditions which is majorly practiced across the globe for feed preparation for ruminants (Gollop et al., 2005). Preserving feed for an entire year is essential for the economic feasibility of animal farming. There are different processes involved in ensilation which are harvesting the crop at the optimal stage of maturity, chopping, loading into a silo, compacting to exclude air gaps, storing and unloading for feeding ruminants. The four processing steps at which biochemical and microbiological incidents can arise are the aerobic, fermentation, storage and unloading stages (Ashbell et al., 2002). Fermentation of crops by microorganisms especially lactic acid bacteria lowers the pH and prevents the growth of undesirable organisms. Impaired silage preparation affects the ruminant health as well its productivity, henceforth for maintaining the silage quality without nutrient loss, silage additives such as chemicals, enzymes and even microorganisms or its derivatives are added to the silage (Duniere et al., 2013). Different feeds like grass, clover, alfalfa, barley, corn, wheat, sorghum (Ashbell et al., 2002) and

various moist "by-products" of the food industry, such as apple pomace, beet pulp and brewer's mash are used for silage preparation (Ajila et al., 2012).

Other than feeding purposes, there are lots of farm-based biogas plants exists in various countries like Germany and Austria where plants are mainly operated with quantities of energy crops as feedstock (Weiland, 2010). For low-loss preservation of whole crop plant material of such energy crops like Maize (*Zea mays*), sorghum hybrid (*Sorghum bicolor*), forage rye (*Secale cereale*) and triticale etc, ensilation is essential to maintain the economic feasibility of the digestion process. Studies have revealed that when discussing the effect of ensiling on methane production, it is of great importance whether storage losses especially dry matters are taken into account (Hermann et al., 2011). It was found that when DM calculation is carried out in terms of dry residue unlike by volatile compounds such as organic acids, alcohols and ammonia, a proper estimation of methane yield can be collected. Mukengele and Oechsner (2007) have found that methane yield of ensiled maize was overestimated by 5–10% without correction of volatile compounds. Effect of variety, harvest and pre-treatment on anaerobic digestion of maize was studied by Bruni et al (2010). It was found that fresh maize gave the highest methane yield/hectare at late harvest and reduction of the particle size of maize silage to an average size of approximately 2 mm increased the methane yield m^3 CH_4 $(kg\ VS)^{-1}$by approximately 10%.

Co digestions with silages are also tried for better energy recovery. Anaerobic co-digestion of concentrated pig manure (PM) with grass silage (GS) at five different PM to GS volatile solid (VS) ratios of 1:0, 3:1, 1:1, 1:3 and 0:1 was evaluated by examining operation stability and methane (CH_4) production potentials. The highest specific CH_4 yields were 304.2 and 302.8 ml CH_4/g VS at PM to GS ratios of 3:1 and 1:1, respectively and on the ratio of 1:1, the system failed (Xie et al., 2011).

Chapter 3

Nutrient removal by floating macrophytes

3.1. Introduction

Major threat faced by the water bodies across the globe is anthropogenic eutrophication. Eutrophication increases rates of primary productivity which eventually shifts plant species composition. The explosive production of aquatic biomass has a massive impact on depletion of water quality and the recreational activities related to the water body like fishing, boating or lake tourism. Eutrophication is triggered with nutrient enrichment process where primary nutrient inputs are Nitrogen and Phosphorous. Nitrogen (N) is needed mainly for protein synthesis, and phosphorus (P), is necessary for DNA, RNA, and energy transfer and they are both required to support aquatic plant growth and are the vital limiting nutrients in most aquatic and terrestrial ecosystems. Ammonia, Nitrate, and Nitrite are the major inorganic forms of nitrogen found in water whereas Phosphorus majorly occurs as orthophosphates which are reactive phosphorus and will be further converted into organic phosphates by biota in the water bodies. The major sources of Nitrogen and Phosphorous are from industrial effluents, runoffs and fertilizers.

Aquatic macrophyte systems can be used effectively to reduce pollutant levels in water bodies (Reddy and De Busk, 1985) and the biomass can be used for the production of gaseous fuels, feed, fiber (Ward et al., 2008), and compost and organic soil amendments (Siracusa and La Rosa, 2006). Many macrophyte species have attracted attention in this aspect because of its ability to grow in heavily polluted water. Several studies have discussed the potential of aquatic plants for reducing N and P levels in wastewater, but most of these studies were limited to one plant, thus no comparative data among different plants grown under the same environmental conditions.

This chapter encapsulates works conducted to evaluate the role of different types of floating aquatic plants available in typical eutrophic lakes for removing N and P.

The experiments were designed to establish the role of these plants in improving water quality by removing nutrients in form of nitrate, ammonia and phosphate. Moreover, this chapter also focuses on the role of microorganisms in the nutrient removal process since the understanding of the diversity of microbes in nutrient removal is critical and least explored.

Therefore the objectives covered in this chapter are 1) to compare the nutrient removal potential of prominent locally available floating macrophytes like *Pistia* sp., *Eichhornia* sp., *Lemna minor* and *Salvinia minima* 2) to study the role of rhizospheric microflora in nutrient removal by macrophytes.

3.2. Materials and methods

3.2.1. Screening of invasive macrophytes for nutrient removal

a) *Nitrate-N and phosphate removal by locally available macrophytes*

Aquatic plants screened in this study were collected from a local eutrophic lake (Akkulam Lake). Water hyacinth (*Eichhornia crassipes*), water lettuce (*Pistia stratiotes*), duckweed (*Lemna minor*) and *Salvinia minima* are the major floating macrophytes found in the Lake. Representative samples of these plants were collected for the experiment, and were maintained in a stocking tank in NIIST campus for conditioning. The plants of uniform size and equal biomasses were selected for nutrient removal experiments. A batch experiment was set to analyze nutrient removal potential by these plants. The concentration range of nutrients (N and P) selected for the batch experiments were based on the field analysis of the local eutrophicated lake. The total nitrogen and total phosphorus level found in the lake were in the range of 10 to 80 mg/L and 0.01 to 1 mg/L respectively. So the nutrient range was set as (all mg/L) 20, 40, 60 and 80 for Nitrogen (Nitrate) and for Phosphorus, it was set in the range of (all mg/L) 0.5, 1, 2.5 and 5 in the form of phosphate. 1000 ppm stock solutions of nitrate-N and Phosphate-P were prepared by dissolving 0.163 gm of KNO_3 and 0.143 gm of KH_2PO_4 in 100 ml. From the stock desired quantities were prepared and added together to enact the nutrient status on the river.

Plastic containers of 35 liter capacity were taken and filled with 20 liters of tap water. Nutrient solutions of N and P in the form of KNO_3 and KH_2PO_4 in different concentrations described were added to the containers. Each concentration was prepared from the stock solutions of KNO_3 and KH_2PO_4. Control basins without plants were also kept to confirm that the removals of nutrients were done by plants. Plants were allowed to grow in the medium for two weeks. Water samples were collected from 5 random points of the container in alternative days from the starting day, for the analysis of nitrate and phosphate. The pH and temperature were also recorded daily using a portable probe and a thermometer respectively. At the end of 12 days, the plants were removed and weighed to determine the biomass buildup for calculating average nitrate-N and Phosphate-P removal by plants. The samples were filtered by using the Millipore GS 0.22µm filter paper. Residual Nitrate and phosphate were checked with the help of Dionex Ion Chromatography system.

b) Uptake of various nitrogen species by Pistia stratiotes

Based on the preliminary screening experiments, *Pistia stratiotes* was selected for detailed nutrient uptake study. Among the nitrogen species observed in eutrophic water, Nitrate-N and Ammonia-N are the dominant forms. So an experiment was set up to check the potential of *Pistia stratiotes* for the uptake of ammonia-N along with Nitrate- N. A concentration of range starting from 20 mg/L , 40 mg/L, 60 mg/L to 80 mg/L were selected for both ammonia-N and nitrate-N. Same concentrations of both Nitrate-N and ammonium-N were added simultaneously and separately to the containers which have the equal plant biomass of 50 gm.

3.2.2. Effect of salinity on nutrient removal

Eutrophic water bodies include brackish water (0.5 ppt to 35 ppt). Considering this, the effect of salinity on the nutrient removal was also studied by adding the four different levels of salt concentrations along with the nutrients. For this study, 0.5 ppt, 1 ppt, 1.5 ppt and 2 ppt of saline solutions were prepared using corresponding

concentrations of sodium chloride. The effect of these salinities on the removal of nitrate (NO_3-N, 40 mg/L) solution and phosphate (PO_4-P, 2.5 mg/L) solution using *Pistia stratiotes*, *Eichhornia crassipes*, and *Salvinia minima* was studied.

Samples for the analysis were collected as described previously. The salinity of the system was monitored using portable salinity probe. The samples were filtered and residual nitrate, and phosphate was checked with the help of Dionex Ion chromatography system. At the end of 12 days, the plants were removed and weighed to determine the biomass buildup for calculating average nitrate-N removal by plants.

3.2.3. Role of Root associated (rhizosphere) microflora in nutrient removal

The role of root associated microflora in nutrient removal by *Pistia* sp. was studied in detail. In an experiment, *Pistia* sp., with surface sterilized and native (without sterilization) root system were compared for both N and P removal. Sodium hypochlorite was used for surface sterilization. Plants with treated and untreated plants were kept in the plastic containers having 4 mg/L of Phosphate-P for the removal studies.

a. Analysis of Microbial Communities in the Root zone.

Isolation of endophytes was done by the modified procedure of Ji et al., (2014). For the isolation of endophytic microflora from the roots of the *Pistia* sp., the roots were collected in petri plates and washed thoroughly in running tap water to remove the dirt particles then washed 2-3 times with distilled water. The roots were placed in the Laminar Air Flow and immersed in 95% (V/V) ethanol for 10 minute. Then it was transferred to 1% sodium hypochlorite solution and immersed in it for 15 min. It was washed with sterile distilled water for three times. Then it was crushed using sterilized mortar and pestle. Suspension was taken and serially diluted (10^{-1} and 10^{-2}), then from each dilution tube 100µl was taken and spreaded onto R2A agar plates and incubated at 37˚C for three days.

b. Isolation and identification of rhizospheric nutrient assimilating bacteria

The Pikovskaya's (PVK) medium selective for isolating P solubilizing bacteria and the medium specific for nitrate reducing bacterium were used for identification of phosphate solubilizing bacteria and nitrate reducing bacteria respectively. The PVK medium was prepared by dissolving 3.13 gm of PVK agar (Hi Media, India) in 100ml distilled water, sterilized and agar plates were prepared. The fresh roots were collected and washed with distilled water. This was repeated for 2-3 times then crushed by using mortar and pestle. The suspension was serially diluted to 10^{-1} and 10^{-2}, and from each dilution, 100µl was taken, spread onto PVK plates by using sterile glass rod. The control plates also being prepared and incubated under 28 °C for 2 to 4 days.

The broth culture of root enrichment also plated on nitrate medium for isolating the nitrate-reducing microbial communities. Serial dilutions (10^{-1} to 10^{-4}) of broth were prepared first and spread 100µl from each dilution to PVK medium and nitrate medium. The colonies with confirmed phosphate solubilizing activity were selected for molecular identification using 16 S DNA sequences.

The bacterial DNA was extracted using Macherey Nagel Nucleospin soil DNA extraction kit from the pure culture broth. The DNA was extracted according to the protocol provided with the kit and is quantified with Nanodrop 2000 (Thermo Scientific, USA). The genomic DNA was amplified with 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-ACCTTGTTACGACTT-3′) using MyCycle Thermal Cycler System (BIORAD, USA) (Lane et al. 1991). Reactions were cycled at the following parameters: 94 °C for 3min; followed by 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and ending with a 10 min extension at 72 °C. PCR amplicon was electrophoresed on Mupid-ex electrophoresis system (Eurogentec, Belgium) to identify PCR product of the appropriate size and were visually confirmed using Gel Documentation system (BIORAD, USA). The PCR product is purified with Macherey Nagel Nucleospin

DNA Purification Kit and was given for sequencing (Scignome, Kochi). The sequence obtained was subjected to NCBI BLAST research for identifying the isolated strains based on similarity index.

Related sequences of the isolated strains and other prominent strains reported to have N and P removal were retrieved from the NCBI GenBank, and a phylogenetic tree was constructed using MEGA software.

3.3. Results and Discussion

3.3.1. Nutrient removal by locally available aquatic macrophytes

Commonly found prominent floating macrophytes like *Pistia stratiotes, Eichhornia crassipes, Lemna minor* and *Salvinia minima* are tested for their potential for removing nitrate-N and phosphate-P together. The nutrient concentrations used in the experiments were similar to the conditions found in local eutrophicated lake.

The result of preliminary screening to evaluate nitrate removal potential of the *Pistia stratio*tes is presented in Figure 3.1.

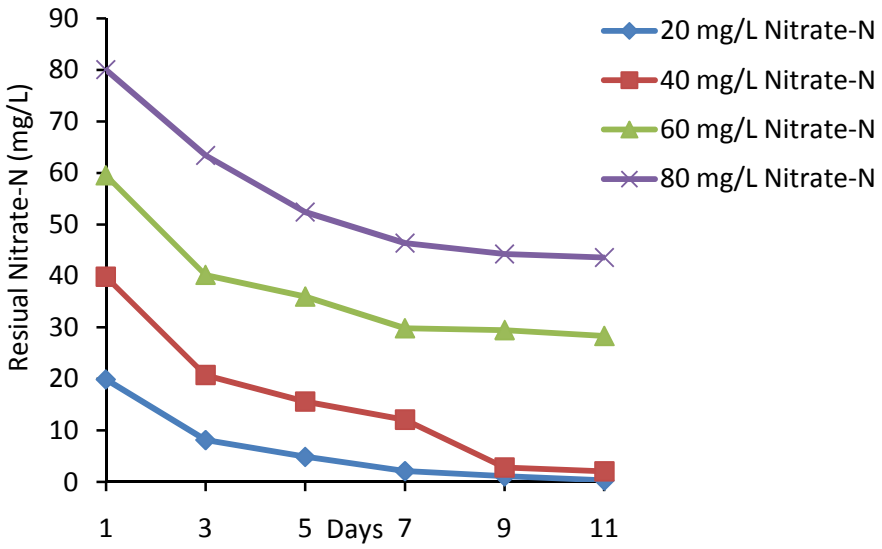

Figure 3.1: Nitrate-N removals by *Pistia stratiotes*

It was found that high removal rate was during the first five days of the experiment. The removal rate was found to be concentration dependent as it was less for higher concentrations of 60mg/l and 80mg/l when compared to the lower concentrations such as 20 mg/L and 40 mg/L. 100 % removal of the plant was observed when 20 mg/L of nitrate-N was given whereas it was 94%, 52% and 45% in the cases of 40 mg/L, 60 mg/L, and 80 mg/L respectively. Several phytoremediation studies have specified *Pistia* sp., as a potential candidate for nutrient removals. Phytoremediation of storm water in the constructed water detention systems was carried out using *Pistia* sp., and the results showed that these plants proved superior to most other plants in nutrient removal efficiency, owing to its rapid growth and high biomass yield potential. It showed 50 % removal of inorganic nitrogen and 31 % removal of Phosphorus (Lu et al., 2010). Similarly it showed 93% of nitrate removal and heavy metals removal from a stream polluted by refinery effluents (Ugya et al., 2015). But on contrary, on a study conducted on nutrient removal from 1:1 diluted anaerobically digested flushed dairy manure waste water, *Pistia* sp., was found to be limited by the high salinity content and other unidentified soluble fractions where as water hyacinth showed a robust growth on the same medium with a removal of 91% and 99 % for TKN and ammonia respectively (Sooknah and Wilkie, 2004).

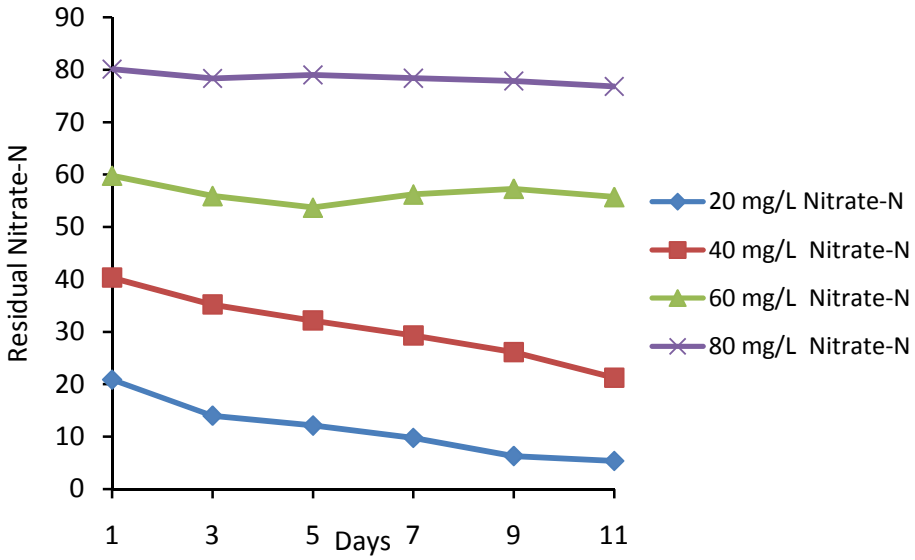

Figure 3.2: Nitrate-N removal by *Eichhornia crassipes*

The nitrate removal of *Eichhornia crassipes* was found to be much compared with *Pistia stratiotes*. A sudden decline in the nitrate concentration was not observed in this experiment (Figure 3.2.). Similar to *Pistia* sp., the removal of nitrate-N was found to be concentration dependent. The removal efficiency was 73% for 20 mg/L of Nitrate-N and 43% for 40 mg/L whereas for 60 mg/L and 80 mg/L; it was 16% and 4% respectively. Remediation performance of wetlands with floating plants was studied by Sung et al., (2015) and was found that the wetlands constructed with *Eichhornia* sp., could remove 50 % of nitrate- N from the retention type ponds. 66% removal of nitrate- N by *Eichhornia* sp. was observed when the plants were used for treating agricultural wastewater (Wenwei et al., 2016). Reddy et al. (1991) have shown in their studies that survival of water hyacinth requires 5.5 mg of N/L and 1.06 mg of P/L, where maximum growth can be achieved by the addition of N, P, and K at the rate of 20 mg N/L, 3 mg P/L, and 52 mg K/L, respectively.

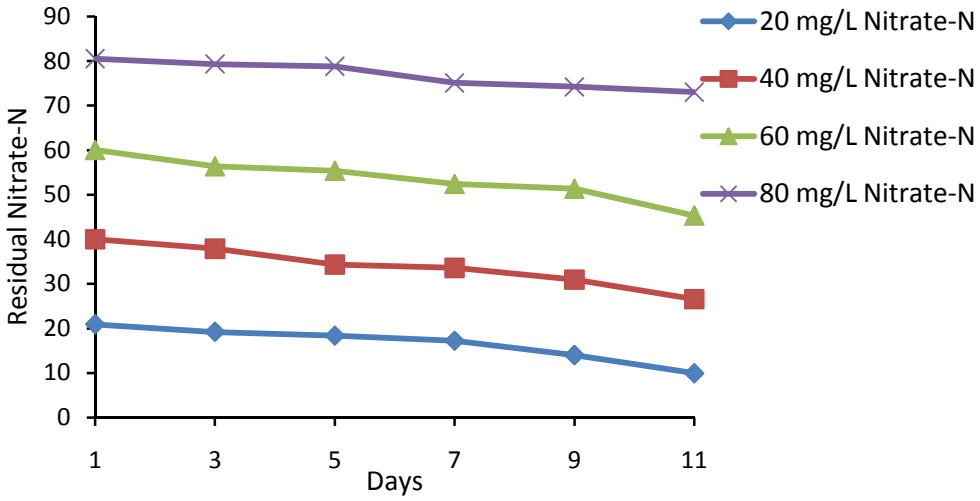

Figure 3.3: Nitrate-N removal by *Lemna minor*

Nitrate-N removing potential of *Lemna minor* was found to be in the range of 20 mg/L which was 51% (Fig 3.3.). It showed almost similar percentage of removal for 40 mg/L and 60 mg/L. The plant was inefficient at 80 mg/L where it showed only 5% removal. In a study using *Lemna* for treating palm oil mill effluent, it was reported that around 7% nitrate-N achieved with *Lemna* alone but along with algae the removal increased to 15% (Kamyabi et al., 2017). On studying nutrient removal from synthetic wastewater, Ng and Chan (2017) have observed that the nitrate removal by *Lemna minor* was found to be showing 6% removal of nitrate removal whereas ammonia removal by the plant was up to 44%.

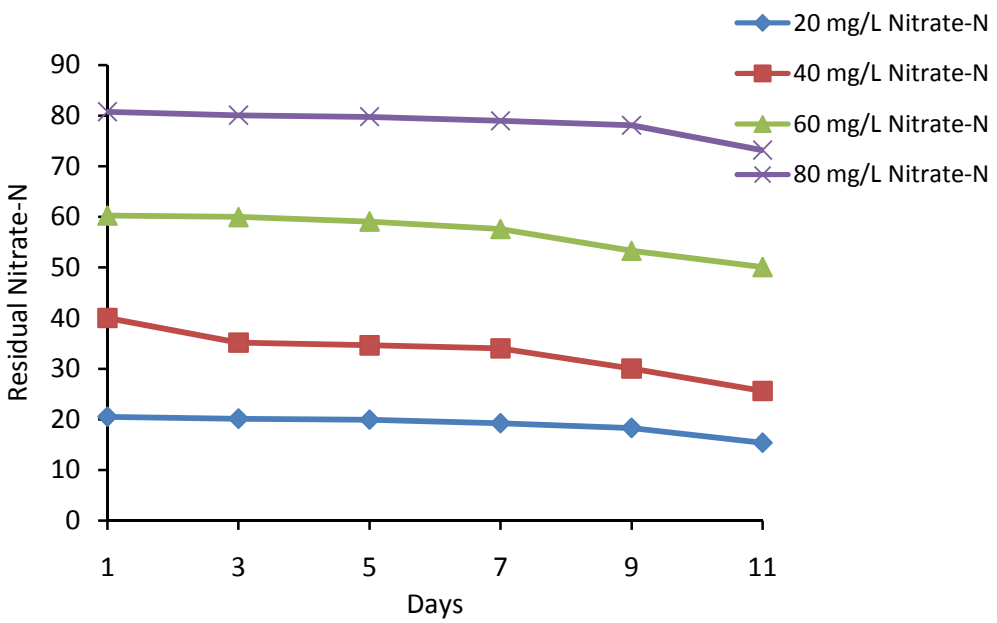

Figure 3.4: Nitrate-N removal by *Salvinia minima*

In the present study, *Salvinia minima* showed 23 % of removal within 12 days at 20 mg/L of Nitrate-N (Figure 3.4). Similarly a lower removal of 3.5 % was found when higher concentrations of 60 mg/L and 80 mg/L of Nitrate- N were given. When phytoremediation by *Spirodela polyrhiza, Salvinia sp.,* and *Lemna* sp. were carried in synthetic wastewater, to evaluate nutrient removal efficiency of NO_3^-, *Salvinia* sp., showed a nutrient removal of 19%, but biomass increment was lower compared to other plants (Ng and Chan, 2017). On contrary, another phytoremediation work by Olguin et al., (2007) indicated that *Salvinia minima* managed to decrease TKN, NH_4-N and PO_4-P up to 97%, 99% and 88% respectively in anaerobic effluents of coffee wastewater.

The performance of nitrate removal by each plant was calculated using the decrease in the initial nitrate in the culture solution and the respective increase in fresh biomass. The average rate of nitrate removal can be calculated by

$$Average\ Nutrient\ Removal = \frac{\{X\}_{initial} - \{X\}_{final}}{biomass_{final} - biomass_{initial}} \times \frac{1}{No:\ of\ days}$$

Table 3.1: Average nutrient removal by various macrophytes (mg of NO_3^-/gm of biomass/day)

	Concentrations of nitrate-N (mg of NO_3^-/gm of biomass/day)			
	20mg/L	40mg/L	60mg/L	80mg/L
Pistia stratiotes	0.3816	0.2638	0.1965	0.1323
Eicchornia crassipes	0.2014	0.1701	0.111	0.0211
Lemna minor	0.0160	0.0202	0.0220	0.0140
Salvinia minima	0.005	0.007	0.006	0.006

Among the four plants tested, *Pistia stratiotes* were found to be having the highest average of nitrate-N removal ranging from 0.38 NO_3^-/gm of biomass/day at 20 mg/L of NO_3^--N (Table 3.1). *Eichhornia crassipes* was also found to be effective compared to *Lemna sp*., and *Salvinia* sp. The nutrient removal capability of *Pistia* sp., and *Eichhornia crassipes* were found to be concentration dependent. As the initial nitrate-N concentration increases, the removal rate decreases. This can be explained by the extent of the nutrient a plant body can absorb. On comparing the removal properties of *Lemna* sp., and *Salvinia* sp., *Salvinia* sp., shows minimal removal ranging from 0.005 to 0.007 NO_3^-/gm of biomass/day and their removal potential is not concentration dependent. The average nitrate removal showed that *Pistia stratiotes* and *Eichhornia crassipes* are the potential candidate for phytoremediation of nitrate than *Lemna* sp., and *Salvinia* sp, so the kinetics of

nitrate-N removal by these two plants is studied. At higher concentrations like 80 mg/L, nitrate uptake was found to be lower for *Lemna minor.* Nitrate assimilation requires energy in the form of NADPH and ferredoxin for the sequential reductions of nitrate to nitrite and then to ammonium and the conversion is mediated by enzymes nitrate reductase and nitrite reductase, respectively (Bloom, 1997). There are reports of repression of nitrate reductase by ammonia which is formed by conversion of nitrate (Joy, 1969). This could be the reason of decreased nitrate reduction at higher concentrations like 80 mg/L by *Lemna minor*. Biomass gain for *Salvinia* was found to be lower compared to other macrophytes used for the study and therefore the average nitrate removal was least for it. Similar observations were reported that when nitrate was given as the sole source of nitrogen, *Salvinia* showed suboptimal growth and leaf yellowing (Jampeetong and Hans Brix, 2009).

a. Nitrate removal Kinetics by Pistia stratiotes and Eichhornia crassipes.

The kinetics of removal rate was studied for following irreversible unimolecular type first order reaction using integral analysis. Integral analyses check the fitting of reaction by putting a rate equation by integrating and comparing the predicted C vs T curve with experimental C vs T curve.

Consider the removal as A $\longrightarrow$ Product

The rate equation of first order reaction is

$$-r_A = \frac{-dC_A}{dt} = kC_A$$

Separating and integrating we obtain,

$$\int_{CA0}^{CA} \frac{dCA}{CA} = k \int_{0}^{t} dt$$

$$\ln \frac{CA}{CAo} = kt$$

A plot of –ln CA/CAo vs time is shown in Figure 3.5., gives a straight line through the origin for this form of rate of equation from which the slope will denote K, rate constant of the first order kinetics (Levenspiel, 1999).

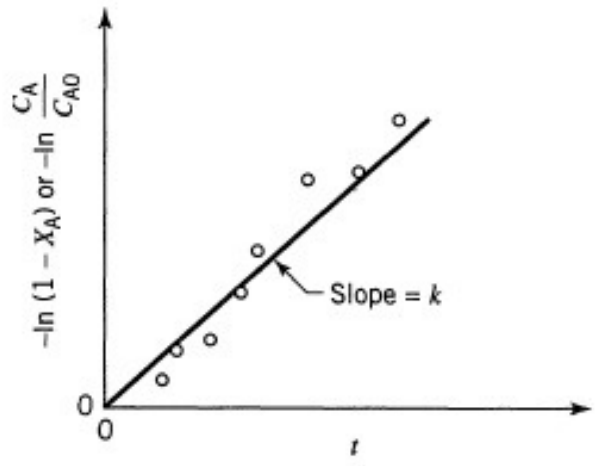

Figure 3.5: Demonstration of first order kinetics reaction.

