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BIOGAS PRODUCTION FROM MARINE MACROALGAE:
MACRONUTRIENT DEMAND IN EXPERIMENTAL SEAWATER ANAEROBIC BIOREACTORS

By:

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Dedication

This thesis is dedicated to my beloved family who, even from far away, did everything to support my success, especially, my indefatigable mother Clavirose Forestil. To Nathalia Elise Menéndez Abovici who's comradeship has been a veritable source of perseverance.

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Abstract

During the last few decades, the anaerobic digestion (AD) process has become a worldwide topic of interest since it is considered as one of the most suitable and cost-effective technologies to address the problem of wastewater treatment while generating bioenergy. Until today, the use of seawater in the operations of anaerobic digestion processes for biogas production was unfamiliar to the scientific community. In this research, we proposed to investigate the dynamics and performance of two multi-stage bench-scale anaerobic bioreactors (MSBSABs), operated under high salinity conditions (an intermediate salinity of 1.0% w/w as a control system, and a high salinity of 3.5% w/w as an experimental system). Both bioreactors were fed with the marine macroalgae, *Sargassum spp.,* as energy biomass during an 18-week period of operations.

The elemental composition of this energy biomass (dry sargassum) was lower than other fresh marine biomasses used in AD process. This was expected since the sargassum feedstock used in our study was harvested onshore and was already dried by the sun (beach wrack blend). The reduction of the volatile solids (VS) content within the control system was greater in the third chamber S3 (6.30± 1.79 g/100ml) than the VS content of the first chamber S1 (9.03± 2.83 g/100ml). A similar pattern was observed in the experimental system, which VS content was 6.90± 2.39 g/100ml in S3, compared to 10.25± 2.65 g/100ml in S1. A significant reduction of the mass fraction of macronutrients (C, H & N) was observed in both systems from the first chamber S1 to the last chamber S3. Nevertheless, the sulfur fraction of the third chamber S3 was higher in both bioreactors when comparing to that measured in S1.

The biogas production was 30% greater in the control system (1.0% w/w) than the experimental system (3.5% w/w). The biogas yield averaged over time was a normalized rate of 91.05 ml of biogas per gram of VS fed per day in the experimental system, compared to a volumetric production of 132.42 ml of biogas per gram of VS fed per day in the control system. The biogas samples of both bioreactors presented a similar chemical composition to that reported for traditional

freshwater anaerobic biodigesters. However, the biogas samples of the experimental bioreactor were of better quality in terms of methane concentration than the control bioreactor (with a methane percentage around 61.28± 1.70 for the experimental bioreactor, and 53.82± 5.10 for the control bioreactor).

Keywords: *Anaerobic digestion; biogas production; proximate analysis; infrared spectroscopy; Sargassum spp., marine macroalgae*

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List of abbreviations and acronyms

A

AD: Anaerobic Digestion

APHA, AWWA, WEF: American Public Health Association, American Water Works Association, and Water Environment Federation

ASP: Aquatic Species Program

ATP: Adenosine Triphosphate

C

C/N: Carbon/Nitrogen Ratio

COD: Chemical Oxygen Demand

D

DEGI: Office of the Dean of Graduate Studies and Research

DO: Dissolved Oxygen

DOD: United States Department of Defense

DOE: United States Department of Energy

E

EIA: United States Energy Information Administration

EPA: United States Environmental Protection Agency

F

FEP: Fluorinated ethylene propylene

FTIR: Fourier Transform Infrared Spectroscopy

G

GHG: Greenhouse Gasses

GWP: Global Warming Potential

H

HOAce Eq: Acetic Acids Equivalent

HPP: Hydrogen Partial Pressure

HRT: Hydraulic Retention Time

K

kcal: Kilocalorie

kJ: Kilojoule

M

MPB: Methane-Producing Bacteria

MSBSAB: Multi-Stage Bench-Scale Anaerobic Bioreactor

N

NADH: Nicotinamide Adenine Dinucleotide

O

OHPA: Obligate Hydrogen-Producing Acetogens (also called Proton-Reducing Acetogens)

OLR: Organic Loading Rate

P

PBDT: *Programa de Becas para Disertacíon, Tesis o Proyecto Equivalente*

PEAF: Formative Academic Experiences Program

pH: Potential of Hydrogen

ppt: Parts Per Thousand

R

RCERE: Research Center for Excellence in Renewable Energy

RCF: Relative Centrifugal Force

RPM: Rotations Per Minute

S

S1: First chamber of the bioreactor

S3: Third chamber of the bioreactor

SAO: Syntrophic Acetate Oxidation

SCFAs: Short-Chain Fatty Acids

sCOD: Soluble Chemical Oxygen Demand

SJBE: San Juan Bay Estuary

SRB: Sulfur Reducing Bacteria

SRT: Solids Retention Time

STP: Standard Temperature and Pressure

T

TAN: Total Ammonia Nitrogen

tCOD: Total Chemical Oxygen Demand

TDS: Total dissolved Solids

TS: Total Solids

TSS: Total Suspended Solids

U

UNFCCC: United Nations Framework Convention on Climate Change

UPRM: University of Puerto Rico, Mayagüez Campus

UPRRP: University of Puerto Rico, Río Piedras Campus

US: United States

V

VFAs: Volatile Fatty Acids (synonyms for VOAs)

VOAs: Volatile Organic Acids

VS: Volatile Solids

W

WWTPs: Wastewater Treatment Plants

Glossary

Anaerobic Digestion (AD): It is a natural breakdown of organic matter in absence of oxygen throughout a series of biological processes, in which biogas and digestate are resulted as end products.