To check if the removal of nitrate-N by *Pistia* sp., and *Eicchornia* sp., a graph of –ln CA/CAo is plotted against Days as T where CA is the residual nitrate and CAo as the initial concentration and T as days taken for removal of the nitrate-N. The pattern approximately fits the trend line which is shown in Figure 3.6 and Figure 3.7 for *Pistia stratiotes* and *Eichhornia crassipes* respectively and thus assumed to be first order reaction.

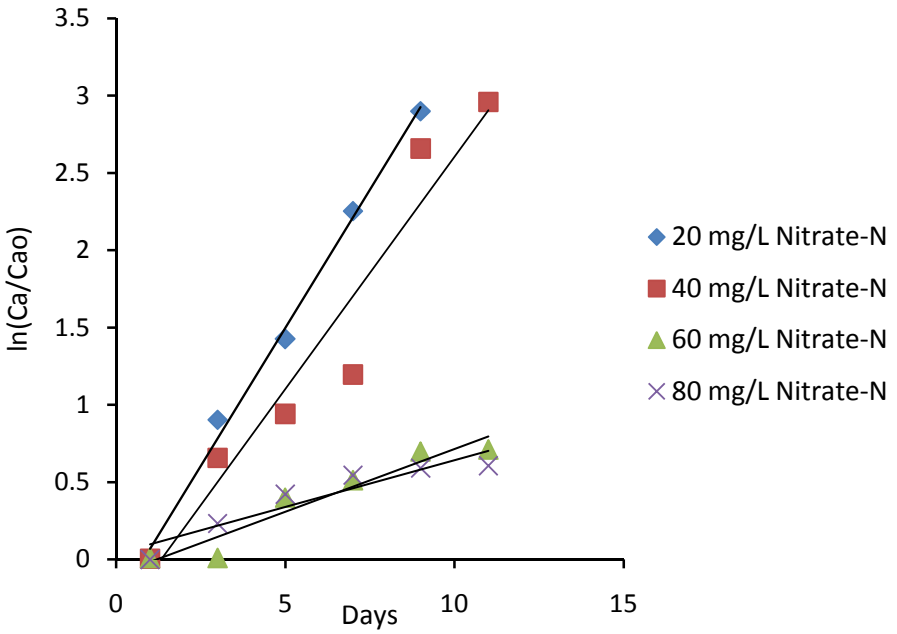

Figure 3.6: Demonstration of first order kinetic removal by *Pistia stratiotes*

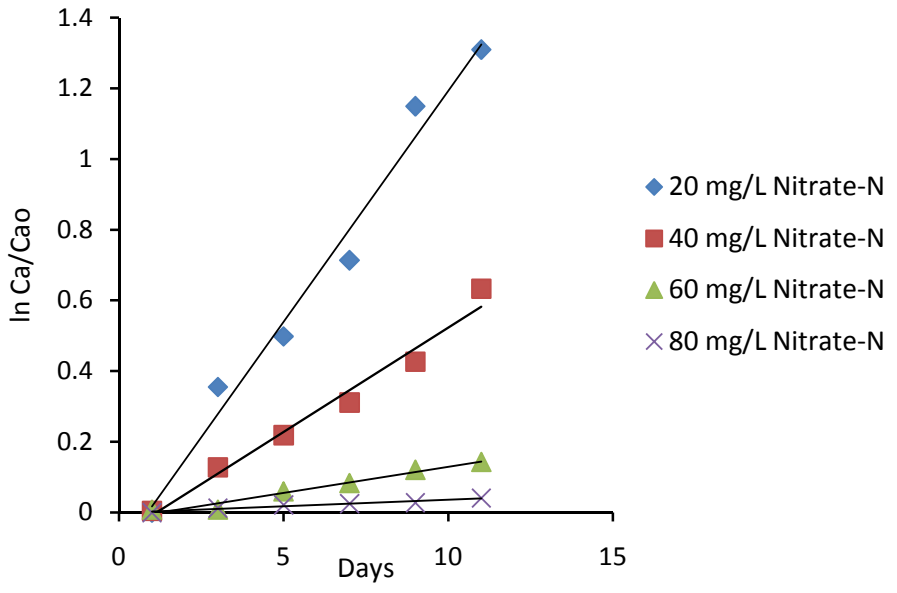

Figure 3.7: Demonstration of first order kinetic removal by *Eichhornia crassipes*

Removal of Nitrate-N followed first order kinetics where the first order rate constant of *Pistia* sp., and *Eichhornia crassipes* found to be concentration dependent. As the initial concentrations of the nutrient increases, the first order kinetic constant, K_m is found to be decreased (Table 3.2). This substantiates the decrease in average nutrient removal when initial concentrations of nitrate-N increases. As the removal rate decreases the $t_{1/2}$ which is the time required to remove half the initial concentration will also increase. So the potential candidate for phytoremediation is supposed to have high Km with low $t_{1/2}$. Among the two plants chosen from having high average nutrient removal, *Pistia stratiotes* is having higher Km for all the nutrient concentrations compared to *Eichhornia crassipes*. 250 gm of *Pistia* sp. can remove 10 to 20 mg/L of Nitrate-N approximately within a day, whereas 30 mg/L to 40 mg/L requires 4 to 5 days. But *Eichhornia crassipes* requires 3 to 5 days for removing 10 to 20 mg/L and the days required for removal of higher concentrations will be longer.

Table 3.2: Kinetic coefficients of Nitrate-N removal by floating macrophytes

Plant species	Nitrate-N conc.	R^2	K (per day)	$t_{1/2}$(days)
Pistia stratiotes	20 mg/L	0.9	0.3816	0.97
	40 mg/L	0.9	0.2638	1.15
	60 mg/L	0.9	0.1965	4.25
	80 mg/L	0.8	0.1323	5.72
Eichhornia crassipes	20 mg/L	0.9	0.2612	3.44
	40 mg/L	0.9	0.1181	5.86
	60 mg/L	0.9	0.0295	23.49
	80 mg/L	0.9	0.0073	94.93

The potential of three floating aquatic macrophytes like Water hyacinth, water lettuce and Pennywort to improve the water quality of anaerobically digested flushed dairy manure wastewater was evaluated by Sooknah and Wilkie and found

that reduction of COD and nutrients by these plants follow first order kinetics (Sooknah and Wilkie, 2004). The kinetic study of nitrate-nitrogen uptake by *P. stratiotes* over a wide range of substrate concentrations in order to determine the maximum rate of uptake and the half-saturation constants according to the Michaelis-Menten expression was done by Nelson et al. (1981) and it was found that nitrate uptake rates were higher after 24 h of exposure to the nitrate source than immediately after exposure. The performance of macrophytes *Eichhornia* and *Typha* were investigated by operating the wetland system at different hydraulic retention times by Rangel-Peraza et al., (2017). According to their kinetic study, the constructed wetland treatment showed a maximum rate of organic load removal of 2.500 mg/L/d, which was considered as a high removal rate.

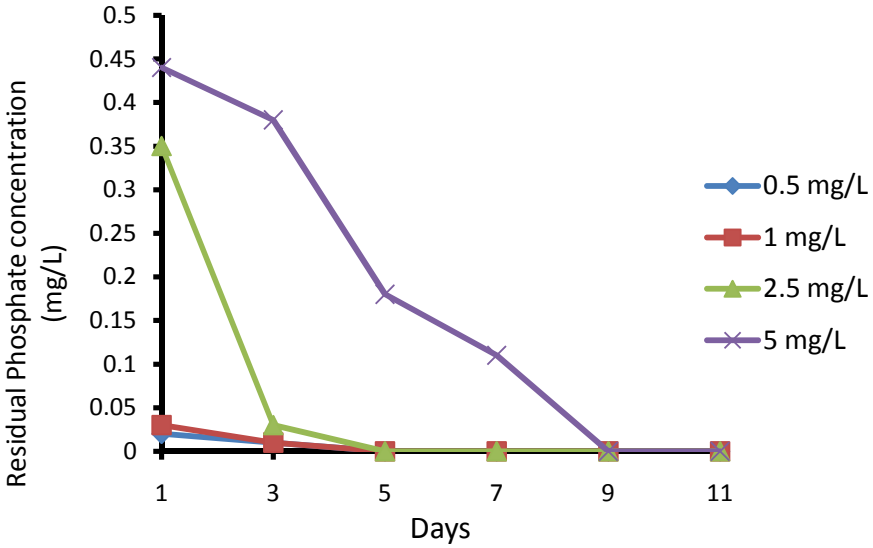

Figure 3.8: Phosphate-P removal by *Pistia stratiotes*

The phosphate removal by *Pistia stratiotes* was shown in Figure 3.8. When phosphate-P of 0.5,1 and 2.5 mg/L were given, 100% removal was found within 3 days. Complete removal of 5 mg/L by plant took 9 days of time. In a phytofiltration lagoon assessment using *Pistia stratiotes*, nearly 73.72 ± 18.5% to 92.89 ± 4.3% efficiency was observed (Olguin et al., 2017).

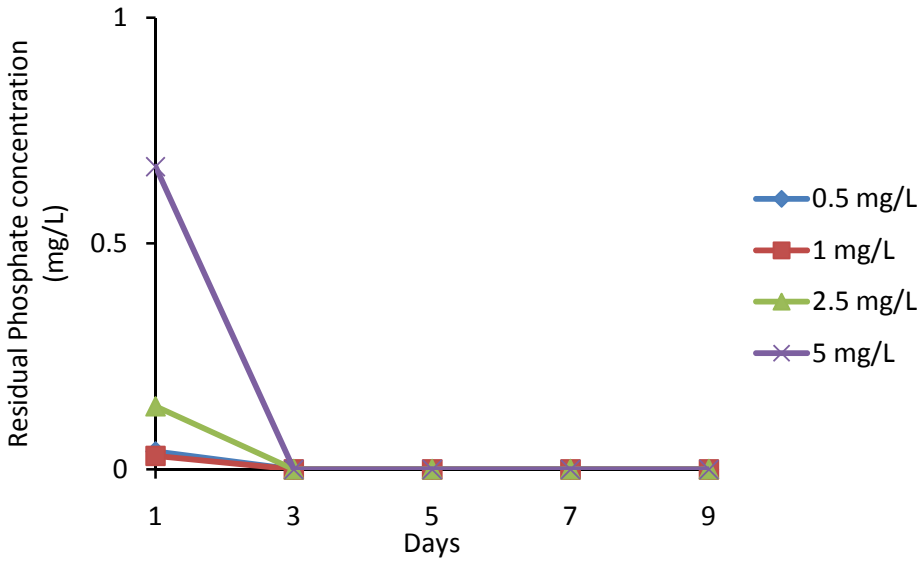

Figure 3.9: Phosphate-P removal by *Eichhornia crassipes*

The phosphate removal was found to be very high for *Eichhornia* sp., as 100% removal was observed within 3 days even for 5 mg/L (Figure 3.9). *Eichhornia* sp., was found to be majorly effective in removal of phosphorus, because within 4 hours the orthophosphate was found to be reduced 0.67mg/L from the initial concentration of 5mg/L. In a domestic wastewater study, *Eichhornia* sp., showed 45% of orthophosphate at 0.8 mg/L range (Rezania et al., 2016).

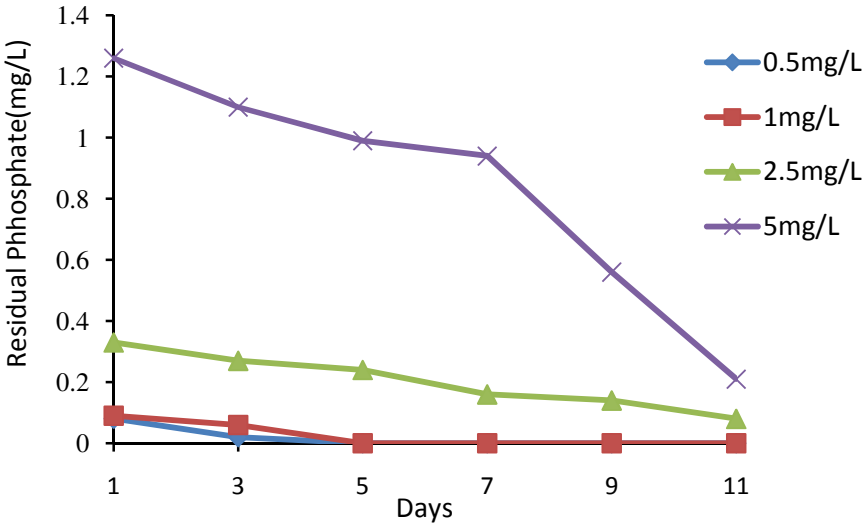

Figure 3.10: Phosphate-P removal by *Lemna minor*

The phosphate removal by *Lemna minor* is shown in Fig 3.10. It was found that 100% removal was found for lower concentrations like 0.5 mg/L. But for higher concentration like 2.5 mg/L and 5 mg/L, up to 63% of removal was only found. Studies by Ng and Chan (2017) on synthetic waste water showed *Lemna* sp., achieving the highest phosphate removal among the macrophytes with 86% removal within 12 days when 3 mg/L of initial concentration was given.

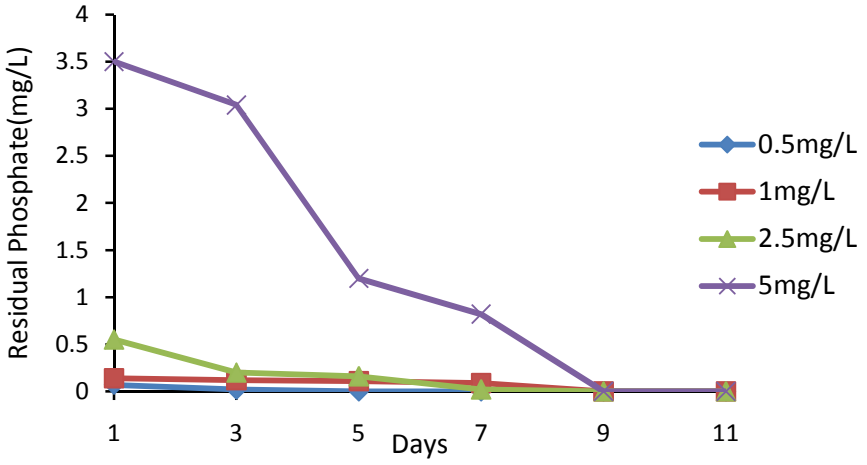

Figure 3.11: Phosphate-P removal by *Salvinia minima*

Salvinia minima showed 100% of removal for all the four concentrations given for orthophosphate. When Phosphate-P was given at 0.5 mg/L range, 100 of removal happened within 5 days whereas the higher concentrations like 1, 2.5 and 5 mg/L took 9 days for complete removal. When *Salvinia molesta* and Duckweeds (*Lemna gibba*) were used for the removal of the heavy metal (Cr, Cu and Fe) and nutrient from industrial wastewater, *S. molesta* and *L. gibba* showed an average total Phosphate removal of 72.63% and 77.28% respectively in an operational period of seven days (Abeywardhana, et al., 2018).

Phytoremediation techniques for treating different types of wastewaters have always been an area of research interest. Different factors involved in the process are identification and implementation of the efficient aquatic plant, uptake of dissolved nutrients and metals by the growing plants and harvest and beneficial use of the plant biomass produced from the remediation system. The most critical factor in implementing phytoremediation is the selection of an appropriate plant. The uptake and accumulation of pollutants vary from plant to plant and also from species to species within a genus depending on its specific growth rate (Marmiroli et al., 2006). During selection, biomass production, growth rate, and easiness of management and harvest should be taken into account. The major aquatic macrophytes available in our study area are *Eichhornia crassipes*, *Pistia stratiotes*, *Salvinia minima* and *Lemna minor*. *Eichhornia crassipes* is a free-floating aquatic plant which is seen on the surface of freshwater and can be anchored in mud, well known for its production abilities and removal of pollutants from water. It can quickly grow to very high densities (over 60 kg/m2); thereby wholly clogging water bodies (Nesic and Jovanovic, 1996). *Pistia stratiotes* is a floating perennial commonly called water lettuce which floats on the surface of the water, and its roots hanging submerged beneath floating leaves. The specific growth rate of *Pistia* sp., is slightly higher than water hyacinth which is 10.5 % but the biomass built up is not so big and heavy (wet weight of water lettuce was under 100g and the width and height of the plant was under 20 cm) (Aoi and Hayashi,1996). Its abundant root system and stolons act as

filters that will trap suspended matters and provides a surface for the adhesion and the proliferation of microorganisms. *Lemna* is a genus of monocotyledonous free-floating aquatic macrophytes which is commonly known as duckweed. They usually grow in stagnant or slow-flowing, nutrient-enriched waters throughout tropical and temperate zones. *Lemna* species are considered as very fast growing, thereby a high turnover and yield with a doubling time in the ranging between 0.7 and two days (Hossell and Baker, 1979). *Salvinia minima* is a free-floating aquatic fern that lacks roots (Hasan and Rina, 2009).The potential of these floating plants as phytoremediation agents can be utilized for removing nutrients, heavy metals, etc. Once harvested, this plant biomass can be used for extracting value-added products through anaerobic digestion, composting, etc.

Many researchers have used different plant species like Water Hyacinth (*Eichhornia crassipes* (Mart.) Solms), Water Lettuce (*Pistia stratiotes* L.), Duckweed (Water Lemna), Bulrush (Typha), Vetiver Grass (*Chrysopogon zizanioides*) and Common Reed (*Phragmites Australis*) for the treatment of water (Water Environment Federation, 2010).

A comparison study by Gupta et al., (2012) analyzed the treatment of wastewater using *Eichhornia crassipes*, *Pistia stratiotes*, and Vetiver grass. It was found that *Pistia stratiotes* can be used as a potential candidate for treatment of wastewater. They can remove up to 70% of TDS, 93% of BOD, 70% of nitrate and total phosphorus by 33%. It is having excellent reproduction potential as it doubles in 5 to 15 days. The growth rate of water hyacinth strongly depends upon the concentration of dissolved nitrogen and phosphorus in the water (Debusk and Reddy, 1987). The specific growth rate of water lettuce is remarkably higher compared to other floating macrophytes (Ismail et al., 2015). But its growth is strongly influenced by the sunlight, so the growth rate is slow in rainy seasons (Aoi and Ohba, 1995). Though it can reproduce in 5 days, they are easily prone to decay. So the efficacy of the wetland construction system using them will be heavily linked

to the proper harvesting of the biomass before decay. Once decomposed, nutrients will be brought back to the system due to the release from the tissue.

From our observation, the second potential candidate for phytoremediation of nitrate and phosphate is Water hyacinth (*Eicchornia crassipes*). Similarly, Gamage and Yapa (2001) used water hyacinth to treat textile effluent and found that they can remove 72 % of total solids, 61% of dissolved solids, 83% of nitrate reduction and 36 % of chloride reduction. It is also known for their resistance to heavy metals like Iron, Zinc, Copper, Chromium, Cadmium, Manganese, Nickel, Mercury and Arsenic up to 10 mg/L (Mishra and Tripathi, 2008). Ayyaswamy et al., (2009) found that nitrate removal efficiency of Water hyacinth was decreased with the increase in nitrate concentration, due to the osmotic pressure.

Hui and Yinghui (2009) examined the growth and kinetics of tropical plants in the presence of macro and micronutrient accumulation. Among the three floating macrophytes, *P. stratiotes* exhibited the highest removal rate for nitrate and phosphate, the highest increase in dry mass and second highest increase in fresh mass. This high growth rate would mean that it would be able to respond quickly to any sudden increase in nitrate and phosphate level in the water body. *S. molesta* has intermediate nutrient removal rate but the highest growth rate for fresh mass while *E. crassipes* had similar nutrient removal ability as *S. molesta*, but a lower nitrate removal rate and much slower growth rate in comparison. But in our experiment, *Salvinia* was found to be showing lower nitrate removal when compared to water hyacinth and water lettuce but higher than *Lemna minor*. *Salvinia* has a wide geographic distribution within the tropical and sub-tropical regions of the world, and the lethal temperatures for this fern are −3°C and 43°C (Whiteman and Room, 1991). It outgrows duckweed (Lemnaceae) in a mixed culture and may reach very high productivities in the range of 5.8 to 11.4 g DW m−2 day−1 when cultivated in a chemically defined medium of Hoagland (Reddy and Agami, 1991).

From our preliminary screening experiment, it was found that *Pistia* and *Eichhornia crassipes* were found to be potential candidates for removing Nitrate-N and Phosphate-P. The applications of these plants as vegetation have various advantages

including better removal of nutrients and easy harvesting. But the primary key point in effective utilization of macrophytes in is their proper harvesting. Plants should be harvested upon the maximum uptake before their decay else the degraded matter will bring back the organics to the water body. This experiment has proved the maximum uptake by 250 gm of various plants when different concentrations of nutrients are given.

b. Uptake of ammonia-N by Pistia stratiotes in presence of nitrate-N

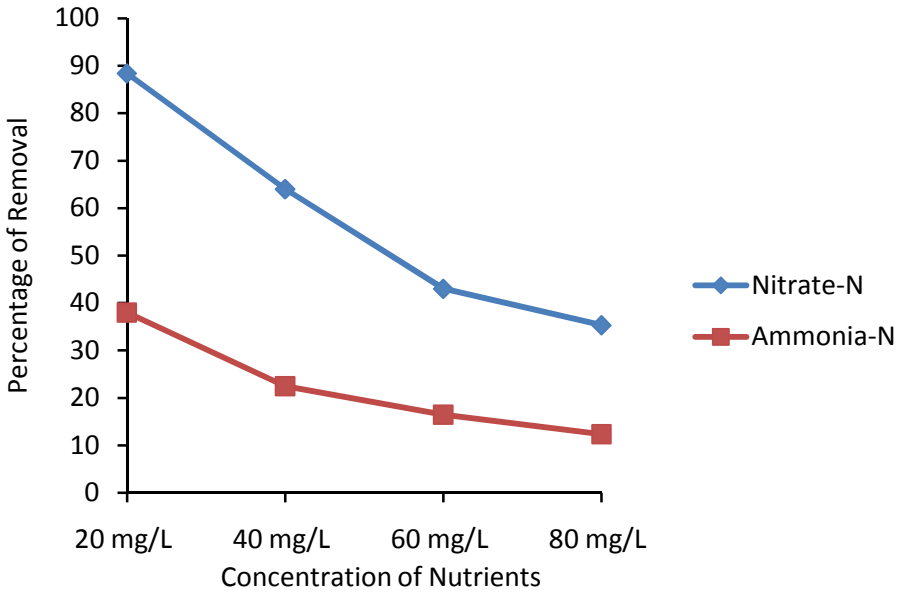

Figure 3.12: Nitrate-N and Ammonia-N removal when given separately by *Pistia stratiotes*

Majority of the water bodies suffer from pollution by organic matters and in most of them the nitrate and ammonia will be present simultaneously. Stripping of these nitrogen forms was tested with *Pistia*, which found to be the best floating macrophyte for removing N and P. Ammonia-N and Nitrate-N were given simultaneously as well as separately to see the difference in uptake by the plant and it was found that *Pistia* sp., removes Nitrate-N better compared to ammonia-N. It

was found that 90 % removal occurred in the case of 20 mg/L of Nitrate-N whereas 53 % of removal occurred for 20 mg/L of ammonia-N. For 40 mg/L, Nitrate-N was removed by 70% whereas ammonia-N was only removed by 35 %. The removal of both Nitrate-N and Ammonia-N was found to be concentration dependent as the removal percentage decreases with increase in initial concentration (Fig 3.12).

When both ammonia and nitrate were given together, it was found that ammonia was preferred more by plant than nitrate-N (Fig 3.13). The uptake of ammonia was 26 %, whereas Nitrate-N removal was only 5 %. Almost five times removal of ammonia removal was found in the case of 20 mg/L nutrient concentration. Removal of 15 % was 3% were found when 40 mg/L of initial concentration of ammonia-N and nitrate was given. But at higher concentrations of 80 mg/L, both of the nutrients were removed equally which can be explained by saturation point.

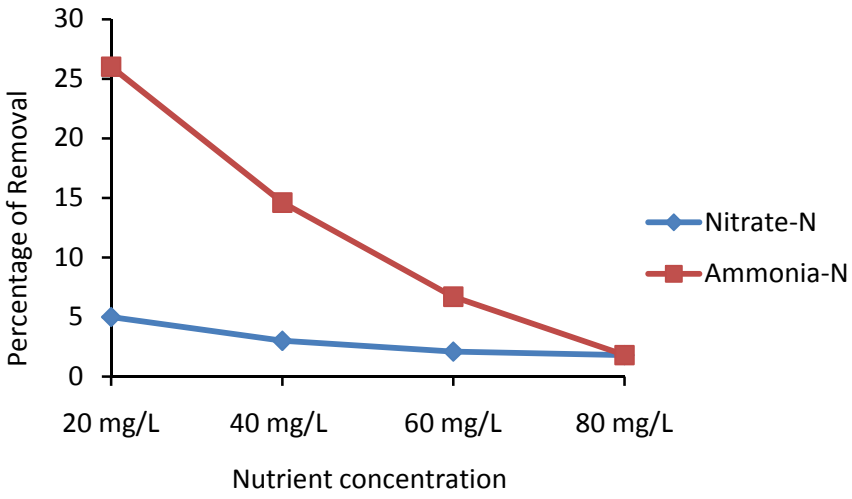

Figure 3.13: Simultaneous removal of Nitrate-N and Ammonia-N by *Pistia stratiotes*

Hillman (1961) suggested that roots of floating macrophytes function mostly as anchors, whereas fronds and leaves are the main organs involved in nutrient uptake. The nitrogen uptake can vary depending upon the plant requirements as well as the ion influx. Toetz et al., (1973) ruled out a possibility that ammonia uptake can also

be happening by passive diffusion. Joy (1969) found that ammonia uptake inhibits the formation of nitrate reductase, a major enzyme involving in the reduction of nitrate for plant absorption and thereby prevent the assimilation of nitrate by plants. Ferguson (1969) found that many plants prefer nitrate only when ammonia was depleted entirely. There were studies which revealed a lag in nitrate uptake after exposure to higher nitrate concentration (Fergusen, 1969, Joy, 1969, Schwoerbel and Tillmans, 1964). A similar observation was made by Ullrich et al. that a floating plant called *Lemna gibba* was found to be taking up ammonia in several folds faster that nitrate and nitrate was only utilized when ammonia was depleted which was explained by a carrier mediated ammonia uniport (Ullrich et al., 1984). This could be the reason for our observation of preferred ammonia uptake and nitrate uptake inhibition.

3.3.2. Effect of Salinity in nutrient removal

Saline tolerant macrophytes are preferred for phytoremediation of brackish water. Inorder to study the effect of salinity in nutrient removal, *Pistia stratiotes, Eichhornia crassipes* and *Salvinia minima* were selected as they are found to be effective in nutrient removal. Among the three plants tested, *Eichhornia crassipes* was found to having the highest average nitrate removal ranging from 0.5 to 1.4286 mg $NO_3^{-/}$gm of biomass/day over a period of 5 days in increasing the salinity concentration of 0.5 ppt to 2 ppt (Table 3.3).

Table 3.3: Nitrate uptake by different floating macrophytes under different salinities

Plants	Nitrate uptake (mg of NO_3^-/g of biomass per day)			
	Salinity			
	0.5 ppt	1 ppt	1.5 ppt	2 ppt
Eichhornia crassipes	0.2935	0.5239	0.8634	1.4286
Pistia stratiotes	0.6306	0.5818	0.8156	0.9929
Salvinia minima	0.3727	0.4019	0.5826	0.8854

Pistia stratiotes was also found to be effective for nitrate uptake but its nitrate removal capability was lower compared to Water Hyacinth at higher salinity levels. *Salvinia minima* shows a comparatively small increasing nitrate removal ranging 0.3727mg NO_3^-/gm of biomass/day to 0.8854 mg NO_3^-/gm of biomass/day. All the three plants were able to absorb the entire orthophosphate supplied. Among the plants tested *Eichhornia sp*., was found to be more salt tolerant and nutrient removal was found to be increased with salinity concentration.

3.3.3. Role of rhizospheric microflora in nutrient removal

The role of rhizospheric microorganisms in nutrient removal was analyzed by comparing the nutrient removal by plants with surface sterilized roots as well as normal root. It was found that plants with unsterilized roots showed the higher removal of phosphate from water compared to the plants with surface sterilized roots. The significant difference in phosphate uptake revealed that the rhizospheric

microorganisms play an essential role in the nutrient removal by solubilizing phosphate and convert it to the inorganic form for plant uptake.

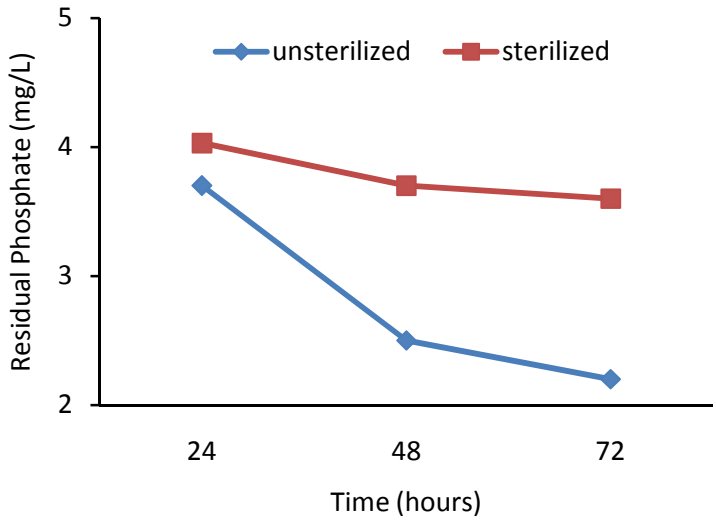

Figure 3.14**:** Nutrient removal by *Pistia stratiotes* with surface sterilized and normal root system

The usage of P-mineralizing and nitrate reducing bacteria on specific agar media resulted in the isolation of respective bacteria. Phosphate-solubilizing bacteria will grow in PVK medium and form a clear zone around the colony, due to phosphate solubilization in the vicinity of the colony. (Figure 3.15). Solubilization of insoluble P by microorganisms was reported by Pikovskaya (1948). Out of several colonies, 3 of them were found to be having the clear zone indicating their P-solubilizing property.

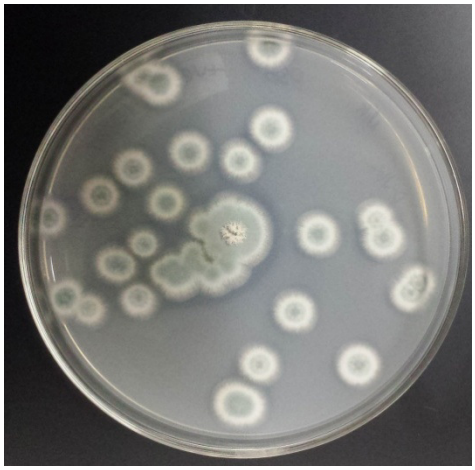

Figure 3.15: Clear zone formation around colonies in PVK medium indicatingthe growth of Phosphate solubilizing bacteria.

Several strains of bacterial and fungal and actinomycetes have been described and investigated in detail for their phosphate-solubilizing capabilities (Sharma et al., 2013). Both bacterial and fungal strains exhibiting P solubilizing activity are detected by the formation of clear halo (a sign of solubilization) around their colonies. . In addition to *Pseudomonas* and *Bacillus*, other bacteria reported as P-solubilizers include *Delftia* sp, *Pantoea*, *Klebsiella* etc (Sharma et al., 2013).

The bacteria that grows on nitrate broth uses nitrate as the energy source and the presence of nitrate reductase enzyme was confirmed by alpha naphtol test (Figure 3.16.).

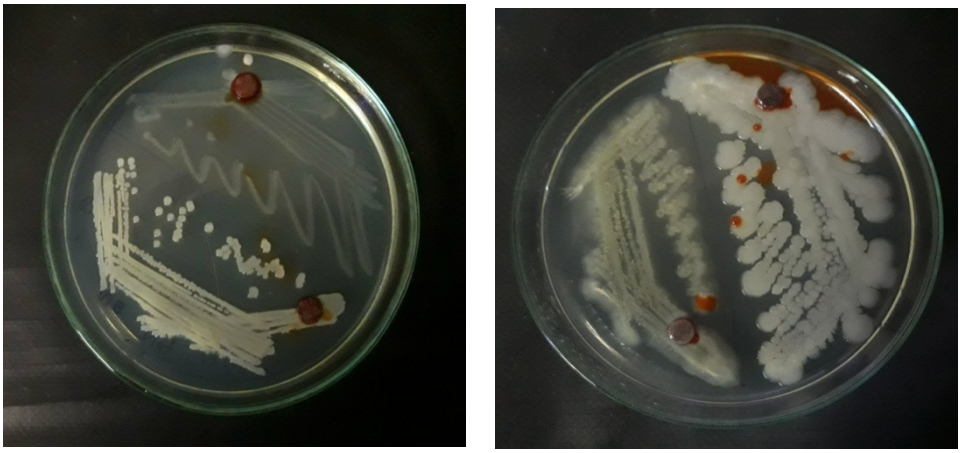

Figure 3.16: Alpha naphtol test for identifying nitrate reductase test.

The phosphate solubilizing organisms were designated as P1, P2 and P3 and nitrate reducing as N1 and N2. The sequences P1, N1 and N2 are deposited in the GenBank as KU230396.1, KU230399.1 and KU230400.1.

Table 3.4: List of identified organisms from the sequences obtained

Sl No	Strain No	Closest similarity (%)	Closest culturable strain (%)	Taxonomic group (class)
1	P1	Uncultured *Pantoea* sp. Clone AV_8R-S-G06 (99%)	*Pantoea agglomerans* strain PCAP5 (99%)	Gamma Proteobacteria
2	P2	Uncultured bacterium clone nbw804c04c1 (99%)	*Pantoea* sp. ATTA33 (99%)	Gamma Proteobacteria
3	P3	Uncultured bacterium (95%)	*Herbaspirillum* sp. DJM4E2 (95%)	Betaproteobacteria
4	N1	Uncultured bacterium clone RS-G28 (99%)	*Stenotrophomonas* sp. CanR-49 (99%)	Gamma Proteobacteria
5	N2	Uncultured *Delftia* sp. clone P234D07 (98%)	*Delftia sp*DM101 (98%)	Betaproteobacteria

The results of BLAST analysis are presented in Table 3.4. P1 was found to be having 99 % similarity with *Pantoea agglumerans* strains. *Pantoea vagans* showed 99% similarity with the P2. *Herbaspirillum sp.* showed only 95% similar to P3. This could be a novel strain which is involving in phosphorus uptake. The N1 was identified as *Stenotrophomonas sp.* with 99% identity with the sequence and N2 as *Delftia sp* with 98% sequence similarity with the species.

Microorganisms are very important in plant growth in terms of circulation of plant nutrients. The significance of rhizosphere associated N2 fixing and P-solubilizing bacteria on leguminous and non leguminous crops were well studied (Schilling et al., 1998). Microorganisms can contribute in global nutrient cycling which makes them an integral part in bioremediation. Helal and Sauerbeck (1989) did a study

which stated the majority of organic compounds excreted by maize (80%) are mineralized by the microorganisms in the rhizosphere to form CO_2, increasing the microbial biomass in the rhizosphere. The isolated *Pantoea* strains from *Pistia stratiotes* can solubilize insoluble P and hydrolyze organic P for plant growth. Studies have reported that *Pantoea* strains were more effective in uptake of tricalcium phosphate (Ca_3 $(PO_4)_2$) compared to iron and aluminium phosphate. It was also found that ammonia having a positive impact in phosphate solubilizing while NO_3^- was found to be inhibitory in nature (Sulbaran et al., 2009). Walterson and Stavrinides (2015) were found that *P.vagans* strain C9-1 is a commercially registered bio control of fire blight, a disease of pear and apple tree caused by *Erwinia amylovora.* *Pantoea* isolated from water and soil has been harvested for industrial purposes including bioremediation and the degradation of herbicides and other toxic products. Some isolates are antibiotic producers and are nitrogen fixers. They are found to be highly versatile group which can host with plants, insects, and humans (Walterson and Stavrinides, 2015) which increases the significance of the isolated species as a potential candidate for augmentation studies.

Stenotrophomonas is a gram negative, rod shaped, gammaproteobacterial reducing nitrate. They can reduce NO_3^- without accumulation of NO_2^- and NH_4^+. There are strains of selenium tolerant bacteria that come under these species (Dick et al., 2013).

Delftia is a plant growth promoting bacteria (PGPB) with chromium removal activity. They are known as nitrogen reducers. They can be used along with hyper accumulating plants for heavy metal contaminated soil remediation (Morel et al., 2010)

Nutrient recycling is majorly supported by lot of microorganism species by different processes like nitrification, denitrification or phosphate solubilization etc. Characterization of these organisms can shed light on different beneficial association in terms of bioremediation.

The phylogenetic relationship of isolated bacteria from the *Pistia* root zone and selected related organisms reported is presented in Figure 3.17. Among the isolates, *Stenotrophomonas* sp NIIST is showing 100 % similarity with already reported strain of *Stenotrophomona*s sp whereas *Herbaspirillum* sp NIIST and *Delftia* sp NIIST didn't showed any resemblance with any reported strain. *Pantoea* sp NIIST 1 and 2 showed 70% resemblance with each other. The study proved that the root rhizospheres are good sources of isolates which have the potential of bioaugmentation for various applications like biofertilizers etc.

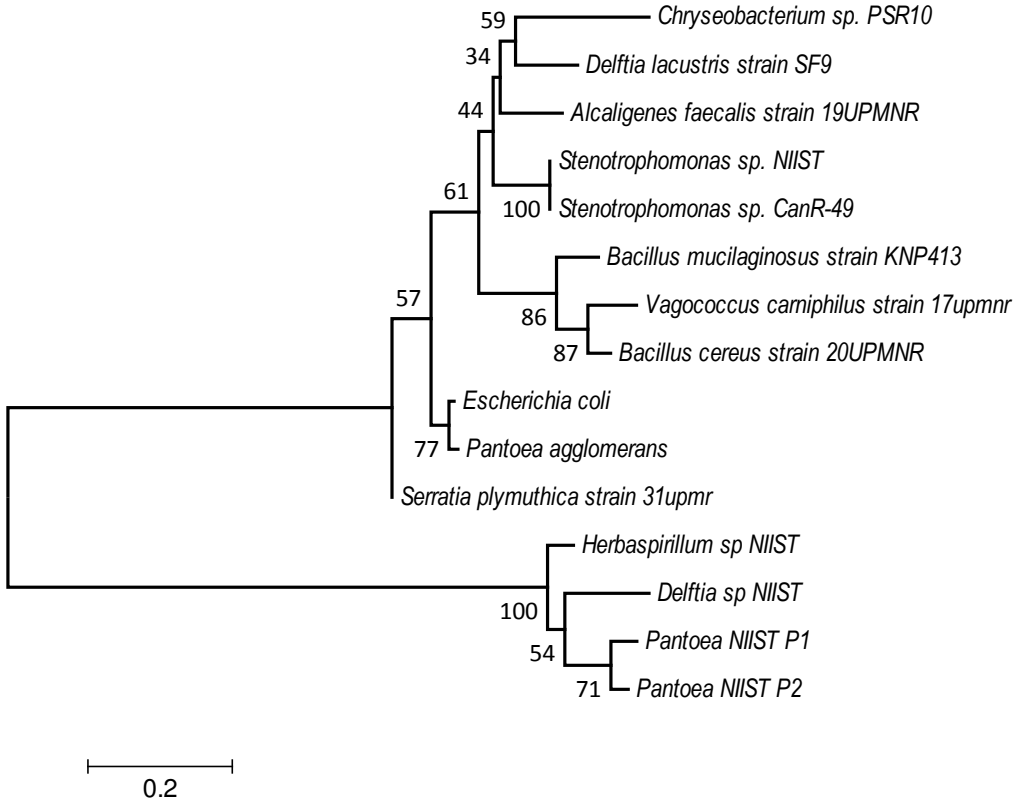

Figure 3.17: Phylogenetic tree showing isolated rhizospheric bacteria and their phylogenetic relation with other nutrient removing bacteria reported.

3.4. Conclusion

The nutrient removal studies with different floating macrophytes showed *Pistia* and *Eichhornia* as good candidates for nitrate, ammonia and phosphate removal. However previous studies on *Eichhornia* sp. showed that it could tolerate a wide range of heavy metals and salinity which make it preferable vegetation for wastewater treatment. The kinetics of removal indicated that the removal is concentration dependent. Moreover environmental variables also found to affect both N and P removal by the plants. Salinity upto 2 ppt has a positive effect on nitrate uptake, but higher salinity (5 ppt) exposure leads to plant necrosis in *Eicchornia* and *Pistia*. This observation can be explored for controlling proliferating *Eichhornia* in brackish water bodies. The present study also revealed the importance of rhizospheric microflora in nutrient uptake. This can be explored further with bioaugmenting selected plants with microbial consortium for improved nutrient removal/phytoremediation approaches.

Chapter 4

Water Quality & Macrophyte coverage in a typical eutrophic lake

4.1. Introduction

The nutrient conditions of many natural water bodies are mostly oligotrophic in nature with limited primary and secondary productivity due to reduced nutrient availability. Due to overpopulation, water is being polluted by sewage and garbage, agricultural development through pesticide and fertilizer application, and rapid industrialization concerning effluent and hazardous waste. Once the nutrients are available in abundance, it will promote the diversity of organisms in the ecosystem which may cause dynamics in phytoplankton's as well as zooplanktons residing in the system.

The primary factor determining the quality of a water body is the run-offs received by the lake. The nutrient inputs degrade the water quality and increase the biological activity inside the lake and subsequently increase the biomass content. The nature and extent of pollution can be determined by assessing the trophic state index of the water body. Eventhough *Eichhornia* coverage is very common in eutrophic lakes, a proper assessment of its quality is lacking even at major sites where it creates problem. This primary data will help in designing proper management plans. In this context, the objectives covered in this chapter are 1) water quality of a eutrophic lake and 2) assessment of the predominant macrophyte (*Eichhornia*) coverage in the lake. Better management of the recovered biomass can be done once an estimation of the biomass is obtained.

4.2 Materials and methods

4.2.1 Study Area

A typical eutrophic lake was selected in this study. The Akkulam–Veli Lake (AV Lake) is located in the South West coast of India. The AV Lake has an area of about 0.76 km^2 and is situated between 8°31'14" and 8°31' 52" North latitudes and 76°53'12" and 76°54'6" east longitudes. It is a shore perpendicular lake with the seaward part abutting the shoreline, and it is separated from the shore by a sandbar

during the non-rainy season. The lake is partially divided into two by the existence of a bund across its length. The western part towards the sea forms the Veli Lake and the eastern part starting from the bund, forms the Akkulam Lake. The silting in the Akkulam Lake affected the free flow of water from the lake to the Veli Lake. For most of the year, the AV Lake remains separated from the sea by a sandbar. The streams that drain through the Akkulam Veli Lake basin include the Kannamoola stream and the Kulathur stream. The T.S. Canal (Parvathy Puthanaar) connects the lake with two estuaries.

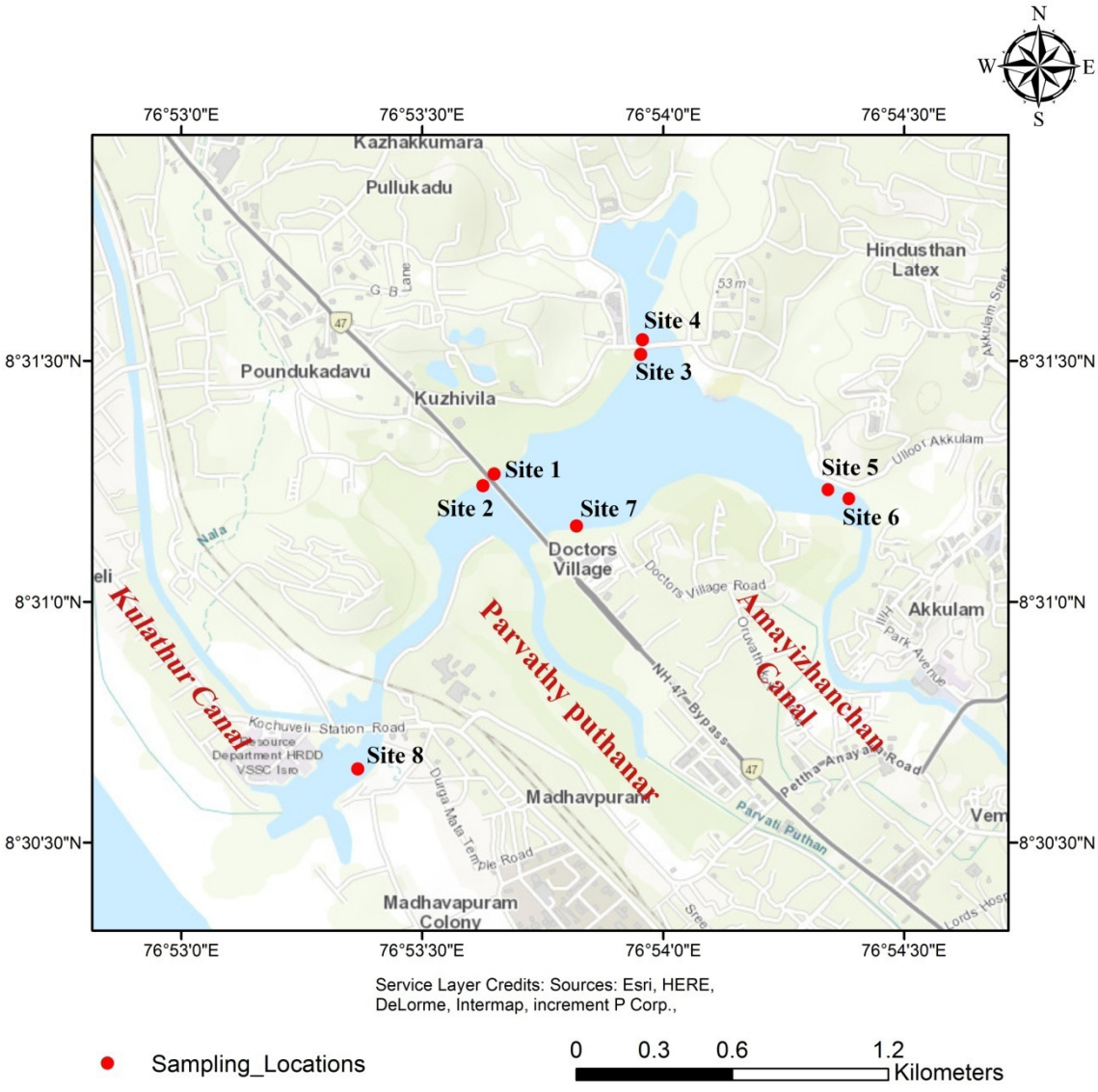

Figure 4.1: Locations of the different water sampling sites

4.2.2 Sampling points and sample collection

To assess the eutrophic status of the lake, water samples were collected from 8 different points in the lake (Figure 4.1). The locations were marked with the help of GPS. The samples were collected from February, 2015 to Jan 2016 covering one-year period. From each sampling points, three samples were collected and the values are expressed in mean ± SD.

4.2.3 Analyzing Scheme

The water quality parameters analyzed include pH, temperature, conductivity, salinity, dissolved oxygen, total nitrogen and total phosphorus. The sample storage and analysis were done as per the standard methods (APHA, 1998). The pH was analyzed with a pH meter (Cole Parmer), and whereas conductivity and salinity were analyzed using ion analyzer (ThermoScientific Orion, Singapore). Dissolved oxygen was measured using a DO probe (Eutech Instruments DO 2700).

Total nitrogen of the water samples was estimated by the Kjeldahl method. The samples were digested with potassium sulfate and cupric sulfate catalyst which converts amino nitrogen of organic materials to ammonium. By adding excess sodium hydroxide, ammonia is distilled from ammonium sulfate absorbed in boric acid and the ammonium:borate complex formed changed the color of the solution from blue to green, which was estimated by back titration with H_2SO_4 (APHA, 1998). The volume of total ammoniacal nitrogen was calculated using the equation

$$\text{Mg/L } NH_3 = \frac{(A-B)\times 280}{mlsample}$$

Where,

a= volume of H_2SO_4 titrated for the sample.

b= volume of H_2SO_4 titrated for blank.

Total Phosphorus analysis of the water samples was done by ascorbic acid method. Fifty ml of the sample was filtered and digested using conc.H_2SO_4 and conc.HNO_3. After neutralization, combined reagent was added which contains sulphuric acid, antimony potassium tartrate solution, ammonium molybdate solution, and freshly prepared ascorbic acid solution. The orthophosphate ion reacts with ammonium molybdate and antimony ion under acidic conditions to form a complex. This complex was reduced with ascorbic acid to form a blue complex which absorbs light at 880 nm. The colored solution should be read within 1-2 hrs. The absorbance was proportional to the concentration of orthophosphate in the sample (APHA 1998).

Monthly water quality data were consolidated into three season's data. Pre-Monsoon data were collected from the month of February, March, April and May. Monsoon data were collected from June, July, August and September. Post Monsoon data were collected from October, November, December and January. The entire study was conducted during February 2015 to January 2016.

4.2.4. Eichhornia coverage on Akkulam Veli Lake during various seasons

A variation on *Eichhornia* growth during various seasons was studied using satellite images from Google Earth during different seasons in a year. Estimating area of the study and the temporal data were calculated using Google Earth 7.1 and ArcMap 10.3 respectively which was supported by visual interpretation method and ground surveying. The Keyhole Markup Language (.kml) shapes of the study area were created in Google Earth using the 'Polygon' tool. These shapes were converted into 'vector' (.shp) files in ArcMap for area calculation. The shapes were converted using the 'Conversion Tool' of the ArcToolbox.

ArcToolbox ⟶ Conversion Tools ⟶ From KML ⟶ KML To Layer

The created shapefiles were reprojected from 'GCS_WGS_1984' to WGS_1984_UTM_43N for the purpose of area calculation. The reporojection was done using the 'Data management Tools' of the ArcToolbox.

ArcToolbox ⟶ Data Management Tools ⟶ Projections and Transformations

⟶ Project

The projected shapefiles were used for the area calculation and was done by using the 'Calculate Geometry' option in the attribute table.

Attribute Table ⟶ Calculate Geometry ⟶ Area.

The area surrounded by the biomass and the approximate biomass obtained was also calculated.

4.3 Results and Discussion

4.3.1 Water Quality of Akkulam Veli Lake over seasons

The water samples collected from Akkulam Veli Lake over different seasons were analyzed and the results were compared with the standard values recommended for organized recreational activities by CPCB and WHO. Sample sites 1 to 4 covered the eastern and western sides of the Aakulam Lake. Sample sites 5 and 6 were from Amayizhanjan Thodu and sample site 7 represented the area where Kannammoola stream joins the lake. The 8th sample site was from Veli Lake. The mean depth of the AV Lake is 0.6 m which means the lake is shallow in nature due to which the dilution of content will be very less. The shallow lakes are more productive than deep lakes and experience less mixing of bottom sediments, as wave action is more

likely to reach the bottom. Since the lake is shallow and its dilution capacity is low, the organic matter content will be high.

The pH of the of the AV Lake during monsoon was in the range of 6.3 to 7.5 whereas, in post-monsoon and pre-monsoon, it was in the range of 6.9 to 7.5 and 6.6 to 8 respectively. The range of the pH was found to be in acceptable with CPCB prescribed pH range. The temperature during monsoon and post-monsoon was in the range of 29-31°C whereas in pre-monsoon which is summer recorded of a temperature of 34 °C. Conductivity which shows the presence of inorganic dissolved solids such as chloride, nitrate, sulfate, and phosphate like anions or sodium, magnesium, calcium, iron, and aluminum like cations varied through all the three seasons. Conductivity was in the range of 450-1150 µS/cm and 210-350 µS/cm in monsoon and post-monsoon respectively whereas in pre-monsoon it was in the range of 350-1505 µS/cm which shows the organic matter content was high in the water.

The major nutrients found in the AV Lake are Nitrogen (N) in the form of nitrate, ammonia and nitrite and Phosphorous (P) in the form of orthophosphate. Eutrophication which is a significant threat to the aquatic systems is a result of the accumulation of N and P from various sources like sewages, factory effluents, organic matters like animal wastes, fertilizers, etc. The nitrogen content in the AV Lake was determined in the form of Total Kjeldahl Nitrogen (TKN) and Total Phosphorous (TP). The nitrogen content of the lake was found to be in the range of 21-80 mg/L during Monsoon and 47-70 mg/L in post monsoon and 11-87 mg/L in pre-monsoon. Phosphorus content in the lake was in the range of 0.1-0.5 mg/L and 0.05-0.2 mg/L in monsoon and post-monsoon whereas in pre-monsoon it was 0.05-0.5 mg/L.

The streams that drain through the AV Lake basin, which are the Kannamoola stream and the T.S. Canal (Parvathy Puthanaar), are passing through the most populated parts of the city which makes Akkulam Lake, a dumping place of the heavy load of sewage wastes. The accumulation of these nutrients has resulted in the enormous mats of macrophytes especially water hyacinth in the lake.

4.3.2 Seasonal variation of physico-chemical parameters in AV Lake.

Hydrological conditions play a key role in nutrient availability by transporting particulate and dissolved nutrients from the bottom to the surface of the lakes and vice-versa. The major factors affecting the growth of water hyacinth are temperature, salinity and nutrients. So variations of different water quality parameters of the lake during three different seasons are studied and are correlated with the macrophyte coverage on the water body.

a) pH and temperature

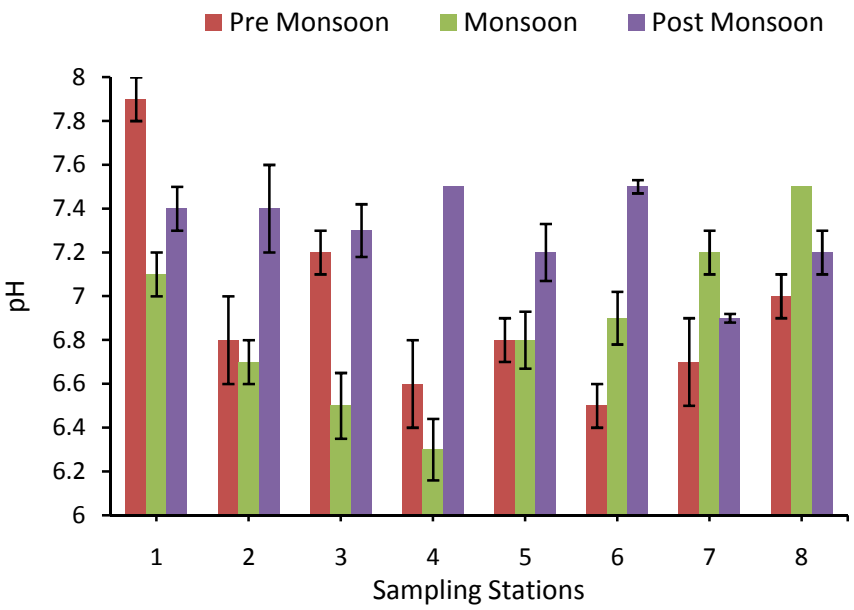

Figure 4.2: Variation of pH over different seasons at the sampling sites

pH variations were observed in sample sites due to the presence of various organic pollutants. This brings about changes in oxygen and carbon dioxide contents which results in changes in pH. The pH range in the AV Lake was found to be in the range of 6.8 to 8 throughout the seasons. Most variations on pH were observed during pre-monsoon period, which could be due to the increased microbial activity due to the higher temperature. The average pH was found to be in the range of 7.2 to 7.3 throughout the post-monsoon season due to sea water ingression. This is similar to the findings of Moundiotiya et al., (2004) in Jaipur, and Okbah and El-Gohary (2002) in Lake edku, Egypt.

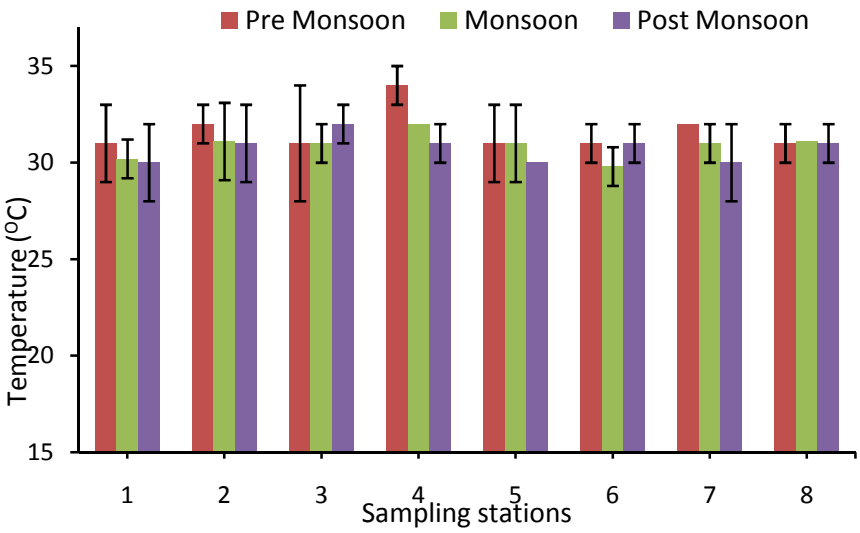

Figure 4.3: Variation of temperature over seasons at the sampling sites

The water temperature influences the decomposition of organic matter by bacterial activity which liberates dissolved gases like oxygen, carbon dioxide, ammonia, hydrogen sulfide and the carbon dioxide and moreover increases the nutrient content of the water body. The temperature range in the AV Lake was from 30 to 34^{o}C during pre monsoon. During pre-monsoon, irradiance is higher and lower water level, and this could be the reason for the raised temperature. The temperature was comparatively lower during the post-monsoon season due to high humidity. The significance of water hyacinth is that it needs tropical climate for maximum proliferation which is present during pre-monsoon period. Since the principal factors affecting the growth of floating macrophytes are the pH, water temperature, light conditions, nutrient concentrations, and predation by zooplankton and fishes, the changes in pH and temperature in the lake are significant factors increasing the biological activities in the lake (Jiang et al., 2014).

b) Salinity

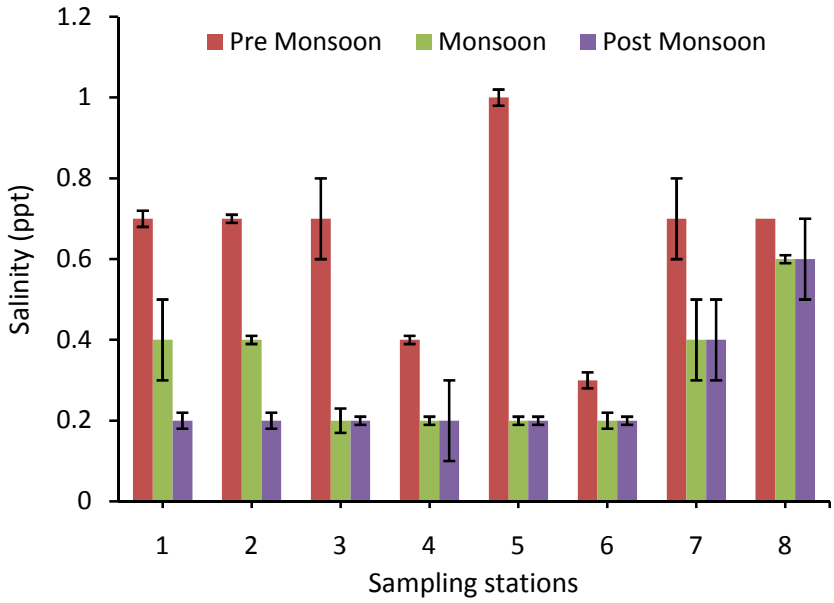

Figure 4.4: Variation of Salinity over seasons at the sampling sites

Salinity is a major factor controlling various physical, chemical and biological processes occurring in the aquatic environment. During the analysis for a year, the major increase in salinity was found at site 5 where the Aamayiyanchan Thodu joins the lake. The major cause for increasing salt concentration could be the increased temperature which concentrates the accumulated organic contaminants. Another possibility of increased salinity during pre-monsoon season could be a saltwater intrusion. The average value of salinity in Akkulam Lake was 0.7 ppt, 0.28 ppt, and 0.2 ppt during pre-monsoon, monsoon and post-monsoon respectively. The salinity over 5 ppt is lethal to the water hyacinth (Olivares and Colonnello, 2000), but the salinity in the lake was found to be only up to 1.5 ppt.

c) Total Kjeldahl Nitrogen (TKN) and total phosphorus

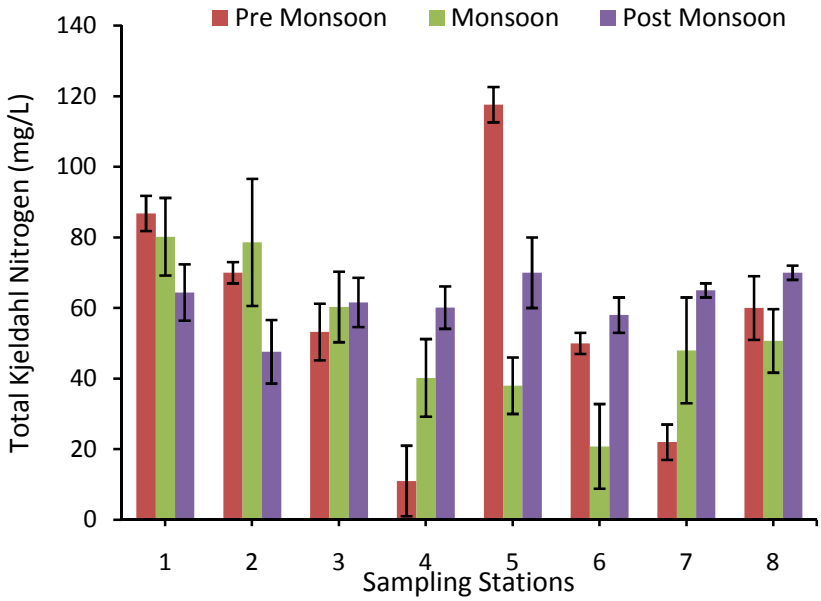

Figure 4.5: Variation of TKN over seasons at the sampling sites

The average TKN and TP of the AV Lake were found to be higher than the CPCB prescribed values. The average concentration of TKN in the water body was 58.6, 52.3 and 60.9 mg/L during pre monsoon, monsoon and post monsoon respectively. Due to high temperature, there will be evaporation of water and concentration of nutrients takes place. The shifts in phosphate and nitrogen during monsoon season are due to the rain inputs of sewage wastes.

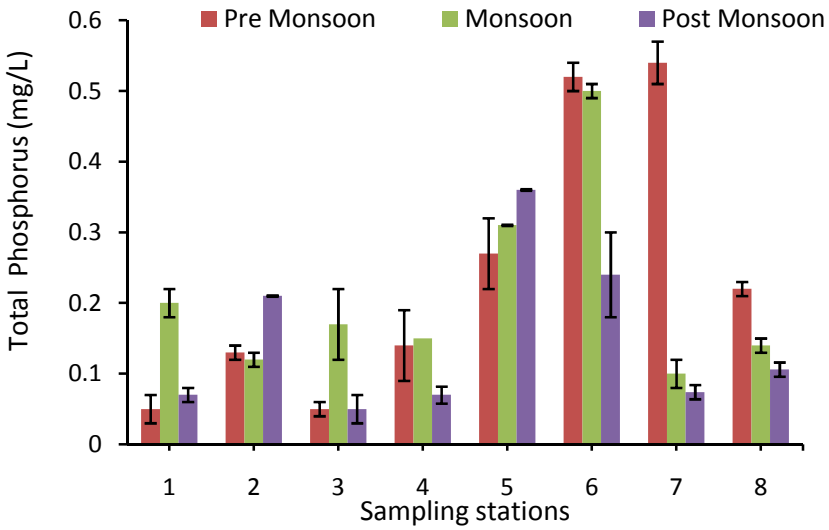

Figure 4.6: Variation of total phosphorous over seasons at the sampling sites

Total Phosphorus at different sampling points was found to be higher compared to previous reports, and it is an essential plant nutrient that stimulates the growth of aquatic vegetation. This may be due to domestic sewage discharged into AV Lake through the Kannammoola stream. The sites 1, 2, 3, and four are found to have the low amount of Total phosphorus and these sites have maximum macrophytic coverage compared to the other sites, and these plants need Phosphorus for their growth.

Table 4.1: Comparison of secondary data with observed values of present study

Parameters	Limiting standard by CPCB and WHO	Moses et al (2011)	Sheela et al (2012)	Sajinkumar et al (2017)	Observed values
pH	6.5-8.5		6.3-7.2	6.3-7.6	6-7.9
Salinity (%)	0.05-3	0.06-1.1	0.07-1.22	0.03-2.5	0.2-1
DO (mg/L)	<6	0-5.6			1-8
Conductivity (µs/cm)	50-800		290-4607	46-4380	210-1466
COD (mg/L)	<10		32-132		390-1130
TKN (mg/L)	<35	5-10	1.8-10	1-8	20-120
TP (mg/L)	<0.1	0.025-0.43	0.2-0.94	0-9.95	0.05-0.5

Few studies have been conducted in the past on the water quality and pollution stress on Akkulam Veli Lake (Sheela et al., 2010-2014). They held a survey in 2010, to assess the Carlson trophic status index of the lake and the values pointed at the hyper eutrophicated status of the AV Lake which is mainly due to the discharge of untreated sewage from the Thiruvananthapuram city to the upstream of the lake. During pre-monsoon, dissolved oxygen, Nitrate-N, total phosphorus, and salinity are very low, whereas ammonia-N and BOD were high whereas in our study total nitrogen and phosphorus was higher. This could be due to the rise in organic pollutants during these years and its concentration due to the higher temperature. In their study, they have also pointed that phosphorus input to the lake is mainly in the

form of fertilizers. It was also reported that during pre-monsoon period, influence of organic loading factor is high whereas in monsoon and post-monsoon its influence is low and the water quality in post monsoon is found to be better compared to other seasons (Sheela et al., 2012). In our study also, findings were similar to the water quality being poorer in pre-monsoon compared to different seasons with higher salinity and nutrient concentrations. Studies explain that AV Lake was in the eutrophic condition in 2007 and then by 2009, it became hypereutrophic and the depth of the lake is found to be considerably decreased due to the accumulation of debris from the sewage dumped. Comparing our results with the previous studies (Sheela et al., 2010-2014), the nutrient concentrations have increased more than ten times and all these points at a lack of management of point sources and nonpoint sources pollution.

4.3.3. Eichhornia coverage on Aakulam Veli Lake during various seasons

Figure 4.7: Aerial View of *Eichhornia* coverage over Akkulam Veli Lake in a) Feb, 2015 b) Aug, 2015 c) Dec, 2015

From the aerial view of AV Lake, it was found that the *Eichhornia* coverage changes with different seasons. The *Eichhornia* coverage was higher during the pre monsoon compared to monsoon and post monsoon (Figure 4.7).

An approximate *Eichhornia* biomass coverage in the lake was calculated using ArcMap from the satellite images and it is presented in Figure 4.8. Estimation of

areas of macrophytic coverage during different seasons was done using satellite images and it was found that there is approximately 3 times increased coverage in pre monsoon compared to post monsoon season. This can be correlated with the water quality parameters of the lake studied during these period. According to a study conducted by Kivaisi (2001), the major factors affecting the growth of water hyacinth is salinity, nutrients (Total Nitrogen and Total phosphorus) and temperature. During our analysis of different parameters of AV Lake over different seasons, salinity, water temperature and nutrient concentration were found to be higher during the pre monsoon season, which can contribute to the proliferative growth of plants during the season.

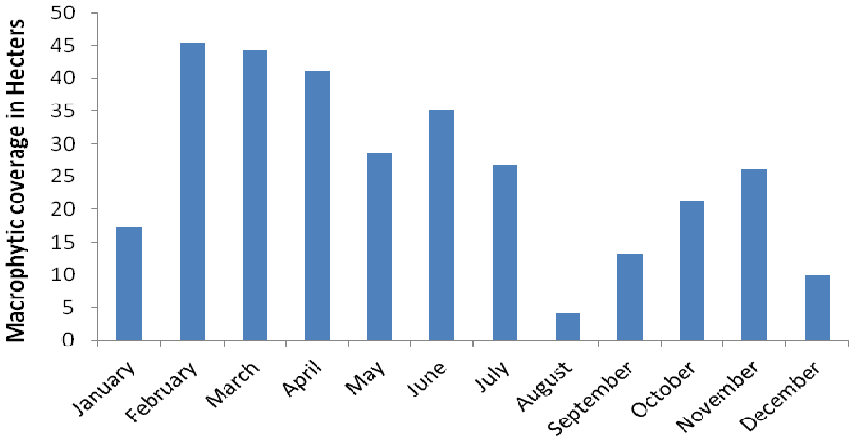

Figure 4.8: Approximate *Eichhornia* coverage of AV Lake during different months

Our field studies in the lake revealed approximately 5 to 6 kg wet weight of *Eichhornia* plants grow per m^2 area. Using this calculation, an approximate wet weight of the *Eichhornia* biomass getting accumulated can be collected is calculated, and it is summarized in Table 4.2.

Table 4.2: *Eichhornia* coverage area and approximate wet weight of the biomass in the lake during different seasons.

Period of Analysis	Coverage (Hectors)	Wet weight of biomass
February,2015	43.30	2864 Ton
August, 2015	6.09	403 Ton
December,2015	10.62	702 Ton

Maximum cover of water hyacinth in Lake Victoria came to about 1800 ha in 1998 (Twongo et al., 1995). In lake Chivero, the water hyacinth mats covered about 3.2 % (83 ha) of the total lake surface during 2003. Estimates made in April 1999 and in August 1999 by Albright et al., (2004) indicated that the input of water hyacinth into Lake Victoria through the River Kagera was 3.5 ha per week. Another study done by Wang et al., (2012) on water hyacinth in Baishan Bay, Lake Dianchi, about 12000 ton of fresh biomass were harvested, that could take away about 19.5 ton of N and 1.7 ton of P from the lake. In our study, it was found that the *Eichhornia* coverage in Akkulam Lake reached approximately 57% during pre-monsoon, and around 8% during the monsoon season and an approximate weight of 2800 ton wet weight can be harvested (Table. 4.2).

To obtain temporal and spatial information on large water bodies, conducting ground surveys are not possible. Henceforth available technologies like remote sensing have been used widely since they are more cost effective. Such studies are conducted based on nondestructive methods using the normalized difference

vegetation index (NDVI) derived from Landsat 5 TM simulated data to collect monthly expansion of WH biomass (Robles et al., 2015). Wang et al. (2011) used hyperspectral remote sensing technology with GPS correction to assess the dynamics of water hyacinth population growth and the amount of nitrogen being assimilated during a large-scale survey in Taihu Lake in China. Estimating biomass like water hyacinth helps in a better management of the weed in terms of valorization for useful products (Yan et al., 2017).

4.4. Conclusion

The extensive coverage of floating macrophytes like water hyacinth in the AV Lake changes during seasons, and that can be positively correlated with factors enhancing plant growth such as water temperature, salinity, and nutrient loading. The assessment of WH accumulation in the lake revealed 5-6 kg wet weight/m^2 area accounting to ~2800 tons over 43 hector area. This huge biomass can be harvested and can be used as a raw material for recovering biogas and manure. This will be a sustainable way of managing and restoring the eutrophic lake. The subsequent chapters of the thesis focus on those aspects. Policymaking regarding sustainable clean water resources makes a vital role in controlling the further pollution of the water bodies which demands proper monitoring of the lake.

Chapter 5

Anaerobic digestion of *Eichhornia* biomass to recover value added products

Priya.P (2018) Bioresource technology, 255, 288-292

5.1. Introduction

Urban landscape waters are more prone and have higher risks for macrophytic overgrowth like *Eichhornia* and algal blooms because of its shallow nature (Kabenge et al., 2016). They form thick coating or mat on the surface of water with several negative impacts, such as depletion of dissolved oxygen, low transparency, and aesthetic disturbance (Dai et al., 2012).The doubling time of *Eichhornia* is 6-12 days, and a mat of medium sized plants may contain 2 million plants per hectare that weigh 270 to 400T of wet weight (Malick, 2007).An effective way of utilising them is for valorisation purposes like biogas production.

Even though the major component of *Eichhornia* and *Pistia* is moisture, its fibrous tissue and protein content provides a variety of useful applications (Adeyemi and Osubor, 2016). Conversion of organic matter particularly lignocellulosic biomass to biogas is a well established source of sustainable energy. *Eichhornia* is the well exploited candidate for these biomass conversions when compared with *Pistia*. Several studies of conversion of the biomasses have been done particularly for the production of biogas, bioethanol, biohydrogen, biobutanol, biopolymer, carbon fiber, as the super absorbent polymer, high calorific fuel, composite, biofertilizers, fish feed/ animal feed, substrate for mushroom cultivation and for effluent treatment (Punitha et al., 2015, Sindhu et al., 2017, Liu et al., 2018).

The biomass conversion processes involves a complex consortium of microorganisms which participates in the hydrolysis and fermentation of organic material and the degradation is facilitated by enzymes like cellulase, lipase, protease etc. The higher volatile fatty acids are converted into acetate and hydrogen by obligate hydrogen-producing acetogenic bacteria (Weiland, 2010). Although many microbial details of metabolic networks in a methanogenic consortium are not clear, some studies suggest that limiting substrate for methanogens is hydrogen (Bagi et al., 2007). At the end of the degradation chain, two groups of methanogenic bacteria produce methane from acetate or hydrogen and carbon dioxide. These bacteria are

strict anaerobes and require a lower redox potential for growth than most other anaerobic bacteria. Only few species are able to degrade acetate into CH_4 and CO_2, e.g., *Methanosarcinabarkeri*, *Methanonococcusmazei*, and *Methanotrixsoehngenii*, whereas all other methanogenic bacteria are able to use hydrogen to form methane. These methanogenic communities though less in abundance, determine the methane yield from the digesters. Due their growth complexities, culture independent molecular techniques like qPCR or Fluorescent In Situ Hybridization (FISH) using domain specific primers can be relayed for monitoring microbial communities in the digester.

Though biomethanation of lignocellulosic biomass has been well studied in the past, there are an inherent technical challenge that affects the feasibility of the process and implementing it at the field. It includes harvesting of the biomass from the water body (Bayracki and Kocar, 2014), seasonal growth of the plants (Newete and Byrne, 2016) and very low biogas yield due to its low solid content (5%-8%) (Nijaguna, 2006). The objectives of the study are

1) to compare the biomethanation potential of *Eichhornia crassipes* and *Pistia sp*. and to select the better candidate for lab scale trials, practical solutions for low biogas yield and ways to ensure continuous supply of biomass for the digester running
2) Biomethanation of WH in lab scale bioreactor and assessment of the value added products (biogas and manure) recovered.
3) To study the microbial ecology of the anaerobic digester through molecular methods like Fluorescent In Situ Hybridization (FISH) and qPCR.

5.2. Materials and method

5.2.1. BiomethanationPotential of Eichhorniaand Pistia

Crushed *Eichhornia* and *Pistia* biomass were used as substrates and anaerobic digestion studies were done in batches. One litre glass bottles (Schott Duran) were used as batch reactors with 400 ml volume. The anaerobic inoculum was collected from an existing food waste biogas plant in NIIST campus. Twenty grams of the plant biomass was used for the batch experiment, by maintaining a sludge ratio of 0.3. Test and controls were kept in triplicates.Bottles were incubated at 25±3°C. Total biogas produced and its methane content (% v/v) was measured dailyusing water displacement set up and GC analysis respectively. The initial pH, prior to the start of experiment, and final pH, at the end of experiment from each reactor was measured using pH meter (Sartorious, Germany). The batch bottle reactors were incubated until no further gas production could be detected.

Figure 5.1: Batch biomethanation set up for digestion of water hyacinth and *Pistia* sp., biomass

5.2.2. Sampling and storage of Eichhornia biomass

The Akkulam Lake is covered majorly by mats of *Eichhornia* (Figure 5.2). Adult and young plants were collected from Akkulam Lake and were temporarily stored in a stocking tank at NIIST as previously mentioned in Chapter 3 (Figure 5.3).

Figure 5.2: Matted growth of Water hyacinth on AkkulamVeli Lake

Figure 5.3: Water hyacinth storage tank

5.2.3. Characterization of Water hyacinth biomass

The composition of water hyacinth biomass in terms of total solids, moisture content, volatile solids and ash content were determined for each plant parts (APHA, 1998).

5.2.4. Comparison of different pre-treatment methods on anaerobic digestion of WH biomass

Different pre-treatment methods like acid hydrolysis, alkali hydrolysis, and mechanical crushing were tested for its effect on biogas yield during biomethanation of *Eichhornia* biomass.

a) Acid hydrolysis

10 gm plant biomass was mixed with 50 ml of 2% sulphuric acid and was kept at 100 ºC for 30 minutes. After the acid treatment, the pH was neutralized to ~7 with 1 NaOH (Kumar et al., 2009).

b) Alkali hydrolysis

NaOH treatment was done by mixing 50 ml of 2% NaOH with 10 gm plant biomass and was kept for 90 min at 121°C (Kumar et al., 2009).

c) Mechanical crushing

10 gm of whole plant was crushed using a rubber sheeting roller machine.

These different pre-treated biomasses were subjected to batch tests for checking their biomethanation potential.

5.2.5. Anaerobic digestion of WH biomass in a two stage anaerobic bioreactor

Based on the results of the batch experiments conducted, a laboratory scale two stage biomethanation system was set up to study biomethanation of WH. The biomethanation system consists of an Anaerobic Leach Bed Reactor (ALBR) connected to a typical Upflow Anaerobic Sludge Blanket Reactor (UASB). The ALBR was made up of a 10 L capacity PVC container. The top portion was tightly capped with an end-cap, with provision for receiving the effluent from the UASB.The soluble organics released in the ALBR was fed to the UASB unit. The UASB unit was made up of glass column of 20 L capacity with gas-solid-liquid separator at top. The liquid from ALBR was pumped to UASB at 30 ml/min (flow rate)using a peristaltic pump (Watson Marlow 505S). The simple design of UASB reactors ensures a uniform distribution of incoming digester feed around the base of the digester, sufficient cross section to prevent excessive biomass entrapment, and

effective separation of gas, biomass, and liquid. The biogas was measured by a wet gas flow meter (Hi Tech, India).

In a typical batch operation, 4 kg (wet wt.) of mechanically crushed WH biomass (0.049 gm VS/gm wet wt) was loaded into the ALBR unit. The seed inoculum used was collected from an existing anaerobic bioreactor in NIIST campus (0.056 gm VS/ gm of wet weight). After 12 days, when the biogas release was almost nil, the digestate from ALBR was removed, and fresh lot of WH biomass was loaded into the ALBR and the cycle was continued. The bioreactor study was continued for a period of one year.

a. Characteristics of WH biomass digestate as organic manure

Once the digestion of WH biomass was over, the digestate from the reactor was tested for its cellulose, hemicellulose, total kjeldahl nitrogen, total phosphorus, sodium and potassium content for its usage as fertilizer.

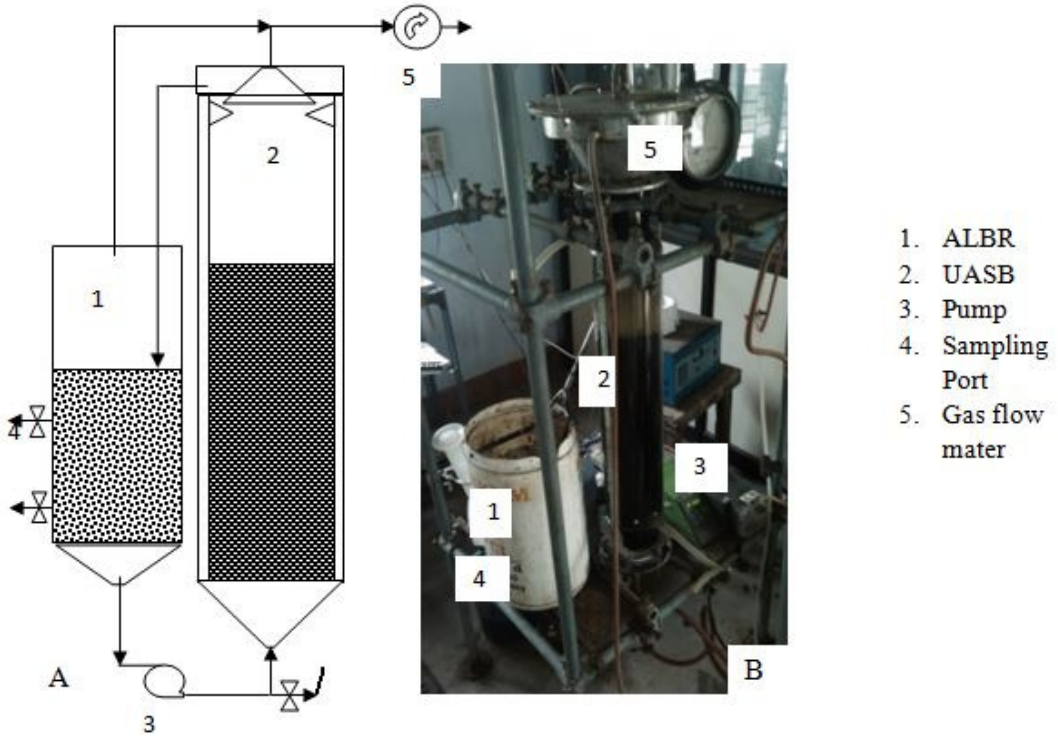

Figure 5.4: A) Schematic representation of the two stage treatment system with closed ALBR and UASB, B) Laboratory scale digester unit.

5.2.6. Microbial community analysis of bioreactor treating WH biomass

The microbial community of the WH treating bioreactor was analysed through molecular methods like whole cell Fluorescent In Situ Hybridization (FISH) and quantitative PCR.

a. Whole cell Fluorescent Insitu Hybridization (FISH) results

Qualitative analysis of the microbial community on the anaerobic digester was analysed through whole cell FISH. 50 ml samples were collected from the sludge bed of UASB reactor digesting lignocellulosic biomass. After mixing, 5 ml of samplewas taken and was washed with sterile MilliQ. Samples were fixed immediately after collection using 4% (w/v) paraformaldehyde (Schuppler et al.,

1998). The Fixed samples were then sonicated at 100% amplitude for one cycle using the UP500H (Dr. Hielscher, Germany) ultrasonic processor. Hybridization was done on 14 well Hydrophobic Teflon Coated (HTC) slides (Cell-line, Erie Scientific Company, Germany) coated with 0.1% gelatin in 0.01% $KCr(SO_4)_2$for adhesion of the specimen and dried at 37^0C. Ten µl of the sample was spotted on alternate wells, heat fixed at 40°C for 10 min and washed in different concentrations of ethanol like 50%, 80% and 100%. Once fixed, the samples were hybridized to domain specific probes tagged with Cy-5 on 5' endpurchased from IDT (USA). The details of probes used for the FISH analysis are listed in Table 5.1.

Table 5.1: FISH Probes used for the microbial community analysis

Probe	Sequence (5'-3')	Domains targeted	Reference
EUB338 I	GCTGCCTCCCGTAGGAGT	Bacteria	Amann et al., 1990
ALF1b	CGTTCG(C/T)TCTGAGCCAG	Alpha proteobacteria	Manz et al., 1992
BET42a	GCCTTCCCACTTCGTTT	Beta proteobacteria	Manz et al., 1992
GAM42a	GCCTTCCCACATCGTTT	Gamma proteobacteria	Manz et al., 1992
MS821	CGCCATGCCTGACACCTAGCGAG C	*Methanosarcina*	Raskin et al.,1994
MX825	TCGCACCGTGGCCGACACCTAGC	*Methanosaeta*	Raskin et al.,1994

Each well of the slides was overlaid with 10 µl of hybridization solution containing 0.9 M NaCl, 20 mMTris-HCl (pH 7.4), 0 to 50% formamide, 0.01% sodium dodecyl sulfate (SDS), and 5 ng/µl of labeled oligonucleotide which was then incubated at 46°C for 2 ½ hrs in a closed hybridization oven (HB Minidizer, UVP, USA). The stringency of the washing step was adjusted by changing the NaCl concentration in the washing buffer according to the formamide concentration. After hybridization, the buffer was rinsed with washing buffer and the slides were kept at 48°C for

30min. The slides were washed with distilled water and air dried. The counterstaining was performed with DAPI (4', 6-diamidino-2-phenylindole) solution (0.5 µg ml^{-1}) and mounted with Vectashield mountant (Vector Laboratories). The prepared slides were examined under an epifluorescence microscope (Leica DM 2500) equipped with CCD camera for imaging.

b. Quantitative PCR analysis of methanogens

Microbial community shifts, in particular to methanogens during the biomethanation of *Eichhornia* were studied using qPCR. Batch biomethanation system were set up as explained previously. Samples were loaded at sample to sludge ratio of 0.3. Samples and controls were kept in triplicates. Bottles were incubated at 25±3°C. The biogas produced was measured using water displacement. The sludge sample for the qPCR analysis was collected at three different periods during the 30 days reactor operation. The first sample was taken during the first day of biomethanation whereas second and third samples were collected on the 15^{th} and 30^{th} day respectively. The sludge samples were washed thrice with MilliQ and the community DNA from the sludge was extracted using Nucleospin soil DNA Extraction kit (Macherey- Nagel, Germany). The purity of the DNA samples was confirmed with Nanodrop.

Three primer sets targeting the methanogenic orders *Methanobacteriales*, *Methanococcales*, and *Methanosarcinales* prepared using PrimerBLAST (NCBI) were used in this study (Table 5.2.). Previous studies have identified that these three domains should cover most methanogens in anaerobic digesters (Yu et al., 2005).

Real-time PCR was performed using CFX Connect Real time system (BIORAD, USA).For a 10µl PCR reaction, 5 µl of Sso Advanced Universal SYBR Green Supermix is mixed with 0.5 µl of 5 µM concentration of forward and reverse primer of each order along with 2 µl of templates. PCR was run as per the following program: 94°C for 4 min of initial denaturation followed by 35 cycles of 10 s at 94°C and combined annealing and extension for 60 s at 58°C for all the primer sets.

Table 5.2: qPCR Primers developed for various target domains

Primer names	Sequences (5'-3')	Domains targeted
metB30F	CTGGCCGTAAACGATGTGGA	*Methanobacteriales*
metB30R	ATGCACCTCCTCTCAGCTTG	
metC92F	GTGGGCTTTTCCGGAGTGTA	*Methanococcales*
metC92R	TGAGCCGCAGGATTTAAGCA	
metS46F	CGGGACCGACAGCAATATGA	*Methanosarcinales*
metS46R	CCTACCGTTGCCCATTCCTT	

5.2.7. Strategies to increase biogas yield during anaerobic digestion of WH biomass.

Three different strategies were tested to increase the biogas yield during the anaerobic digestion of water hyacinth biomass.

a. *Increasing WH biomass solid content to improve biogas yield*

Sun drying was adopted as a simple strategy to increase the solid content of WH biomass. 10 kg (wet weight) of WH plants were exposed to direct sunlight for different durations from one hour to one week. Periodically samples were taken and solid content (TS and VS) and its biomethanation potential were tested. The biomethanation potential was tested in 1000 ml batch experimental unit as described earlier. The substrate to inoculum volatile solid ratio was kept at 0.5. The amount of biogas produced was measured after one day through a water displacement set-up.

The composition of biogas was analyzed using a micro GC system (Agilent 490 Micro GC with narrow-bore capillary GC column).

Figure 5.5: Sun drying of water hyacinth biomass in open terrace

b. Ensilation of Water hyacinth biomass and biomethanation of ensilated biomass

Mechanically crushed WH biomass was exposed to four hours sunlight and was used for the ensilation experiment. Ensilation was carried out in one liter plastic container with air tight lid. The partially dried biomass was thickly packed in the bottle upto lid without any air space. Three replicate were kept for silages. The weight losses along with TS and VS change in the silage before and after preservations were checked. The bottles were maintained at room temperature for six months. Biomethanation potential tests were performed with silage and control samples (wilted fresh biomass) in triplicate. The biogas yield and its composition were periodically monitored as described earlier.

Figure 5.6:Six month old silage of sun dried water hyacinth

c. Co-digestion of water hyacinth biomass

Mechanically crushed WH biomass was used for the co-digestion studies. Food waste and waste activated sludge (WAS) from a sewage treatment plant were used as substrates for the co-digestion study. The pH and solid contents (TS and VS) of the food waste and WAS were tested (APHA, 1998). The food waste and WAS were added to water hyacinth biomass in the VS ratio of 1:1 for co-digestion. Co-digestion studies were carried out in 1000 ml batch tests for 15 days. The seed inoculum used was from an existing anaerobic digester (0.049 gm VS/ gm of wet weight. Triplicates of treatments and control bottles without the substrate were also kept in parallel.

5.2.8. Analysis

Total Kjeldahl Nitrogen (TKN), Total Phosphorus (TP), cellulose, Klason (insoluble) lignin and hemicelluloses of plant biomass were estimated. TKN was estimated using distilled/ titrimetric Kjeldahl's method and TP was estimated using molybdate-ascorbic acid method (APHA, 1998). Cellulose was estimated using Ethanol: Toluene soxhlet extraction (Sun and Sun, 2002; Sun et al., 2004). The

hemicelluloses and the Klason lignin content were extracted using the method prescribed by Weihe and Philips (1947) and Hatfield et al., (1994) respectively. COD was estimated by open reflux method (APHA, 1998).

The volatile fatty acid analysis was done by titration method prescribed by Anderson and Yang (1992).Gas Chromatography techniques of the gas samples were done using Agilent 490 Micro GC with Narrow-bore Capillary GC column and micro-machined thermal conductivity detectors (μTCD).

The volatile fatty acid composition of the silage was analyzed through HPLC. Plant tissues of ensilated and wilted fresh biomass were crushed using mortar and pestle, and the leachate was collected. The collected liquid samples were acidified with H_2SO_4 to pH 3 and filtrated through 0.45 μm polypropylene filters. The content of C1-C6 VFAs (including isoforms of butyric and valeric acid), lactic acid, succinic acid, and ethanol were determined using high-performance liquid chromatography (HPLC) using Shimadzu – LC-10AD with RID 6A RI detector) using 0.008N H_2SO_4 buffer and Bio-Rad Aminex HPX-87H (300 × 7.8 mm) column at 50°C. Results were analyzed using Autochro 3000 software. The statistical analysis was done using MS Excel and values were reported as an average of minimum three values with standard deviation.

5.3 Results and Discussions

5.3.1. Biochemical Methane Production Potential of Eichhornia and Pistia biomass

Preliminary studies in batch units revealed that biogas production continued till day 10 and maximum biogas was produced during 3-6 days. The initial solid content of the WH and *Pistia* biomass were 0.052gm VS/gm and 0.08 gm VS/gm of biomass respectively. The cumulative biogas generated by the batch experiment using mechanically crushed WH biomass and *Pistia* were 138±5 ml/gm VS and 102±8

ml/gm VS respectively. The profile of biogas generation during the biomethanation of both WH and *Pistia* is presented in Figure 5.7. A 26 % increase in biogas yield was observed with WH biomass compared with *Pistia*. This can be justified with the high solid content of WH biomass (7%) compared to 5 % in *Pistia*.

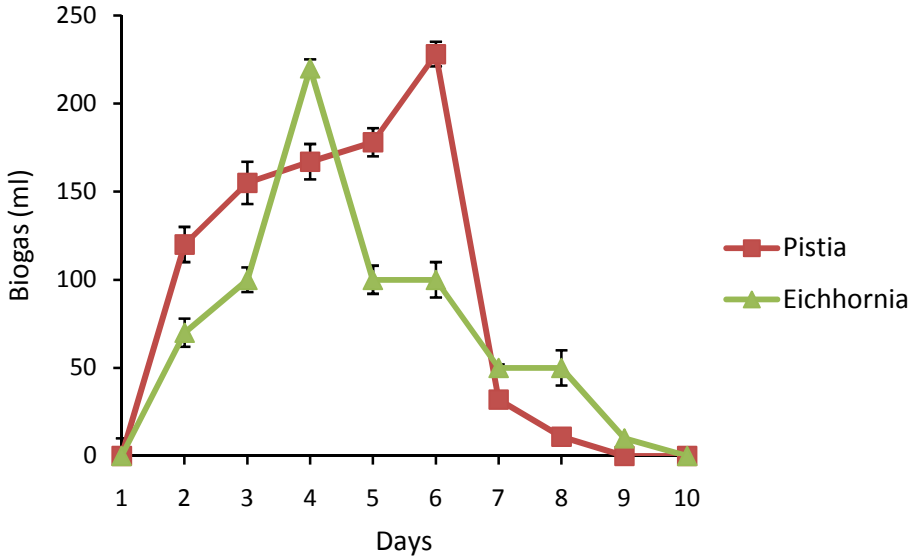

Figure 5.7: Profile of biogas production by*Pistia* and *Eichhornia* in batch biomethanation

The lower biogas production with *Pistia* (27% of Dry weight) compared to WH (30% of dry weight) was reported in a previous study (Sivashankari and Raveendran, 2016). Different studies on biomethanation of WH and *Pistia* were done previously and there were variations in biogas yield and time. Dipu et al., (2011) reported biogas yield of 195 ml/ gm VS from *Pistia* and 205ml/gm VS from WH.

As presented in Chapter 3, our nutrient removal studies have revealed *Pistia* as better candidate compared to WH (25% more efficient). However, the biomethanation studies revealed more biogas yield from WH. Moreover, in natural water bodies WH proliferation is noted as a serious environmental problem. Therefore WH is selected over *Pistia* for more detailed biomethanation studies.

5.3.2. Characterization of water hyacinth biomass

The moisture content, solid and ash content of WH plant are presented in Table 5.1. The root and stem have almost similar composition such as moisture content, total solids, volatile solids and ash content. On the other hand, the moisture and ash content of the leaf was found to be lower than that of the stem and root. The total solids and volatile solids were higher in leaves than that of stem and root.

Table 5.3: Composition of different parts of WH plant was given in the table

	Root	Stem	Leaf	Whole plant
Moisture Content (w/w %)	94±0.7	94±0.5	83±1	95±3
Total Solids (%)	6	6.5	17	5
Volatile solids (% of Total Solids)	76.5	79	86.5	77
Ash Content (% of Total Solids)	23.5	21	13.5	23

Proximate analysis of the WH biomass was done prior to anaerobic digestion. It revealed that 77% of the total solid present is volatile solids (Table 5.4). The nitrogen content of the WH biomass and Phosphorus content was in the range of 53 mg and 15 mg per gram of dry weight respectively. The hemicellullose content of the WH biomass was higher compared to cellulose and lignin.

Table 5.4: Proximate analysis result of WH biomass

Parameter	Value
Moisture Content (w/w %)	92±3
Total Solids (%)	8±3
Volatile solids(% of Total Solids)	77±3
Ash Content (% of Total Solids)	23±2
TKN (mg/g Dry weight)	53±12
TP(mg/g Dry weight)	15±5
Cellulose content(% of dry weight)	18±5
Hemicelluloses content(% of dry weight)	30±4
Lignin content (% of dry weight)	12±3

Lignocellulosic biomass is an attractive raw material for anaerobic digestion. For WH, 18 to 35 % of total solids are cellulose where as 30 to 48 % is hemicelluloses and 7 % to 12 % is lignin (Bhattacharya et al., 2016). A similar range of values was also reported earlier (Nigam, 2002; Gunnarsson and Petersen, 2007; Mishima et al., 2008).

5.3.3. Comparison of different pre treatment of WH biomass for biomethanation

The biogas produced from differentially treated biomass from batch tests were summarized in Figure 5.8.

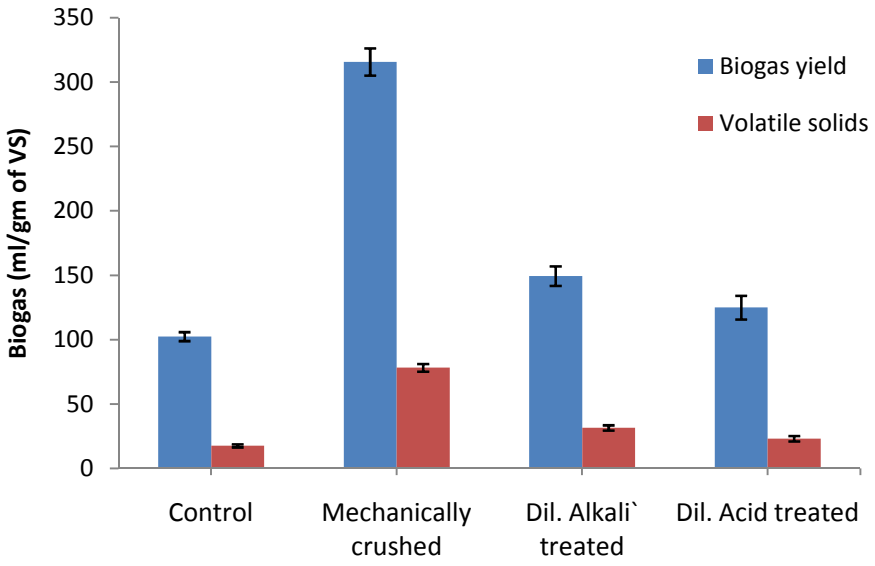

Figure 5.8: Cumulative biogas production and VS content after different pre treatments

Among the pre treated samples, the mechanically crushed water hyacinth biomass produced 380 ml biogas per gram VS, whereas minimum yield (100 ml/gm of VS) was from acid hydrolysis. The higher biogas yield from mechanically crushed WH could be due to the increase in surface area of the biomass which can cause an effective enzymatic decomposition by cellulolytic microorganisms. The lowest biogas yield was observed in the control where the biomass digestion was done without any pre-treatment.

The VS content of the biomass after different pre treatments was also checked. It shows that mechanical crushed biomass is having more volatile solids contents (50 % of dry matter) which mainly contribute for the biogas production during anaerobic digestion. During treatment with diluted acid and alkali, the VS content of the biomass was found to be approximately 17% and 23% compared to the control (17.6 gm).

Alkali pretreatment decreases polymerization and crystallinity and destroys links between lignin and other polymers. This pre treatment works better for low lignin content biomass like water hyacinth biomass (Sun and Cheng 2002; Badiei et al., 2014).Acid pre treatment results in the disruption of the various bonds in the biomass components, which consequently causes the solubilization of hemicellulose and the reduction of cellulose (Li et al.,2010). The main reaction that occurs during acid pretreatment is the hydrolysis of hemicellulose, especially xylan, as glucomannan which is more stable but lignin is unaffected by acid pre treatment. Mechanical pretreatments of lignocellulosic material is an important step for improving the bioconversion efficiency, particle densification and distribution, enzymatic accessibility, and overall transformation of lignocellulosic material into biofuels without the generation of toxic side streams (Barakat et al., 2015). This could be the reason for increased biogas yield compared to other pre treatment techniques. A comparative study conducted between untreated and hot air oven pretreated water hyacinth revealed that the hot air oven pretreated water hyacinth showed the highest methane yield of 193 ± 22 ml CH_4/g VS whereas untreated water hyacinth could showed the methane yield of only 143 ± 14 ml CH_4/g VS (Barua and Kalamdhad, 2017).

5.3.4. Biomethanation of WH biomass in a two stage anaerobic bioreactor

A two-stage anaerobic process unit was used in this study to minimize the direct inhibition of hydrolytic products (organic acids) on methanogens. The ALBR ensures the proper hydrolysis of the biomass which is the rate-limiting step in the biomethanation process (Noike et al., 1985). The pH was not controlled at any stage of the process, and it was found to be in the range of 4 to 8. The digester unit was maintained at an ambient temperature of 30±3°C. A number of previous studies on the anaerobic digestion of organic wastes containing lignocelluloses reported that two stages or multistage process without pH control are more effective than single stage system (Abbasi et al., 1992, Paixao et al., 2000, Demirel and Yenigun, 2002).

The removal efficiency of COD and VFA in the system was about 70% and 60% respectively. UASB was found to be very effective in removing 90% of COD and 92% of colour from textile industry waste water (Somasiri et al., 2008). A pilot scale waste water treatment study was conducted using a UASB followed by duckweed pond by El Shafai et al., (2007) and found that temperature affects COD removal. During a study on the long term performance of UASB treating starch waste water, 80-99% of COD removal was found and they explained sludge floating as the major factor determining the performance of the UASB (Lu et al., 2015). The profile of COD, VFA, and biogas yield during a typical batch (completed in 12 days) digestion is shown in Figure 5.9.

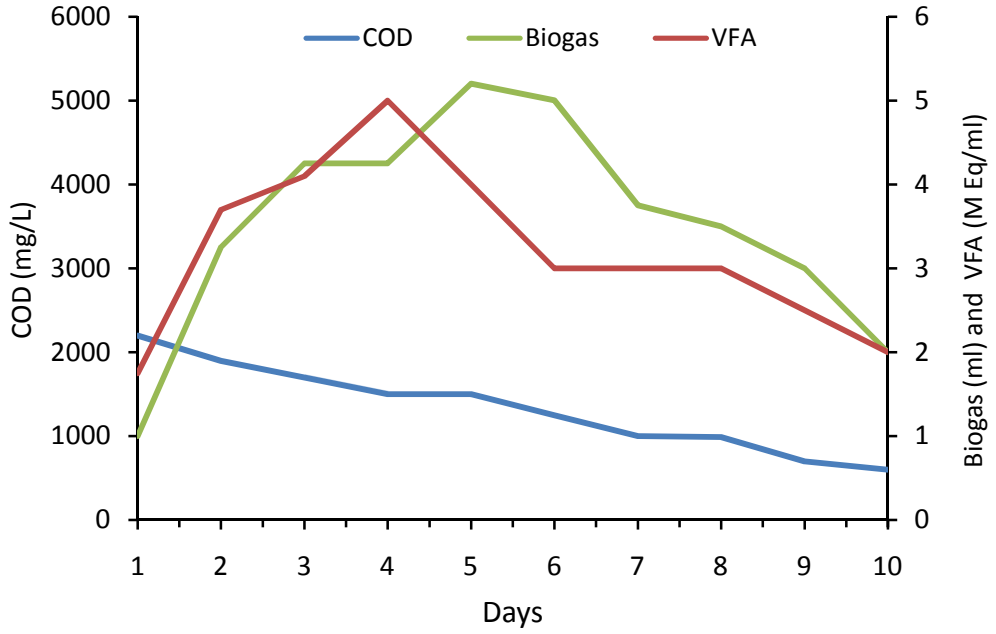

Figure 5.9: COD, VFA and biogas production profile of a typical batch operation of WH biomass digestion

In this study, 36.5 L biogas was produced from 4 kg (wet weight) mechanically crushed whole plant WH biomass in 10 days. This biogas yield was equivalent to 141±6 ml biogas/gram VS. Anaerobic digestion of WH (alone) was reported in few

previous studies. Hernández-Sheket al., (2016) have observed 114 ml biogas/g VS from shredded WH biomass. In a more recent report, the macerated equal ratio of roots, leaves and, stem of WH yielded 193±14 ml methane/g VS (Barua and Kalamdhad, 2017). The VS content of the plants is different for parts like shoot, leaves, and roots which are 77±0.4, 86±0.7 and 35±0.3 respectively. The biogas yield from the present study is lower compared to the reported studies. It is due to the usage of crushed whole plants as the feed and VS content of the whole plant is 4.5±1.5%. The methane content in the biogas was in the range of 63-68% (v/v) which is comparable with the values reported (67%) in previous studies (Barua and Kalamdhad, 2017). The average biogas yield from the digester during the one-year period of operation was around 8.85 l/kg wet weight WH. The pH of the ALBR and UASB were found to be between 6.2 to 7.2 without the aid of any external pH regulators. It was reported that optimum pH for methanogenesis is between 6.8 to7.2 (Sreekrishnan et al., 2004).In the present study, VFA production was higher in the Leach Bed (2-7 meq/L) where as VFA concentrations on UASB were lower (1-2 meq/L). The VFA generated in ALBR is converted into methane at a high rate in UASB which already contains large methanogenic biomass.Previous reports on the biomethanation of water hyacinth are consolidated on Table 5.5.

Table 5.5: Previous reports of biogas yields from anaerobic digestion/ co-digestion of Water hyacinth

Sl. No.	Scale of study	Substrate	Pretreatment Used	Biogas/ Methane yield	Methane %	References
1	Lab scale	Fresh WH+ cow dung	Chopped	180.7 L/kg of TS	65%	Chanakya, 1992
		Dried WH+ cow dung	Chopped and dried	147.7 L/kg of TS	68 %	
2	Lab scale	WH	Untreated	190 L CH4/ gm VS	-	Chynoweth et al.,1993
3	Pilot scale	WH shoots + cow dung	Sundried and powdered	441 ml CH4/gm VS	80%	Kivaisi et al., 1997
4	Pilot scale	WH + cow dung	Sundried and powdered	267 ml biogas/gm VS	50 %	O' Sullivan et al.,2010
5	Lab scale	WH+ Poultry litter	Sundried and powdered	390 ml biogas/gm VS	60 %	Patil et al.,2011
		WH+ cow dung	Sundried and powdered	240 ml biogas/gm VS		
6	Lab scale	WH alone	Shredded	114 ml biogas /gm VS	-	Sheikh et al.,2016
		WH+ fresh vegetable waste	Shredded	230 ml biogas /gm VS	-	
7	Lab scale	Equal ratio of root, stem and leaves	Macerated	193±14 CH4/gm VS	67%	Barua and Kalamdhad, 2017
		WH + cow dung	Hot air oven pre treated	193±22 ml CH4/ gm VS		
8	Lab scale	WH alone	Mechanically crushed	141±6 ml biogas/gm VS	63%-68%	Present study
		WH+ Waste sludge	Crushed and powdered	148 ± 5 ml biogas/gm VS		
		WH + food waste	Crushed	394±12 ml biogas/gm VS		

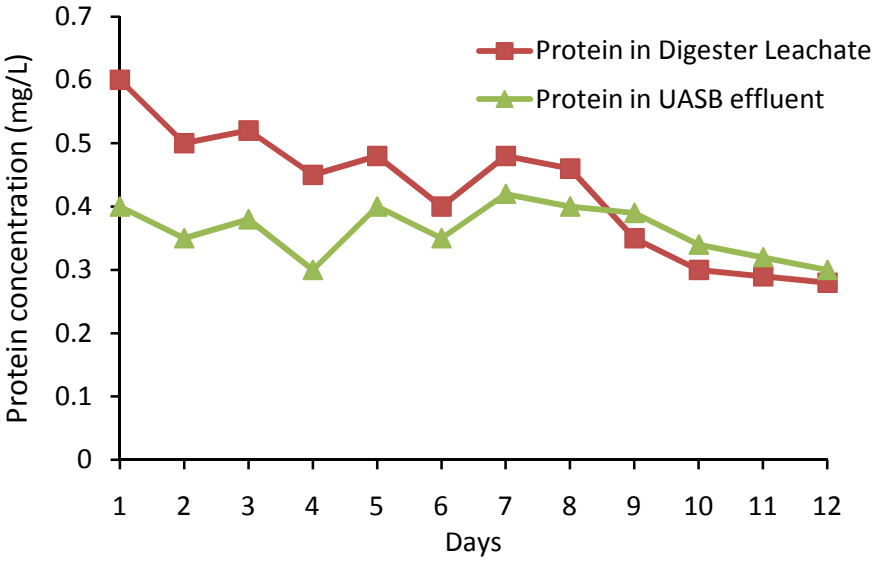

Figure 5.10: Soluble protein concentration in the batch operation

Volatile solids of the WH biomass consist of the biodegradable portion which includes carbohydrates, fats and protein which will be converted into methane by bacterial activity. Ammonia (NH_3) content in biogas is used to indicate digestion efficiency, since ammonia is an end product of the breakdown of complex organics such as proteins, which are composed of nitrogen based compounds. Ammonia built up occurs in digesters when proteins are being used up which causes inhibition of methanogenesis in the digester. So the utilization of protein for methane production was checked on the digester.As the digestion and methanogenesis progress, the protein concentration on the digester will be decreased (Figure 5.10). The concentration of protein in the effluent was lower than that of the ALBR leachate, which indicates that the protein released in the digester was consumed within the UASB.

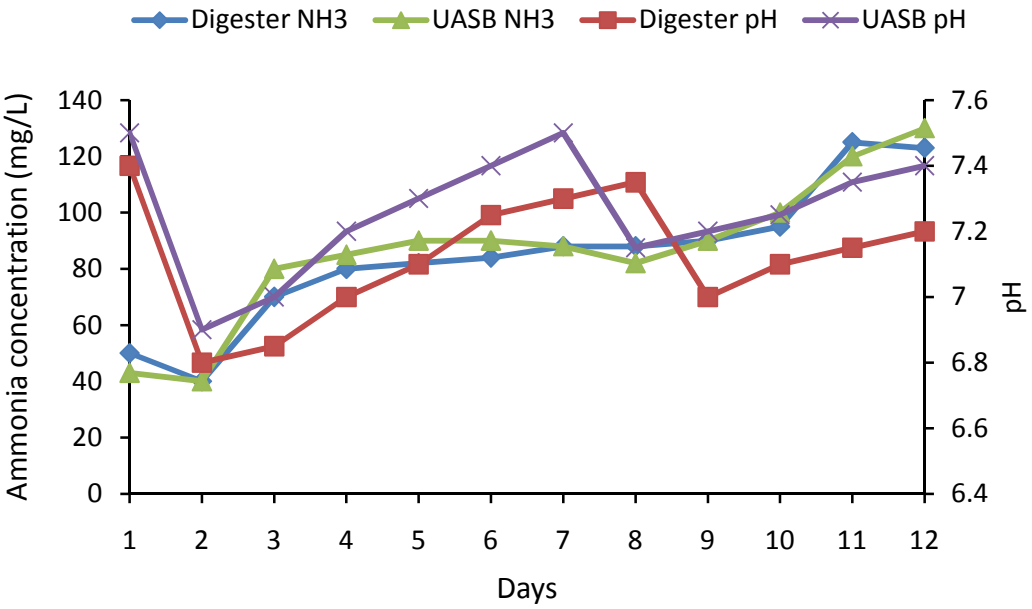

Figure 5.11: Ammonia and pH profile in the ALBR and UASB during WH biomass digestion

The ammonia concentration in the digester and UASB effluent was found to be gradually increasing and the concentration was maximum towards the end of the process (124-127mg/L) (Figure 5.11). The concentration of ammonia in the UASB was higher than that of the ALBR which can be explained on the basis of protein removal by the microorganisms. Initially, high concentration of protein and low concentration of ammonia was observed in the ALBR. This is because, protein hydrolysis is the major source of ammonia in the system, so the initial higher protein concentration indicates that there was no considerable hydrolysis. As the hydrolysis progresses, the soluble protein concentration get decreased from 0.6 mg/L to 0.25 mg/ml, resulted in an increase in ammonia concentration from 40 mg/L to 120 mg/L towards the end of the process. As the ammonia concentration increases, the pH of the system also increased from 7 to 7.5.

Ammonia has been regarded as one of the most significant inhibitors in AD processes, because it directly inhibits microbial activities by means of proton imbalance in the cell due to its permeability to microbial membrane (McCarty and McKinney, 1961).Concentrations of

free ammonia above 100 mg N/L inhibit methanogenic communities though different species of methanogens have different tolerances to ammonia (De Vrieze et al., 2012).The studies on the effect of ammonia on methanogens showed that it is having relatively more negative effect on acetate-consuming methanogens than on hydrogen consuming methanogens (Yenigün and Demirel, 2013). The biogas yield from the digester was recorded for one year and the result is presented in Figure 5.12.

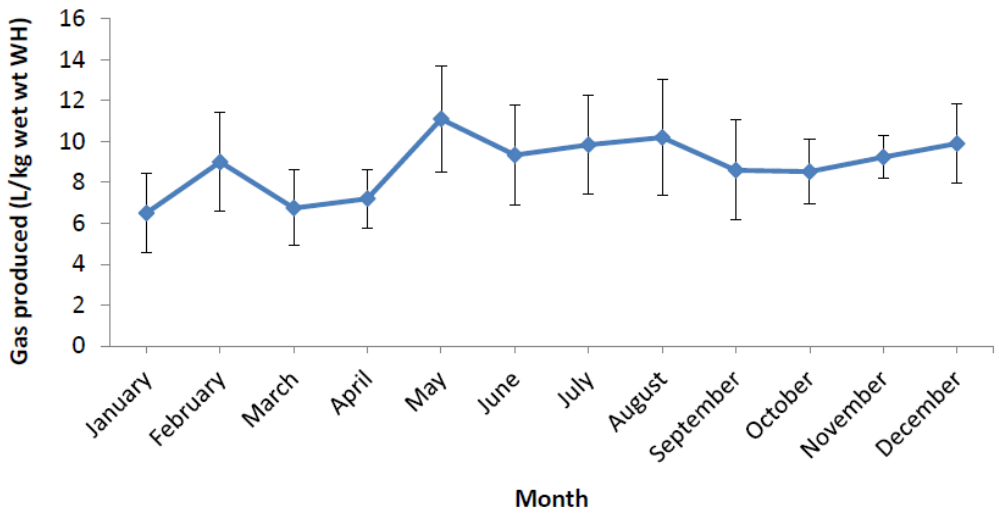

Figure 5.12: Monthly average biogas yield per kg of wet WH biomass during one year of operation of the two stage bioprocess unit.

Different studies on anaerobic digestions including two stage biomethantion systems were reported for various effluent or waste water treatments. A study was conducted in a two stage UASB (an acidogenic UASB coupled with a methanogenic UASB) for treating palm oil mill effluent using (Borja et al., 1996). Some studies suggested that when acetogenesis and methanogenesis occurs on separate reactors, it becomes possible to increase the rate of methanogenesis by designing the second reactor with biomass retention scheme and rate of hydrolysis in the first stage by increasing microaerophilic conditions (Weiland, 1992;

Kublerand Wild, 1992; Capela et al., 1999; Wellinger et al., 1999). Sreekrishnan*et al*. (2004) reported that, in the first stage of biomethanation (hydrolysis step), insoluble organic material and compounds like lipids, fats, proteins, and polysaccharides are broken down into soluble monomers, such as amino acids and monosaccharides, which can be used as a source of energy. This stage is enzyme driven and is more efficiently carried out by strict anaerobes, it is clear that, ALBR provided a suitable environment for the action of anaerobes in the hydrolysis stage. So having a two stage biomethanation system ensures higher rate of hydrolysis which is the rate limiting step and better methanogenesis which will improve biogas yield.

5.3.5. Characteristics of WH biomass digestate as organic manure

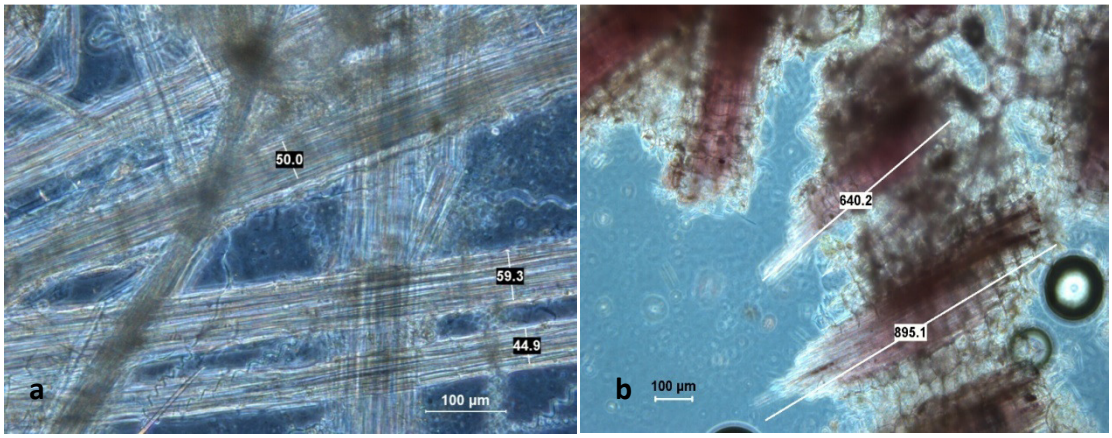

Figure 5.13: Phase contrast image of the digested WH biomass (Leica DM 2500)

A) Fibres in the digestate b) Digested WH plant

The digestate (slurry after the treatment) was studied in detail to identify its composition and itsmanure value. Digestate was found to contain 95% of moisture and 5% of total solids. The cellulose content was found to be 22% where as hemicellulose was found to be 7.6% of total solids. Total phosphorus was found to be in the range of 0.78% whereas TKN was 8.7%. Sodium and potassium content on thedigestate was found to be 0.46 % and 3.8% of total solids. Penhallegon (2005) reported that, most of the effective organic manures with slow and immediate nutrient/nitrogen releasing ability had a Nitrogen, phosphorous and

potassium concentration of 0-42%(N), 0-55%(P), and 0-27%(K) respectively. The nitrogen, phosphorous and potassium concentration of the slurry was also found to satisfy the preferred limit, which indicated the potential application of the digested WH slurry as an organic manure, because potassium and phosphorous are the primary nutrients in plant growth, and Nitrogen is the nutrient needed in largest quantities (Tilmanetal.,2011).

5.3.6. Microbial community analysisof WH treating bioreactor

a. Whole cell Fluorescent in-situ Hybridization (FISH)

FISH analysis revealed the presence of alpha proteobacteria, beta proteobacteria, gamaproteobacteria and archae like *Methanocaeta* and *Methanosarcina* (Figure 5.14.a and b). The presence of Eubacteria was found positive using the probe EUB338. The presence of different types of proteobacteria, which the largest subgroup of eubacteria were confirmed using ALF1b, BET 42a and GAM 42a. Methanogenic archae populations like Methanocaeta and Methanosarcina are confirmed using probes like MS 821 and MX 825 respectively. Qualitative analysis of the sludge from both UASB and ALBR showed the presence of Eubacteria and archae.

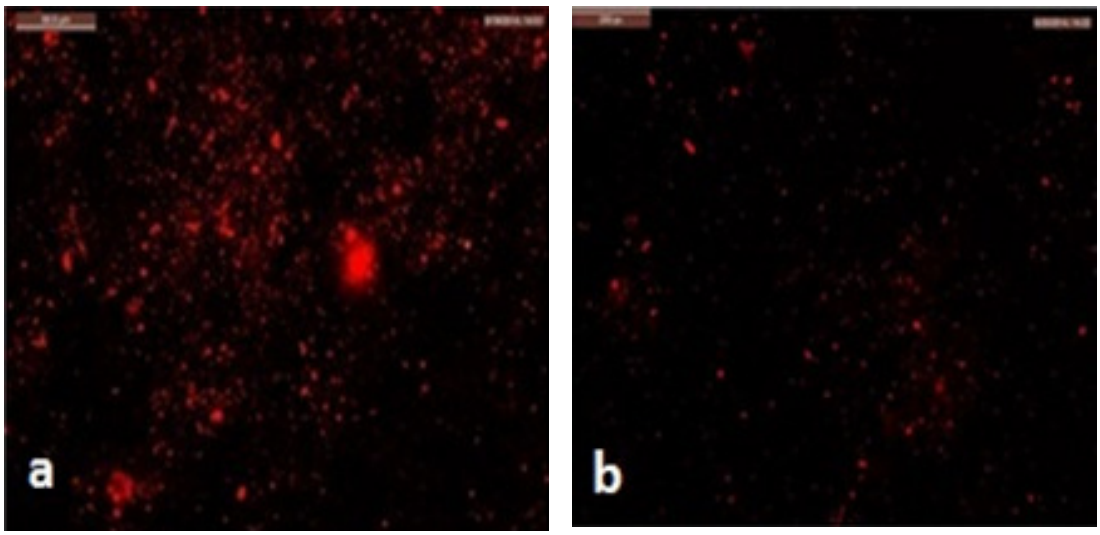

Figure 5.14: a. FISH images of sludge using different probes (40X objective).

a. EUB 338 b. BET 42a

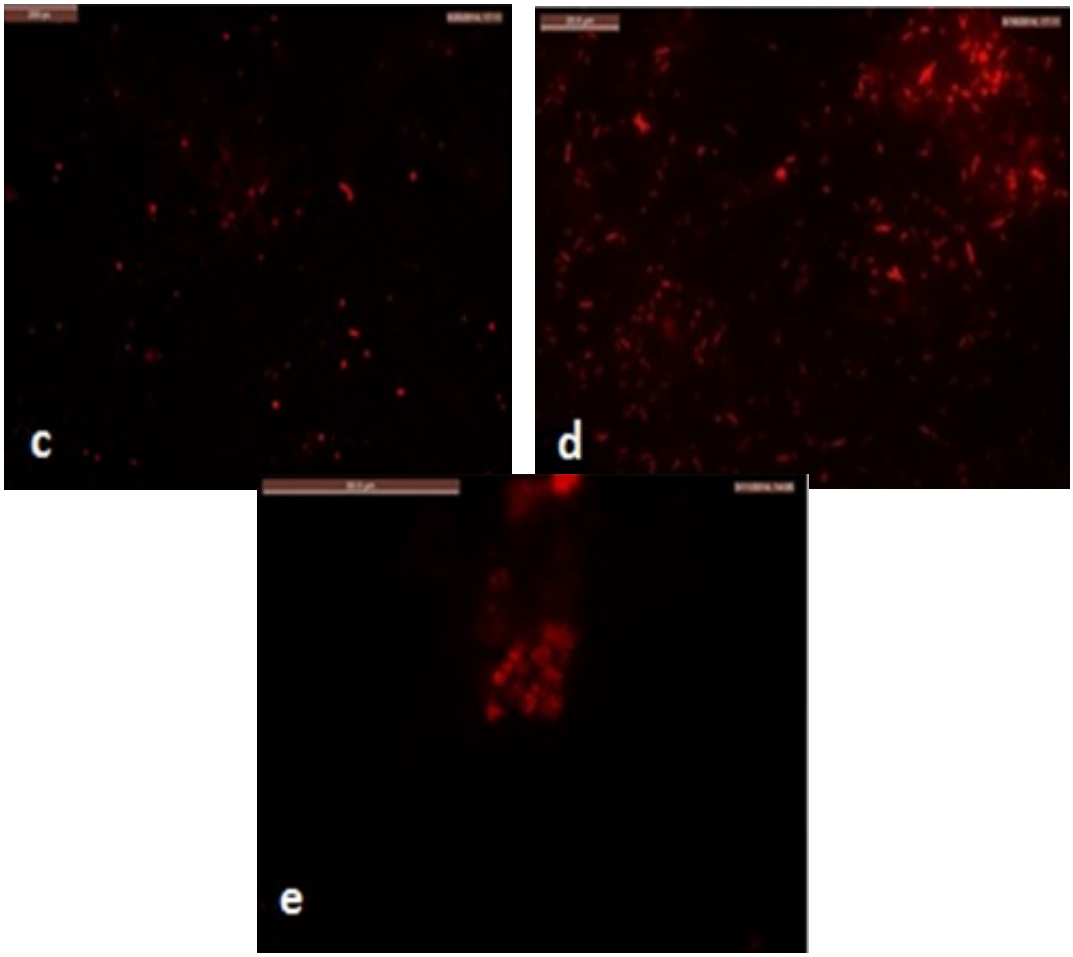

Figure 5.14: b. FISH images of sludge using different probes (40X objective).
c. GAM 42a d. MX 825 e. MS 821

Micro-organisms with the ability to degrade the components of lignocellulosic materials are found among a wide range of taxonomic groups. The conversion of lignocellulose to methane is mediated by four microbial populations, including cellulolytic microbes, noncellulolytic saccharolytic microbes, syntrophic hydrogen-producing bacteria and methanogenic *Archaea* (Chynoweth and Pullammanappallil, 1996). FISH studies on community changes during start up in methanogenic bioreactors done by Calli et al., (2005). They found that *Methanosaeta*like organisms in filamentous forms as the prevailing organisms on the startup

period but on exposure to elevated levels of free ammonia nitrogen, *Methanosarcina* species were the most dominant species. *Methanosarcina* is highly resistant due to their high volume to surface ratio and formation of big clusters.Duringmeta analysis of microbial diversity observed in anaerobic digesters, it was found that the obligate acetoclastic*Methanosaeta* was the most predominant archael genus with 55% prominence and *Methanosarcina* only showed 5% presence among the total genus found (Nelson et al., 2011). In a study on the microbial activity and microbial community structure of full scale anaerobic digesters treating different substrates using FISH probes, the major active population observed was proteobacteria, whereas most abundant archae were hydrogenotrophic methanomicrobales and acetoclastic methanosarcinales (Regueiro et al., 2012).

b. qPCR analysis of methanogenic community

The results of the qPCR of major methanogenic communities prove little variations during the period. The major observation was the absence of *Methanococcales* in the startup period and its presence along with increased biogas production. *Methanosarcinales* were found to be the dominant organism throughout the batch experiment. Though *Methanomicrobiales* are a major group of methanogens found in anaerobic digesters, they were absent in the present reactor.

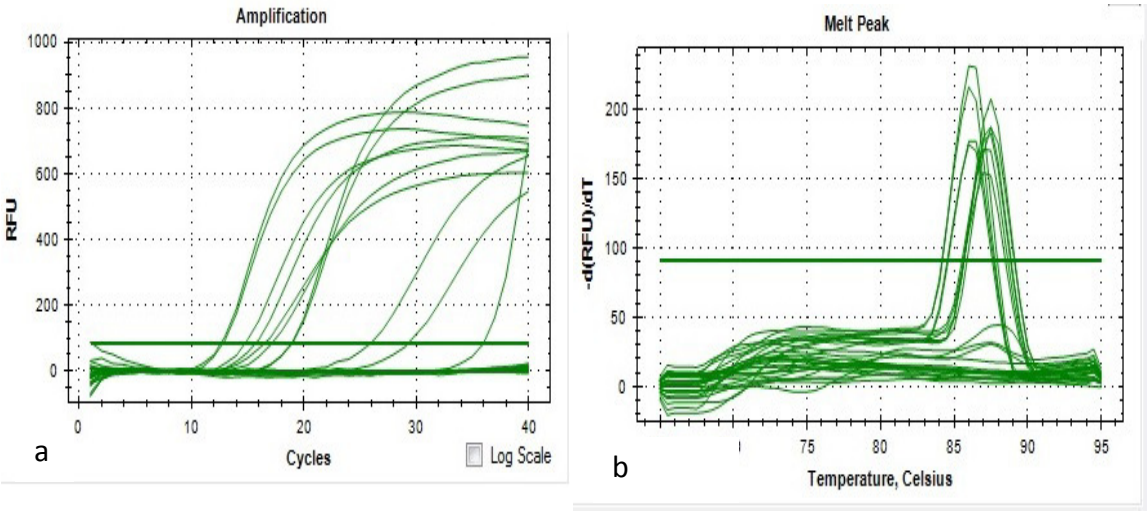

Figure 5.15: a. Amplification curves of the methanogenic communities' b. Melt curve summary of methanogenic amplicons

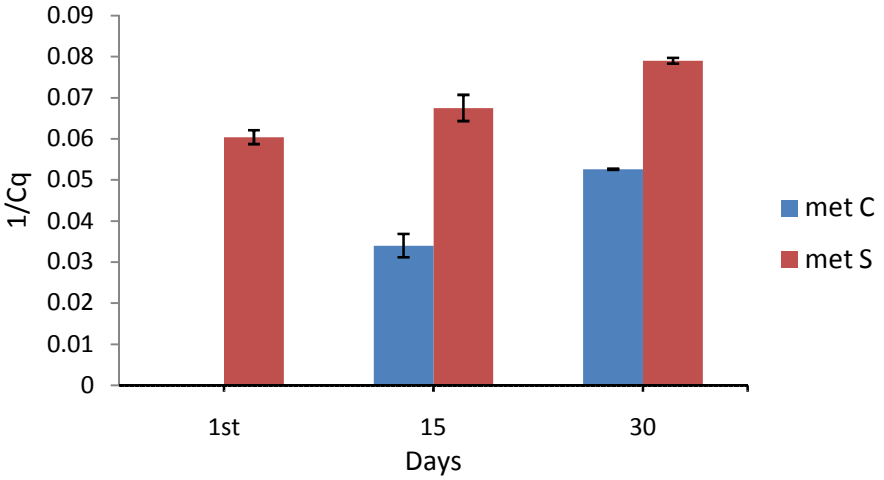

Figure 5.16: Community variations of methanogens in the digester during 30 days

Methanococcales are hydrogenotrophic methanogens which are found in either mesophilic or thermophilic digesters, whereas *Methanomicrobiales* are hydrogenotrophic methanogens found only in mesophilic anaerobic digesters. *Methanosarcinales*can be strict acetoclastic*Methanosaetacea* or hydrogenotrophic*Methanosarcinaceae* found in mesophilic or thermophilic digeters. The population diversity (or qualitative community structure) of

methanogens is known to be less variant compared withhydrolytic bacteria in steady state anaerobic digesters (Zumstein et al., 2000; Akarsubasi et al., 2005).In the other hand, as expected, the quantitative structures of methanogenic communities changed over treatment time in all trials. Corresponding to this, the variations in relative abundances of methanogenic populations were observed. In an anaerobic digester fed with microcrystalline cellulose, assessments of methanogens revealed the dominance of *Methanosarcinales* and absence of *Methanomicrobiales,* which was similar to our observation (Bartell et al., 2015). It was explained that the nutrients and conditions within the tested digester are more suited to the metabolism of *Methanosarcinales* spp. than *Methanomicrobiales* spp.

5.3.7. Strategies to improve biogas yield for WH biomass anaerobic digestion

The biogas yield from WH biomass is lower due to its lower solid content (~5% VS). It limits its usage as a feed stock for producing biogas in field units. The lower economic feasibility of the large scale digestion can be overcome by different strategies like

1) Increasing solid content
2) Preservation by Ensilation to ensure continuous availability of the biomass and
3) Co-digestion where the energy value of the substrate is improved by other commonly available waste substrates like food waste or waste activated sludge.

a. Increasing WH biomass solid content to improve biogas yield

Sun drying as a simple dehydration approach practised in this study. One hour of exposure to active sunlight increased the TS and VS content from 6% to 10 % and 4.6 % to ~ 9% respectively (Figure 5.10.). Drying for 24 hr increased the solid content to 80%, but solid content remained same beyond 24 hr drying up to one week. This indicates 80% could be the maximum solid content achieved by dehydration. Dehydration of WH is a prominent pre treatment method for fibre extraction for paper and board making (Punitha et al., 2015). A study conducted on sun drying yielded a relationship between moisture content and drying time that enabled the prediction of drying rates for the range of drying temperature used

(Innocent et al., 2008). The TS content of solid waste influences anaerobic digestion performance, especially biogas and methane production efficiency (Pavan et al., 2000).

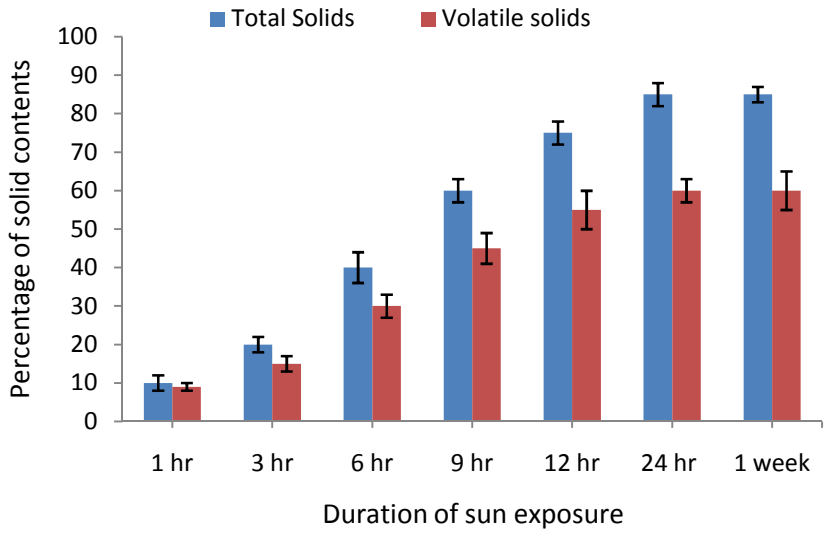

Figure 5.17: Profile of solid content of biomass with wilting duration

In the present study the gas production was found to increase with increase in total solid content from 140 ml/gm of VS to 160 ml/gm of VS till the solid content reached 40 % which was 14 % increase in gas production. After that, the gas production started decreasing and reached the level of 66 ml/ gm of VS. This variation could be due to disrupted C/N ratio and VFA content after which VFA buffering will be interrupted. Excess VFAs may inhibit the methanogenesis reaction. Another reason could be lower moisture content of the biomass which could lead to decreased bacterial degradability and hence lower gas production (Yong et al., 2015).

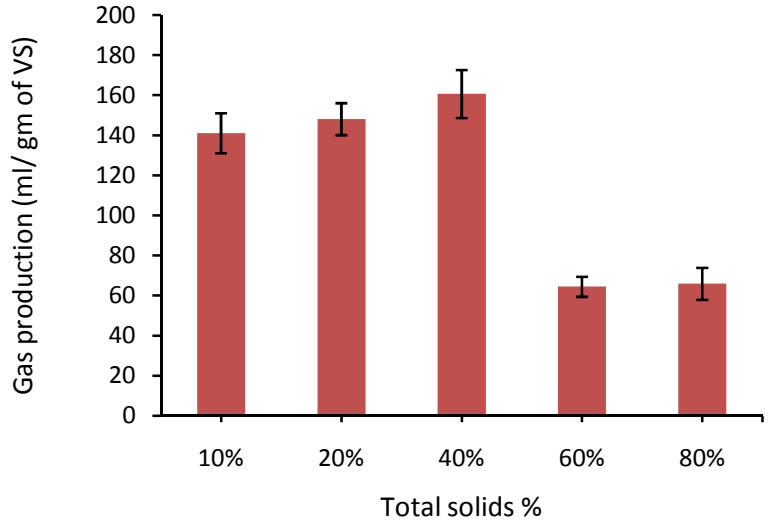

Figure 5.18: Biogas yield from differentially wilted biomass

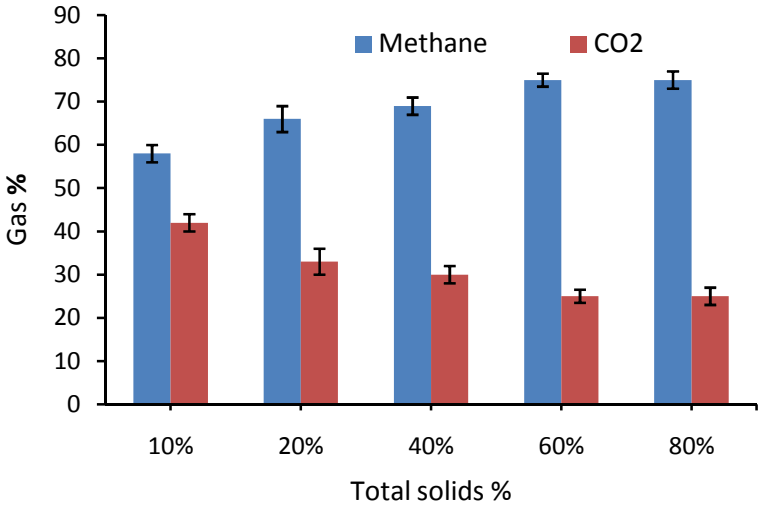

Figure 5.19: Percentage of Methane and CO_2 in the biogas

The methane content in biogas from wilted WH biomass was 70% to 75% at 40% dry matter content and there was not much increase after that (Figure 5.19).From these results, it can be concluded that the optimum dry matter content of WH biomass for anaerobic digestion

should be kept in the range of 20 - 40% and it can be achieved by 4 - 6 hrs of active sunlight exposure.

b. Ensilation of WH and its biomethanation

Ensilation is the process of fermenting high moisture crops under anaerobic conditions where the fermented biomass is called silage and the storage structure is called silo. This is mainly practiced for feeding purpose for ruminants like cattle , sheep or buffalo where fermentation of microbes produces acids which prevents further spoilage of the feed like hay, rye grass etc. Harvested grass is chopped into even smaller pieces and then compacted to get out as much oxygen as possible because the presence of oxygen accelerates the growth of decaying bacteria which will reduces the quality of the silage. The ensilation of WH biomass and its subsequent biomethanation was not reported so far. The amounts of TS or dry matter (DM) and VS are often used to characterize the ensiled material and to assess methane content in the biogas. The drying of WH under sunlight for 4 hrs increased the TS content to 30 - 40 % which was found ideal for biomethanation from the present study. It was found that there was approximately 10% loss in TS for three months old silage and 19% loss for six months old silage which reflected in the biogas production. Compared with wilted sample (160 ml/g VS), the three months and six months old silage yielded 3% and 20% less biogas than the fresh biomass which is 154±5 ml/ gm of VS and 125±6 ml/ gm of VS respectively (Figure 5.21).

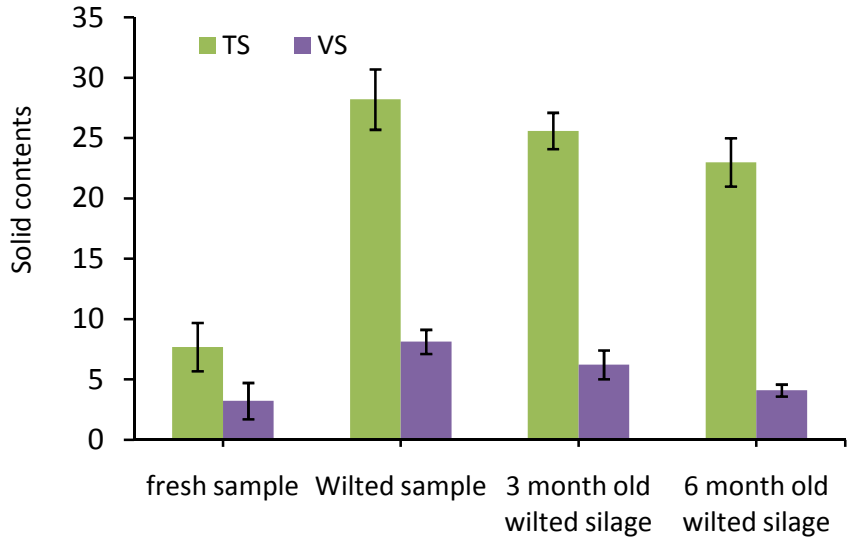

Figure 5.20: Profile of solid contents in fresh sample, wilted sample and silages.

The methane content from wilted sample and silages ranged in 65% to 73% and was comparable with the biogas from fresh biomass. The pH of the silage ranged from 3.8 to 4.5 which is optimal for the silages since acidic condition reduces further bacterial action on it (Idler et al., 2007). A weight loss between the wilted sample and silage of maize and sugar beet was observed in a previous study (Kreuger et al., 2011). A VS loss of 18% to 35% was also reported in silage preparation of wheat and barley (Pakarinen et al., 2008). The reasons for decreased biogas in six-month silage could be because of lower volatile solid content compared to 3 month old silage (Figure 5.20).

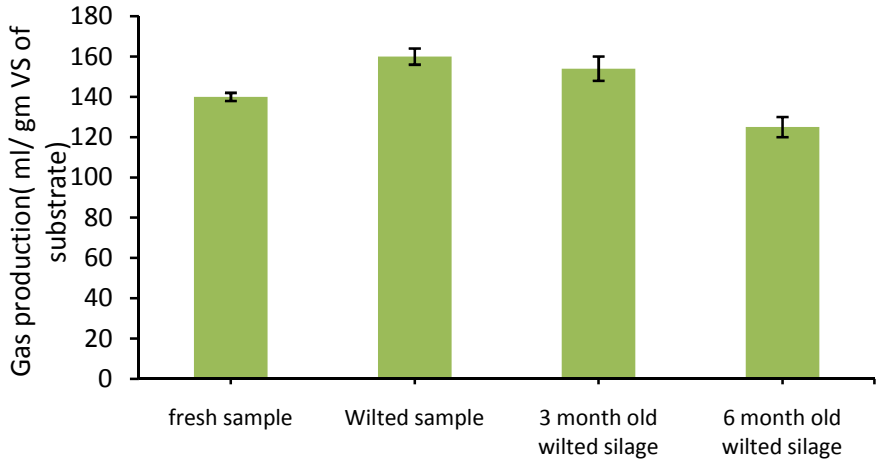

Figure 5.21: Comparison of biogas yield from fresh sample, wilted sample and silages

HPLC analysis of VFA revealed citric acid, lactic acid and acetic acid as major acids formed and the prominent sugars were Glucose and Fructose (Fig 5.22). The VFA profile was comparable between fresh biomass, wilted biomass, and silages. The citric acid which is a fermentation inhibitor was found to be higher in 6-month-old silage which is due to the fungal growth of the silage. The VFA profile between chopped plant and whole plant were similar which indicates that mechanical alteration of material has no effect on preservation.

Analysis of different silages showed homofermentative process leads mostly to lactic acid and ethanol whereas heterofermentative process shows lactic acid, acetic acid and ethanol; whereas whole plant silage leaves a large amount of free sugars (Herrmann et al., 2011). Different studies have shown that an organic acid is one of the most effective silage additives for preventing mould growth (Filya et al., 2004; Koc et al., 2009). The studies by Danner et al. (2002) showed that acetic acid as the sole organic acid responsible for increased aerobic stability by inhibiting the growth of spoilage organisms and also reported butyric acid showing similar activity. Therefore lactic acids and acetic acids are used as silage additives for improving silage nutritional quality (Herrmann et al., 2011). Lactic, acetic and formic

acids have been shown to inhibit the in vitro growth of enterobacteria and *Listeria monocytogenes* (Ostling and Lindgren, 1993) whereas Formic acid increases the initial rate of decline of enterobacteria in grass silage and is effective in reducing *E. coli* O157:H7 (Byrne et al.,2002).

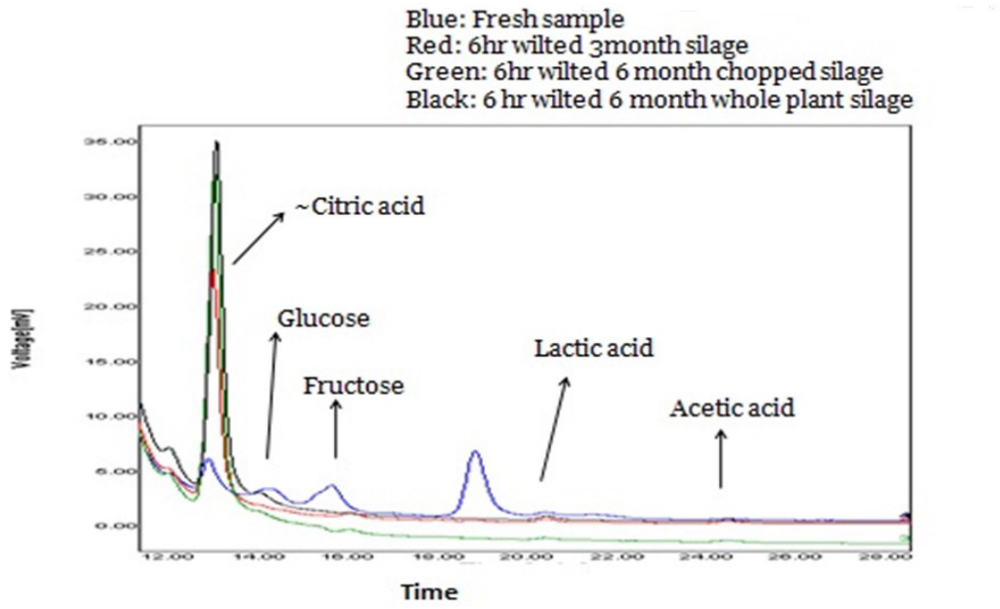

Figure 5.22: HPLC profile sugars and acids of silage and fresh biomass

Acids, alcohols, Ketones, Esters and aldehydes are the major volatile organic components found in the silages. Among the different groups, acids are the most stable form and its volatile loss is highly unlikely from the silage. Production of different VOCs is determined by possibly climate, management, or crop characteristics. VOC pattern and emission helps in determining the effective silage management plans (Hafner et al., 2013).

c. Co-digestion of WH with WAS and food waste

Co-digestion of WH was done in previous studies also and the biogas yield varied depends on the nature of co-substrate used. In the present study WAS and food waste were selected due to their availability in bulk as well as both have a management problem. There were no previous reports on WAS or food waste co-digested with WH biomass. The pH of the

substrates was found to be in the range of 6.2 to 6.4. The results of co-digestion studies are summarized in Table 3. When WH biomass was digested with WAS, the biogas yield increased to only 4.2%. On the other hand, around 63% increases in biogas volume (394.6±12 ml/g VS) was observed when WH co-digested with food waste, which could be due to the higher solid content (VS) in it. Most of the co-digestion studies of WH was done with animal waste (cow dung, poultry litre etc.), and the biogas yielded varied from 147.7 to 390 ml/g VS (Chanakya, 1992; Kivaisi et al., 1997; O' Sullivan et al., 2010; Patil et al., 2011; Hernández-Sheketet al., 2016; Barua and Kalamdhad, 2017). To improve the energy content of the*Pistia* sp., a co digestion study was conducted by Zennaki et al., (1998) using cow dung which yielded 612 ml/ gm VS. Different pre-treatments such as sun drying followed by powdering, shredding, macerating, chopping, and drying, etc. were followed in these reports. Other than animal wastes, co-digestion with fresh vegetables was also reported and the biogas yield was 230 ml/g VS (Hernández-Shek., 2016). Therefore, it can be concluded that the lower biogas yield during anaerobic digestion of water hyacinth alone is due to low solid content, and it can be overcome by co-digesting it with a suitable organic waste like food waste or waste activated sludge.

Table 5.6: Solid content profile of digestates and biogas yield by co-digestion

Digestates	TS %	VS %	Substrate for co-digestion	Biogas production
Water hyacinth	7.1	5.6	Water hyacinth alone	142.8 ±10 ml/ gm of VS
Waste aerobic sludge	6	4.3	Water hyacinth and municipal sewage sludge	148 ± 5 ml/ gm of VS
Food waste	25	22	Water hyacinth and food waste	394.6 ± 12 ml/gm of VS

5.3.8. Techno economic feasibility of biomethanation of water hyacinth biomass

For the techno-economic feasibility of the biomethanation of WH biomass, setting up of a unit for treating 100 kg/day was considered.

1. Calculation of Biogas and manure yield from 100 kg WH biomass:

COD of the water hyacinth biomass	=	16.7 gm/kg
Theoretical biogas yield/ gm of COD is 700 ml (with 50% of methane).		
Biogas yield per kg of water hyacinth is 11.7 Litre,		
Therefore, from 100 kg WH biomass, the biogas yield	=	1170 litres.
LPG equivalence for 1.17 m^3 of the biogas	=	0.52 kg/day
	=	Rs 16200 per year (Cost of LPG is calculated as Rs 85/kg of LPG).
Organic manure recovery will be	=	~ 1.8 ton/year
	=	Rs. 18000 (@ Rs 10/kg)
Therefore, total profit	=	Rs 34,200/ year

2. Estimated cost of CSIR-NIIST model biogas plant treating 100 kg is Rs 3 Lakhs

3. Considering the process unit cost (for 100 kg) and the recovery in terms of biogas and manure, ROI is expected to be ~ 8.77 years.

   It should be noted that the cost for WH biomass recovery from the lake is not accounted in this calculation.

5.4. Conclusions

Biomethanation of invasive macrophytes like *Pistia* and *Eichhornia* can generate biogas. The practical approaches tested in this study such as increasing the WH biomass solid content through sun drying, preservation of WH biomass by ensilation to ensure continuous WH biomass availability for anaerobic digestion and co-digestion with waste organic matter proved to be effective for improving the net biogas yield as well as methane content, making the biomethanation of WH more feasible. Programmed harvesting of WH biomass followed by postharvest treatment of the biomass as proposed in this study will be a sustainable solution to address the eutrophication of surface water bodies.Evaluation of its techno economic feasibility on a scale of 100 kg per day also showed a return of investment in 9 years. The microbial ecology study of the anaerobic digester revealed the dominance of *Methanosarcinales* as the major group of organisms which comprises the acetatoclastic and hydrogenotrophic methanogens *Methanocaeta* and *Methanosarcina* respectively. *Methanococcales*were also present but their dominance was lesser compared to the *Methanosarcinales*. Significant levels of active methanogenic community are required for the reactor performance and culture independent molecular techniques can be used for its monitoring.

Chapter 6

Biomethanation of Water hyacinth biomass:

Community dynamics and ecological niche of protozoa

Priya P (2016) Renewable Energy, 98, 148.

6.1. Introduction

In the present study, as discussed in the previous chapter, the harvested biomass was mechanically pre treated and anaerobically digested using a two stage bioreactor unit for recovering biogas. The process of anaerobic digestion involves a series of metabolically interacting microorganisms converting organic matter into methane, carbon dioxide, and reduced nitrogen and sulfur compounds. The sequential steps involved in the digestion process are hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Among this, hydrolysis is often identified as the rate-limiting step where the complex organic matter is broken down to simpler organics by hydrolytic enzymes (Noike et al., 1985; Adney et al., 1991). Previous studies on the microbial ecology of anaerobic digester focused mainly on the diversity and involvement of Bacteria (Demirel and Scherer, 2008) and Archaea (Nelson et al., 2011; Pycke et al., 2011) in the breakdown of organics and methane production. But, in addition to bacteria and archaea, higher trophic organism such as protozoa and micro-metazoa like rotifers, nematodes, etc. are also regular inhabitants of anaerobic environments including treatment systems (Priya et al., 2007; Ginoris et al., 2007; Bayane and Guiot., 2011, Covarrubias, 2012; Chouari et al., 2017). However, compared to aerobic treatment systems, the higher trophic organisms do not receive much attention in anaerobic treatment systems. Among the higher trophic organisms, protozoa are a major group, but studies on the diversity, population dynamics and ecological niche of protozoa in anaerobic environments confines to rumen (Morgavi et al., 2010) as well as anaerobic natural environments (Hobson and Stewart, 2011). Meanwhile, limited studies have addressed protozoa in anaerobic bioreactors for wastewater treatment (Henze et al., 2001; Lee and Oleszkiewicz, 2003; Hailei et al., 2006; Nimi et al., 2007; Priya et al., 2007; 2008, Spychala et al., 2015).

The present chapter exclusively focus on the diversity, population dynamics and role of protozoa during the anaerobic digestion of pre-treated water hyacinth biomas

6.2. Materials and Methods

The activity of major hydrolytic enzymes and the population dynamics of protozoa during biomethanation of water hyacinth biomass were studied in the lab scale two stage bioreactor unit discussed in Chapter 5.

6.2.1. Analysis of different parameters in the digester

The activity of hydrolytic enzymes such as cellulase, xylanase, pectinase and amylase involved in the anaerobic digestion of lignocellulosic biomass was monitored. Quantitative analysis of these enzymes in the UASB and ALBR were done by estimating the amount of reducing sugars released from different substrates like carbohydrates and polysaccharides using DNS assay (Marsden et al., 1982) as described in Chapter 5.

Representative sludge sample from both UASB and ALBR were periodically withdrawn, and filtered through a 0.22 μm filter. One ml of clear filtrate was used for the enzyme activity. To localize the hydrolytic enzymes activity in the reactor, the activities were analyzed separately in bulk liquid (in suspension) as well as bound to flocks. After uniform mixing, approximately 100 ml sludge was withdrawn from the reactor. To estimate cell-free enzymes, 10 ml sludge was centrifuged, and the supernatant was used for enzyme assay. For estimating flock bound enzymes, 10 ml sludge was centrifuged, and the pellet was resuspended in 10 ml phosphate buffered saline (pH-7.0). The suspension was sonicated (Ultra-Turrax T25) for one minute, centrifuged again and the supernatant obtained was used for the enzyme assay. 3 ml reagent was added to one ml each of sample and distilled water in a test tube and placed in a boiling water bath for 15 min and cooled before analysis. The absorbance was then measured at 575 nm. From the standard curve for cellulase, xylanase, pectinase and amylase, the concentrations were calculated (Saqib and Whitney, 2011).

The volatile fatty acids measurement in the digestion process was calculated using titrimetric method (Sun et al., 2017). The volume of titrant (0.02 N H_2SO_4) consumed by 50 ml of sample can be calculated from the deference of the distribution at pH 5 and pH 4.4 for the components involved in titration. The biogas production from the digester was measured using gas flow meter (Hi Tech, India) throughout the 10 days of digestion period.

6.2.2. Population dynamics of protozoa in the digester

The population diversity and dynamics of protozoa in the ALBR was followed through periodic analysis of sludge samples during one digestion cycle that was completed in 10 days. In this study, both total and individual protozoa counts were correlated with various digester parameters like hydrolytic enzyme activities, volatile fatty acids and methanogenesis. The identification of different protozoa present in the sludge was done through direct microscopic observation (Leica DM 2500) under live, fixed and stained conditions according to the schemes summarized by Patterson (1995) and Foissner and Berger (1996). For detailed morphological observation, the protozoa motility was arrested by fixing in Schaudinn's fixative (A mixture of $HgCl_2$ saturated in 0.9% saline, 60 ml; ethanol, 30 ml and acetic acid, 10 ml) (Martindale et al., 1982). The staining was done with 1% Lugol's iodine to identify ciliates and flagellates (Patterson, 1995).

The protozoa number was assessed through manual counting on a Neubaur counting slide. The reactor sample was fixed using Schaudinn's fixative. One ml of fixed reactor sample, diluted four times with distilled water and counting under the low power (10X & 20X) objectives of the microscope (Leica DM 2500) under phase contrast mode. 10 µl of the sample was taken for counting. The counting was repeated three times and the average number was accounted with variations. The number of protozoa was expressed as number/ml.

6.2.3. Statistical analysis of the data

The relation between protozoa dynamics and enzyme activity was assessed by regression analysis using MS Excel accounting total and individual protozoa count; different enzyme activity and concentration of VFA build up in the digester as well as biogas produced in the reactor. The analysis was repeated for three different batch cycles and the average values were accounted. The counting of protozoa and analysis of other parameters were done on the same day to establish the relationship between them.

6.3. Results and Discussion

6.3.1. Analysis of hydrolytic enzyme activity in the digester

Cellulase, xylanase, pectinase and amylase were the major hydrolytic enzymes monitored in both reactors units (ALBR and UASB) in this study. The activity of different enzyme in both reactor units is presented in Table 6. 1. Between the two reactors, cellulase, xylanase and pectinase activity in ALBR was slightly high compared to UASB whereas pectinase was found to be same in both the reactors. A probable reason for this could be WH biomass was directly loaded to ALBR where the initial digestion takes place and hence more enzyme activity takes place. The soluble organics released here was transferred (pumped) to the UASB unit for its subsequent biomethanation. The continuous circulation of liquid between ALBR and UASB provided mixing up of enzymes in both systems keeping the enzyme activity more or less uniform in both the systems.

Table 6.1: Average activities of hydrolytic enzymes in ALBR and UASB reactors during a typical batch digestion of water hyacinth biomass.

Source	Cellulase (U/ml)	Xylanase (U/ml)	Pectinase (U/ml)	Amylase (U/ml)
ALBR	26±6	21±10	25.4±8	9±2
UASB	24.5±3	20.5±7	19±7	9.6±3

Compared to bulk liquid (cell-free), higher enzyme activity was associated with the sludge flocks (cellulase – 83.7%, xylanase – 77%, pectinase – 78.7% and amylase – 77.6%). As observed in our study, high concentrations of extracellular enzymes immobilized in flocks are reported by few previous studies. For example Higuchi et al., (2005) have indicated that cell-bound alpha-amylase is mainly responsible for the hydrolysis of digested sludge. Similarly studies done by Yu et al. have reported that most of the extracellular enzymes (except α-amylase) were present as bound on pellet and Extracellular Polymeric Substances (EPS) (Yu et al., 2008). According to Frolund et al. (1996) the extracted solution of WAS contains negligible amount of enzymes, representing that nearly all the enzymes are immobilized on sludge flocs. The studies conducted by Ayol et al in 2008 stated that EPS controls the release of extracellular hydrolytic enzymes in activated sludge and increasing degradation of EPS can increase the reactor performance.

6.3.2. Population dynamics of protozoa in the digester

The analysis of reactor sludge sample revealed that ciliates and flagellates were the dominant protozoan present in the reactor. The ciliate community was dominated with *Metopus, Cyclidium* and *Colpoda*. Among flagellates, *Menoidium,*

Rhyncomonas and *Bodo* were the major types. A species level identification of these protozoa was not done at this stage. The population dynamics of different protozoa during a typical batch digestion of Water hyacinth is presented in Figure 6.3. Among the protozoa, the population of flagellate *Menoidium* (99000±200) dominated in the digester sludge, followed by *Cyclidium* (24000±110), *Metopus* (13000±130), *Colpoda* (9000±67), *Rhyncomonas* (5000±40) and *Bodo* (4000±80). Flagellates like *Menoidium* are considered as lower forms of life compared to ciliates and they have more opportunity to grow and multiply within short life span (generation time 6 to 18 hours) and that may be the reason of their dominance in the reactor (Ekelund et al., 2002).

During one typical batch operation completed in 10 days, as digestion progressed the number of many protozoa increased, reaching to a maximum on day 5–6 and then declined gradually till day 10. Only limited studies have reported the diversity and community dynamics of protozoa in anaerobic bioreactor for waste treatment (Priya et al., 2007; 2008). The ciliated protozoa were reported in a combined UASB–activated sludge system in waste water treatment plant in southeastern Brazil like *Aspidisca cicada*, *Vorticella* spp., *Gastronauta aloisi*, *Acineria uncinata*, and *Epistylis plicatilis* complex (Siqueira-Castro et al., 2016). Protozoa are highly sensitive organisms and any fluctuation in the reactor condition like pH, dissolved oxygen, substrate concentration (both dissolved and suspended), etc. can have direct effect on their population dynamics. Such a study was conducted on textile sewage activated sludge system by dos Santos et al. (2014). *Epistylis rotans, Vorticella microstoma, Aspidisca cicada* and *Arcella* sp. were the most frequent protozoa identified in the system.

Protozoa grazing on the WH biomass is shown in Figure 6.1 and images of some of the protozoan found in our biphasic reactor are shown in Figure 6.2. and a brief description about the dominant organisms are described on Table 6.2.

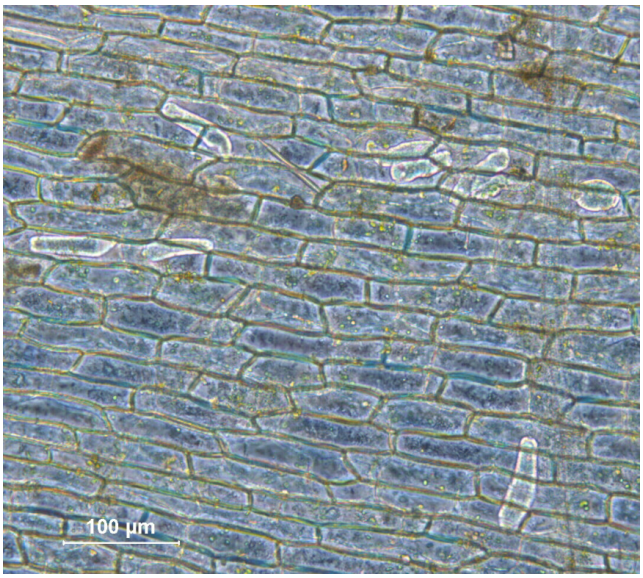

Figure 6.1: Anaerobic protozoa *Metopus* grazing on water hycinth biomass

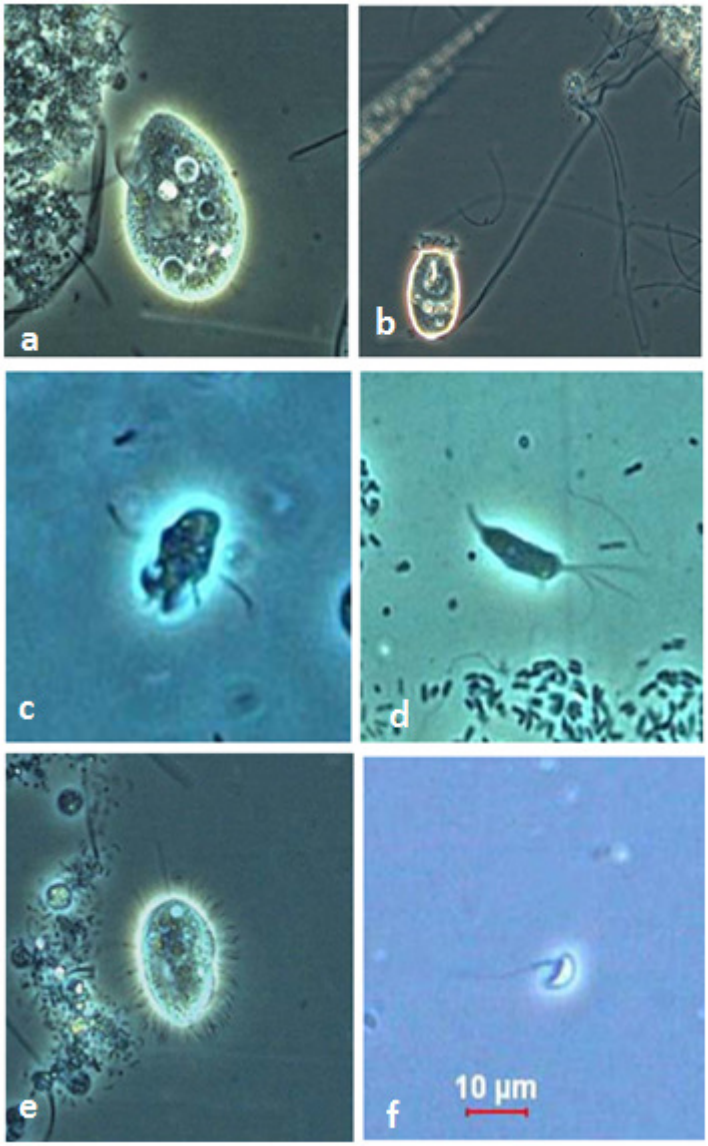

Figure 6.2: Phase contrast images of protozoa observed in the reactor;

a. *Tetrahymna* b. *Vorticella* c *Trepomonas* d. *Cercomonas* e. *Cyclidium* f. *Bodo.*

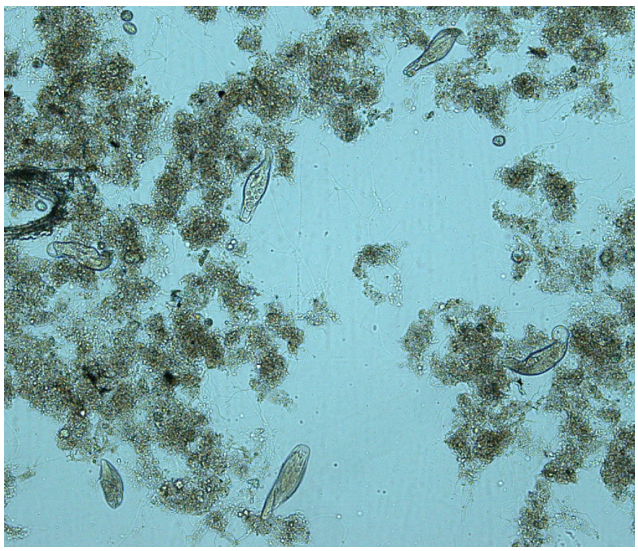

Figure 6.3: Grazing *Metopus* in the digester sludge

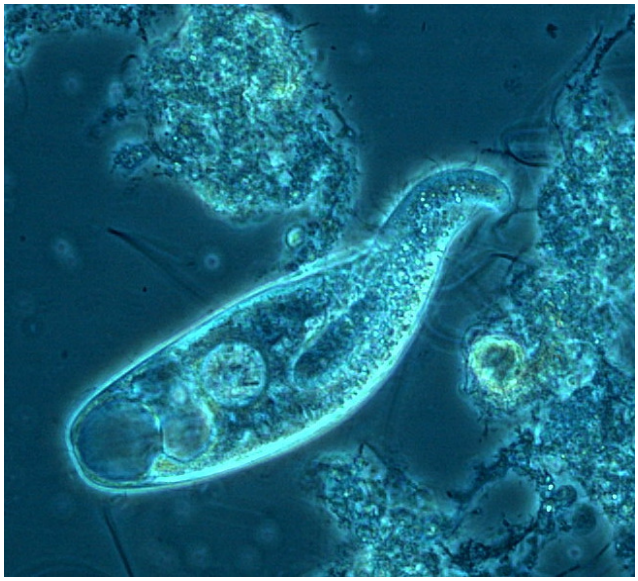

Figure 6.4: Phase contrast image of a single *Metopus* sp.

Table 6.2: Dominant protozoans in the digester

Protozoa	Genus	Description	Mode of nutrition
Ciliates	*Colpoda*	Colpodids ranges 16-30 µm in size and are motile	Suspension feeder using organelles
	Cyclidium	Size ranges from 21-32 µm and can move moderately fast by rotating around body axis	Suspension feeder
	Metopus	Larger ciliates with 80-115 µm in size and is highly motile	Feeding using membranelles
Flagellates	*Menoidium*	Euglenoid flagellate with single emergent flagella with a size of 30-60 µm	Osmotrophic nutrition
	Rhyncomonas	Bodonid with a single trailing flagellum with a size of 5-10 µm	Uses mouth to prise bacteria
	Bodo	Small cells of size range 5-15 µm with two flagella	Bacterivorous and ingest individual attached particles

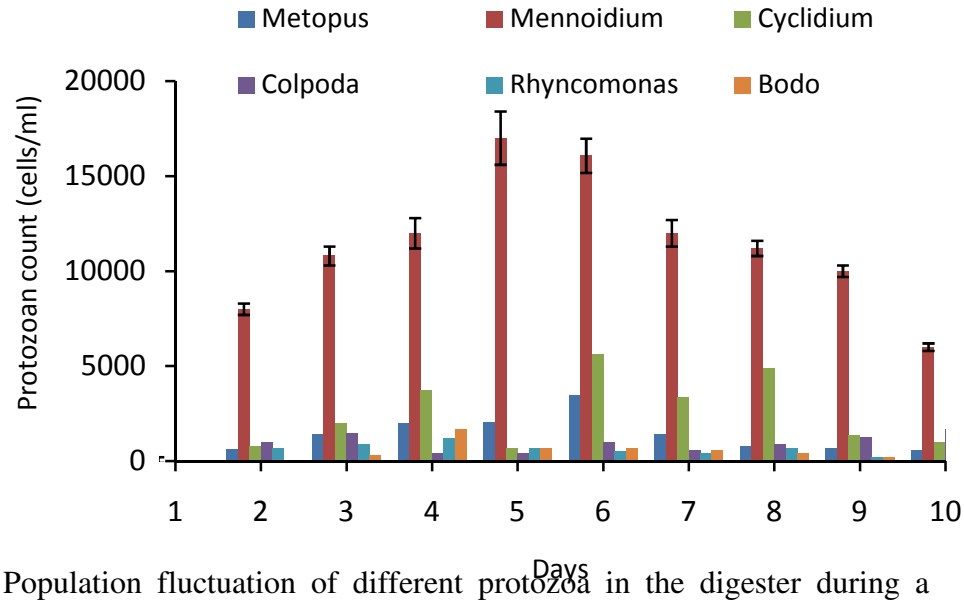

Figure 6.5: Population fluctuation of different protozoa in the digester during a typical batch operation.

a. Influence of protozoa on hydrolytic enzyme activity

When total protozoa count was analyzed with different enzyme activities, only cellulase showed significant positive correlation (R^2 =0.71; P=0.004). Meanwhile, pectinase (R^2 = 0.50; P=0.02), xylanase (R^2 = 0.34; P=0.09) and amylase activity (R^2 =0.53; P=0.02) had only weak correlation with total protozoa count (Figure 6.6.).

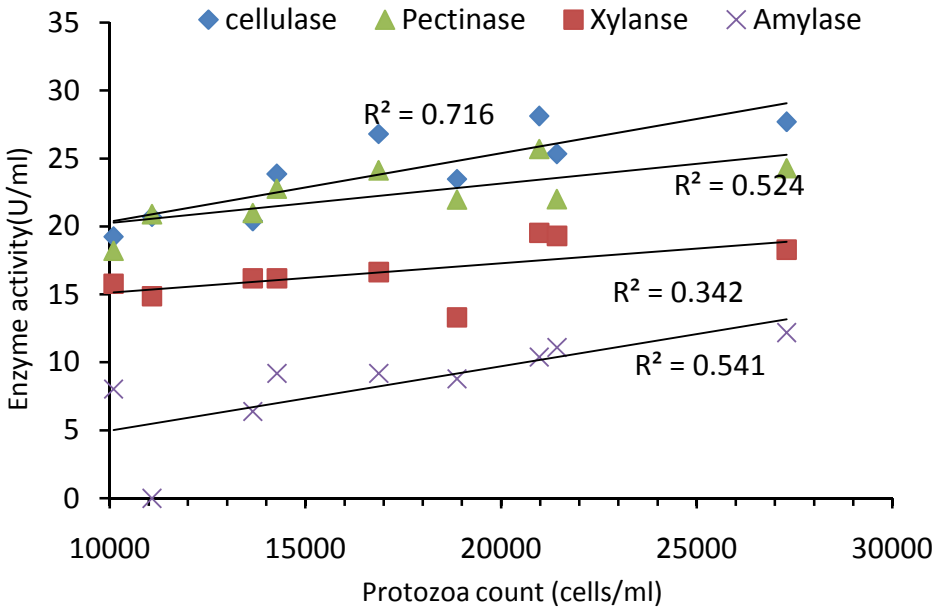

Figure 6.6: Regression plot showing the relationship between total protozoa count in the digester and different hydrolytic enzyme activity.

In anaerobic digesters, protozoa are known to play a major role in flocculation, nutrient mineralization and also as grazers but the full extent of their contribution are not fully quantified. The role of protozoa in hydrolysis of lignocellulosic feeds in the rumen, which is a model anaerobic environment, was studied by Santra and Karim (2002). They found that absence of ciliate protozoa decreased nutrient digestibility and ammonia production along with increased VFA. The major protozoan communities observed in the system were *Isotrichidae* and *Entodinomorphid*. Rumen microorganisms including protozoa contributing for acidogenesis in a two stage bioprocess for cellulose degradation was reported earlier (Bera- Maillet et al., 2005). But the present study discloses further into the population dynamics of protozoa community and their functional importance in the

hydrolysis process by suggesting the relation between hydrolyzing enzymes and population dynamics.

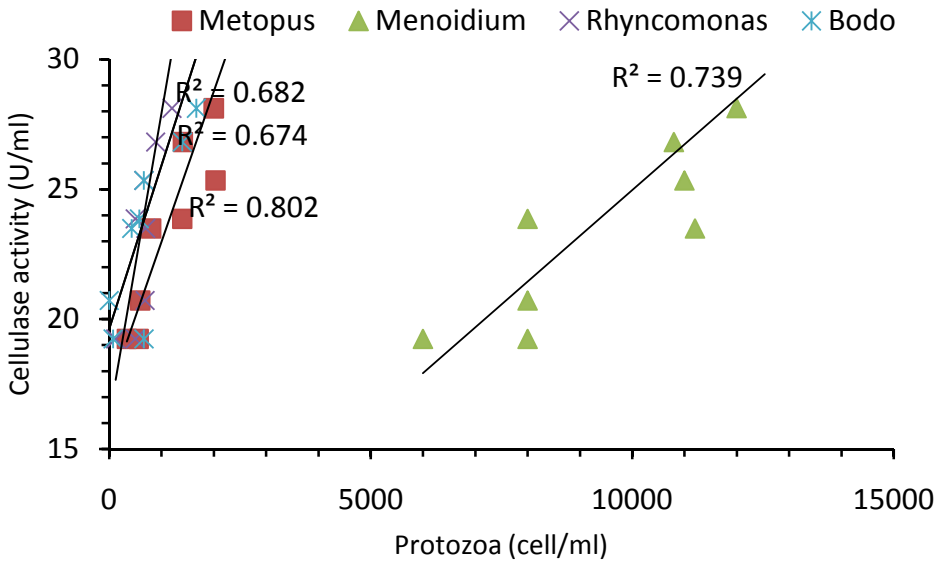

Figure 6.7: Regression plot showing the relationship between cellulase activity in the digester and different protozoa count.

Analysis of individual protozoa count and assessment of different enzyme activity revealed significant relation between many of them in the digester (Figure 6.7). As observed with total protozoa count, all individual protozoa count also had significant positive correlation with cellulase activity, such as *Metopus* (R^2 = 0.80; P = 0.005), *Menoidium* (R^2 = 0.74; P = 0.02), *Rhyncomonas* R^2 = 0.68; P = 0.04) and *Bodo* R^2 = 0.67; P = 0.03). Rumen anaerobic fungi and protozoa were found to be capable of efficient hydrolysis of cellulose in bovine rumens in which *Epidinium* and *Polyplastron* were the major cellulolytic protozoa observed (Dai et al., 2004; Tomme et al., 1995). Santra and Karim (2002) observed that the elimination of rumen protozoa resulted in the reduction in carboxymethyl cellulase activity, which lead to lower cellulose digestibility in defaunated lambs.

The pectinase activity in the digester was found to be influenced mainly by *Menoidium* count (R^2 = 0.73; P = 0.003), followed by the population of *Rhyncomonas* (R^2 = 0.66; P = 0.004) and *Metopus* (R^2 = 0.54; P = 0.02). But, no meaningful effect could be traced between the populations of *Cyclidium*, *Colpoda*, *Bodo* and pectinase activity. Pectolytic enzyme activity was studied extensively in bacteria, fungi, yeast and certain eukaryotes (Arunachalam and Asha, 2010). The occurrence of polygalacturonase activity was observed in rumen ciliates *Eremoplastron* and *Ostracodinium* in an earlier study (Gijzen et al., 1991).

Among the protozoa only *Metopus* count showed significant relation with xylanase activity (R^2 = 0.88; P = 0.0004). Microorganisms such as bacteria, Fungi and yeast are usual candidates capable of producing extracellular xylanase. Xylanase activity was also reported rarely in some of the rumen protozoa, *Polyplastron multivesiculatum, Eudiplodinium maggii,* and *Entodinium* sp (Bera Maillet et al., 2005).

Amylase activity in the digester was found to be positively correlated on *Menoidium* (R^2 = 0.73; P = 0.003) and *Metopus* (R^2 = 0.44; P = 0.05) populations in the digester. Ciliates are already reported to possess significant fibrolytic, amylolytic, and proteolytic activities (Williams and Coleman, 2012), and are believed to contribute to ruminal recycling of microbial Nitrogen (Jouany, 1996). Amylase activity on rumen of cattles was found to be correlated with the dominance of Entodiniomorphid protozoa on different diets (Hristov et al., 2001). Among the two types of ciliates, Holotrichid ciliates are primary users of soluble sugars, while entodiniomorphs use a large variety of substrates. All entodiniomorphid ciliates have high amylase activity to digest engulfed starch granules (Nagaraja, 2016). There are reports that ciliates alter the course of ruminal carbohydrate metabolism by competing with bacteria for excess carbohydrate, by maximising reserving of carbohydrate synthesis and minimizing energy spilling (Teixeira et al., 2017).

Though the mechanism of contribution to amylase production is not known, uptake of iodine stained starch molecules by protozoa from aerobic granular sludge particles was also reported by De Kreuk et al (2010). But in bioreactor studies, amylase activity of protozoa was not yet reported.

b. Involvement of protozoa in VFA generation

By contributing in hydrolytic enzymes activity in the digester, the protozoa community directly involve in VFA production in the digester. This view was supported by the positive correlation observed between VFA concentration and protozoa count in the digester (Figure 6.6). The limited studies on the relation between protozoa dynamics and VFA production were confined to rumen environments, where mainly the ciliates reported to involve in fermentation and VFA production (Wereszka and Michalowski, 2012). In the present digester, the protozoa community was dominated by flagellates. During the digestion process, maximum VFA level coincide with maximum *Menoidium* count in the digester (on day 5). Simultaneously, flagellates can also consume VFA produced that can help to avoid VFA build up and to maintain favorable condition for methanogenesis to proceed. Therefore, flagellates may not be contributing significantly in hydrolytic enzyme production, but can play major role in the digestion by consuming VFA.

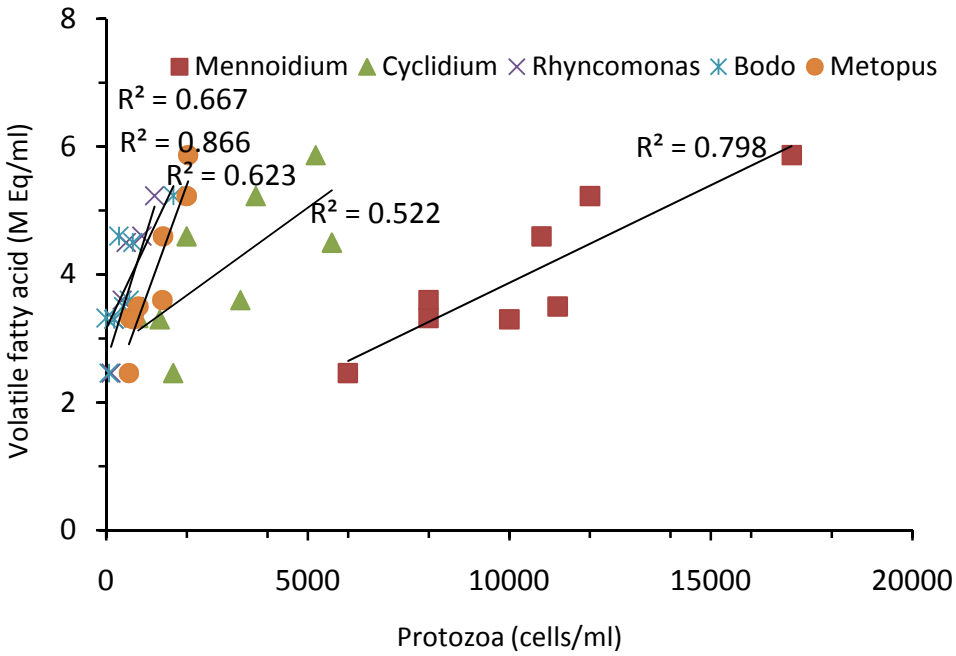

Figure 6.8: Regression plot showing the relationship between volatile fatty acid level and the count of different protozoa in the digester

c. Involvement of protozoa in methanogenesis

The present study also demonstrates the functional importance of different protozoa in biogas production during anaerobic digestion of lignocellulosic matter. A significant level of correlation was observed between biogas produced in the UASB and number of protozoa in the digester (Figure 6.9). The ciliates like *Metopus*, *Cylcidium* and *Colpoda* showed correlation of 0.9, 0.7 and 0.4 respectively with gas production whereas flagellates like *Menoidium*, *Bodo* and *Rhyncomonas* showed 0.7, 0.9 and 0.7 respectively. In our study, flagellates and ciliates were found to be equally important in methanogenesis. In a previous study conducted in our lab, high ciliate count could be correlated with enhanced methane production in an anaerobic CSTR fed with synthetic waste water (Priya et al., 2007). In a study with microbial fuel cell using rumen microflora including protozoa, an increased VFA and biogas was observed (Wang et al., 2012). Nguyen et al., (2016) studied the effects of

presence or absence of rumen protozoa on rumen fermentation characteristics and methane production; they found that the refaunated rumen with protozoa was found to be having higher methane production. In a similar study an interesting observation has been made by Belanche et al (2014), that the inoculation of cattle rumen with holotrich protozoans increased acidogenic bacterial diversity and thereby increased rumen methanogenesis.

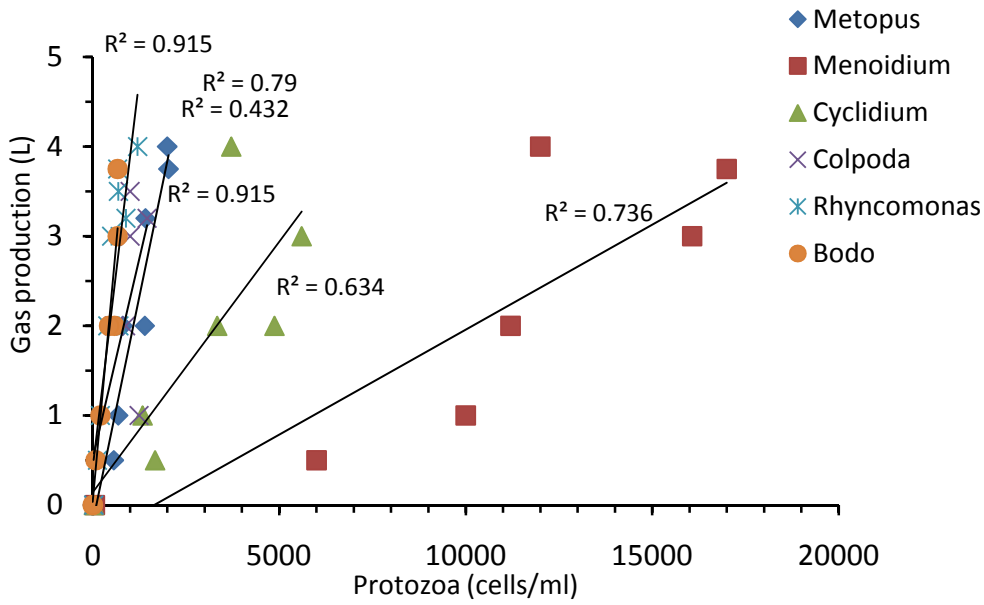

Figure 6.9: Regression plot showing the relationship between biogas produced in the UASB reactor and the count of different protozoa in the digester.

d. Hypothetical role of protozoa in anaerobic digesters

From our observations and previous reports, there could be three possible ways through which protozoa in anaerobic reactor can enhance biogas yield (Figure 6.10). One possibility is the endosymbiotic methanogens in certain protozoa can increase net biogas release. Anaerobic ciliates like *Metopus*, *Plagiopyla*, *Trimyema*, *Caenomorpha, Brachonella* and *Cyclidium* have the ability to harbor endosymbiotic

methanogens (Embley and Finlay, 1993; Fenchel and Finlay, 1991; Finlay et al., 1997; Nimi et al., 2007). A second possibility is that extracellular hydrolytic enzymes from protozoa can enhance the breakdown of complex organics leading to more VFA that can subsequently enhance methanogenesis. Results of the present study also support an increased enzyme activity and VFA level associated with high protozoa count. A third possibility is that large protozoa like *Metopus* can ingest particular organics and hydrolyze them intracellularly. The soluble organics released by the fermentative metabolism of the complex organics can contribute to the pool of substrate such as organic acids, CO_2 and hydrogen for the methanogenesis. In a reactor environment, it will be practically difficult to assess the contribution of protozoa alone in the release of various enzymes among the rich bacterial/archaeal community in the sludge.

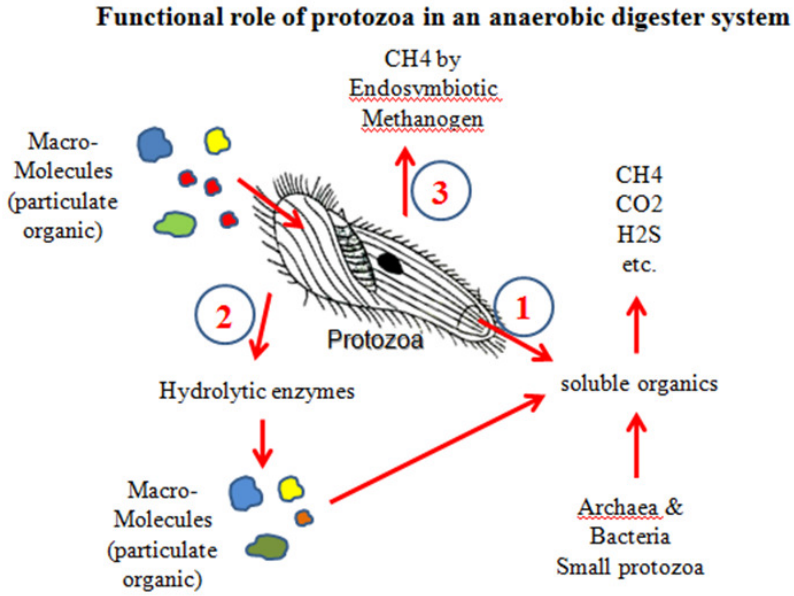

Figure 6.10: A hypothetical scheme of role of protozoa in anaerobic digestion of complex organics.

6.4. Conclusions

In summary, the presence of ciliates such as *Metopus*, *Cyclidium* and *Colpoda* and flagellates such as *Menoidium*, *Rhyncomonas* and *Bodo* in an anaerobic digester for biomethanation of a typical lignocellulosic waste like water hyacinth biomass. In addition to the release of hydrolytic enzymes, both ciliates and flagellates were found to be important for acidogenesis as well as methanogenesis. More specifically the activity of some the enzymes, volatile fatty acid accumulation and biogas production can be well correlated with the population of specific protozoa in the sludge. The information about the role of anaerobic protozoa will help to design and develop anaerobic digesters harboring higher trophic community.

Chapter 7

General Discussion

The present study has demonstrated the potential of invading floating macrophytes like *Eichhornia*, *Pistia*, *Salvinia* etc in nutrient uptake. This potential can be explored further for their practical application to control eutrophication and restore polluted water bodies through nutrient over load. Engineered ecosystems like constructed wetlands including floating wetlands have been reported from different parts of the world for nutrient removal purpose. These wetlands units are planted with selected, locally available plants with nutrient uptake property. Similarly, as proved in the present study, dominant plants like *Eichhornia* and *Pistia* can be used for nutrient removal purpose under controlled conditions. The detailed microbiological analysis of *Eichhornia* and *Pistia* has revealed the functional role of root associated microorganisms in nutrient uptake. This is a new observation especially in the case of *Eichhornia* and *Pistia*, and it has a practical relevance. An enrichment consortium with the isolated P solubilizing and nitrate reducing bacteria can be prepared and the same can be used for bio-augmenting the plants for enhanced nutrient uptake. More research may be needed this area for developing a biological system.

The water quality parameters (especially the organic and nutrient level) of the eutrophic lake covered in this study illustrates its eutrophic status and justifies the luxuriant growth of water hyacinth (WH) in the lake. The quantitative assessment of WH biomass in the lake provides valuable information about a feed stock for valorization. Quantitative information like this is very scare particularly in Indian context, and the practical relevance of such data is that it will help in designing proper technology/process for taping value added products from the same. Even though there are reports on anaerobic digestion of WH biomass, the feasibility is often expressed as doubtful or even not feasible. This is mainly due to the very low solid content of WH biomass (~5%). This aspect was very seriously addressed, and two practical solutions (drying to increase the solid content and co-digestion) were empirically validated in this study. Furthermore, the practical difficulty of handling seasonal bulk availability of the plant was also addressed separately through ensilation. These practical solutions will make biomethanation of WH biomass more feasible. It is estimated that the nearly 2800 tons of WH biomass (wet weight) accumulates during the peak seasons, which is equal

to ~140 ton dry solids (5% solid level), sufficient for recovering biogas and organic manure in a feasible way. The ensilation approach practiced in this study can be adopted for similar organic feed stocks for preservation and biomethanation.

Among the different pretreatment studies tested, mechanical treatment was found effective for more biogas yield. This is more attractive than thermos-chemical (acid/alkali) treatment. In this study, anaerobic digestion/biomethanation is established as a sustainable way of recovering value added products like biogas and organic manure. Moreover, the co-digestion approach validated proved to be very effective for recovering attractive levels of biogas, making the process feasible. This will also solve the problem of managing food wastes as well as waste activated sludge, which are very serious problem especially in urban/ semi-urban areas.

The proposed two stage bioprocess unit in this study has advantage of managing the VFA build up which is a major reason for anaerobic process failure. Moreover, the process can be operated as batch/fed batch or even as continuous feed mode. The selection of UASB has the advantage of low foot print and it can handle high organic levels. An integrated treatment system including pretreatment followed by mixing with suitable co-substrate and preservation through ensilation, followed by anaerobic digestion can generate biogas and manure continuously.

The novel information generated in this study about the microbial ecology of the anaerobic digesters, on the diversity and functional role of protozoa is an area of basic research where limited information is available. This information has a high practical relevance, that new engineered bioreactors can be designed which promotes the proliferation and sustained activity of these organisms that will improve the process efficiency and biogas yield.

In short the entire study brings out the possibility of exploiting the potential of problematic weeds in eutrophic water bodies for its restoration and simultaneously recovering high value products, thus establishing a sustainable way of managing eutrophication.

Chapter 8

Summary and Conclusions

In the present study, common floating macrophyte like *Eichhornia*, *Pistia*, *Salvinia* and *Lemna* found in eutrophicated lakes were screened for their nutrient (N and P) removal property, and found that among the plants *Eichhornia* and *Pistia* were ideal for both N and P removal applications. The nutrient removal was affected by environmental variables including concentration and nature of the nutrient species. Evaluation of rhizospheric microflora indicated the presence of P solubilizing and nitrate reducing bacterial strains, functionally supporting in plant associated nutrient removal.

An identified eutrophic lake was studies for its seasonal water quality (in terms of eutrophication) and an assessment of the accumulation of predominant macrophyte (*Eichhornia*) coverage. The macrophtytic coverage was found to increase three times in pre monsoon season than post monsoon, which can be correlated with increased salinity, temperature and nutrient content on the lake. The maximum quantity of water hyacinth accumulated in the lake was estimated to be round 2800 Tons wet weight/43 hector at peak seasons (December to February, 2015) from the whole lake.

Biomethanation potentials of the commonly occurring macrophytes like *Eichhornia* and *Pistia* were studied and found that *Eicchornia* can be considered as better candidate for biomethanation due to its bulk availability, higher biogas yield and better adaptation to natural conditions. Among the different pretreatment tested, mechanical crushing was found more effective for maximum biogas yield. Mechanically crushed whole water hyacinth plant biomass in a two stage biomethanation system yielded ~ 9 L biogas/kg of wet weight. The characterization of slurry (digestate) proposes its application as organic manure.

To improve the biogas yield from WH biomass, different strategies were tested like increasing the solid content through simple drying and co-digestion with waste residues like food waste and STP secondary sludge. Ensilation was found very effective for preserving WH biomass to address the seasonal bulk availability of the plant. The quality of the silage remains up to 6 months by storing it anaerobically.

Application of molecular techniques like FISH revealed the presence of α, β and γ proteobacteria along with archael population in the WH treating dogester sludge. Moreover qPCR analysis showed that *Methanosarcinales* as the predominant methanogenic archael population in the reactor. Other than the prokaryotic communities, the higher trophic organisms like protozoans were also studied, since it is a least explored area. It was found that the protozoa community in the digester was majorly represented by ciliates (*Metopus, Cyclidium and Colpoda) and flagellates (Rhyncomonas, Menoidium* and *Bodo*). The importance of both ciliates and flagellates in anaerobic digestion process, more specifically, the contribution by individual protozoa in hydrolysis, which is the rate limiting step in anaerobic digestion was elucidated in this study.

In conclusion, the present study brings out the potential of invading macrophytes for nutrient removal, thereby restoring eutrophic water bodies. However, this can be achieved through "programmed harvesting" of selected macrophytes (like Eichhornia). This can be employed through engineered ecosystem, possibly with bioaugmentation for enhanced nutrient/ pollutant removal. This study further demonstrates the application of anaerobic digestion (biomethanation) as a successful approach for recovering biogas and manure from the harvested WH biomass. The practical problem associated with low biogas yield can be addressed through the simple approaches empirically validated in this study. Therefore, programmed harvesting followed by mechanical pre treatment, drying and ensilation can ensure continuous WH biomass supply for field level biomethanation units, making the whole process chain a sustainable way of managing WH biomass and eutrophication.

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Contributions to Academic Conferences (poster presentation)

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***Priya P** and Krishnakumar B (2016). Rhizospheric Bacteria inspired N&P uptake in locally available aquatic macrophytes. Awarded **second best paper** in National Seminar on Biodiversity of microbes and Climate Change Mitigation, Catholicate College, Pathanamthitta.*

***Priya Prabhakaran**., Bhasi, A., Ali, S., Narayanan, N., Balakrishnan, M. V., & Bhaskaran, K. (2015). Community dynamics and significance of anaerobic protozoa during biomethanation of lignocellulosic waste. International Conference on New Horizons in Biotechnology (NHBT 2015), Thiruvananthapuram.*

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List of Publications

***Priya P**., Bhasi, A., Ali, S., Narayanan, N., Balakrishnan, M. V., & Bhaskaran, K. (2016). Community dynamics and significance of anaerobic protozoa during biomethanation of lignocellulosic waste. Renewable Energy, 98, 148-152.*

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