Biogas: It is a mixture of fuel gas formed by the breakdown of organic matter by anaerobic microorganisms. This mixture is mostly composed of methane (50-75%), carbon dioxide (25-50%) and other trace gasses including hydrogen sulfide, water vapor nitrogen and hydrogen (0-10%).

Biomass: Also mentioned as energy feedstock in this document, biomass is referred to any carbon source available on a renewable basis for energy conversion into electricity or other forms of energy.

Bioreactor: Also called digester; it is a system in which biological conversion is achieved through the activities of enzymes, microorganisms, and animal or plant cells. For the purpose of our research, the term “bioreactor” refers to a controlled anaerobic system in which organic matter (sargassum) is converted into biogas and derived products (effluents) via microbial reactions.

Buffering Capacity: Ability of a solution to resist massive changes in pH. The buffering capacity is expressed as the required molarity of sodium hydroxide (NaOH) to increase the pH of a given sample by 1.0.

Carbon/Nitrogen Ratio (C/N): The carbon/nitrogen ratio is a measure of the relative amount of organic carbon and nitrogen present in the feedstock.

Chemical Oxygen Demand (COD): Measurement of the amount of material that can be oxidized (combined with oxygen) in the presence of a strong chemical oxidizing agent.

Colloid: Any substance consisting of particles substantially larger than atoms or ordinary molecules but too small to be visible to the unaided eye. The size of the colloidal particles is comprised between 1 nm to 0.1 μm.

Control system: Also called *control bioreactor*; in this research, the term "control system" refers to a 15L multi-stage bench-scale anaerobic bioreactor (MSBSAB), working with a salinity of 1.0% w/w sea salts.

Dissolved Oxygen (DO): Dissolved oxygen refers to the level of free, diatomic oxygen (O_2) present in water or other liquids.

Dissolved solids (DS): Particles with size less than 1 nm found in the water column including inorganic and organic materials.

Experimental system: Also called *experimental bioreactor*; in this research, the term "experimental system" refers to a 15L multi-stage bench-scale anaerobic bioreactor (MSBSAB), working with a salinity of 3.5% w/w sea salts.

Greenhouse Gasses (GHG): Chemical compounds found on the Earth's atmosphere that absorb infrared radiation and trap the heat in the atmosphere. Some of them occur naturally: water vapor (H_2O), carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O); while others are exclusively human-made: fluorinated gasses or F-gasses including Chlorofluorocarbons (CFCs), hydrochlorofluorocarbons (HCFCs), hydrofluorocarbons (HFCs), perfluorocarbons (PFCs), and sulfur hexafluoride (SF_6).

Hydraulic Retention Time (HRT): Sometimes referred to Residence Time; it is defined as the time necessary to pass one reactor's volume worth of liquid through the bioreactor at a given flow rate. It is a key parameter used in anaerobic systems to evaluate the average length of time that a soluble component is retained in a digester, in contact with bacterial mass.

Organic Loading Rate (OLR): It refers to the amount of organic dry solids loaded per m^3 of digester volume per unit of time. In the present study, the organic loading rate is expressed as kg/m^3*d or equivalent g/L*d.

Potential of Hydrogen (pH): The pH is the measure of the acidity or alkalinity of a solution. Aqueous solutions at 25 °C with a pH less than seven are considered acidic, while those with a pH greater than seven are considered basic or alkaline, and those with a pH equals to 7 are considered neutral.

Relative Centrifugal Force (RCF): Also known as G-Force, it is a measurement of the acceleration that indirectly causes weight. It refers to the force generated by various centrifuges on the basis of the speeds rotation and distances from the rotation center.

Short-Chain Fatty Acids: (View Volatile Fatty Acids).

Solids Retention Time (SRT): Parameter used in the design of water and wastewater treatment plants, relating to the growth rate of microorganisms and the effluent concentrations. The retention time of the solids is defined as the average length of time that a unit mass of suspended solids is resident in the bioreactor. It is a function of the hydraulic retention time as well as recirculation rate of solids and liquids within the bioreactor. The SRT as well as the HRT may be adjusted by the system operator to maintain a satisfactory rate of biodegradation.

Total Solids (TS): Measure of the combined suspended, colloidal and dissolved solids of all inorganic and organic substances contained in water sample. This refers to the material residue left in the vessel after evaporation of a sample and its subsequent drying in an oven at 103 or 105 °C. The TS can be further broken down into total suspended solids and total dissolved solids.

Total Suspended Solids (TSS): Particles that are larger than 0.1 μm found in the water column including inorganic and organic materials.

Volatile Fatty Acids (VFAs): Also known as short-chain fatty acids, they are low-molecular mass carboxylic acids with an aliphatic tail of less than six carbon atoms.

Volatile Solids (VS): An estimate of the organic fraction of the total solids of a given biomass or energy feedstock. In this study, the volatile solids content is performed at 550 °C.

PART I: RESEARCH BACKGROUND, STATEMENT AND PURPOSE

1.1. Introduction

One of the major challenges facing industrialized countries is securing sustainable energy supplies for the future. During the last few decades, global energy demand has been rapidly increasing while new discoveries of fossil fuel reserves are decreasing, which causes a long-term trend towards energy price growth. Meanwhile, fossil fuel consumption increases the amount of greenhouse gasses (GHG) in the atmosphere. These specific gasses represent the major driver of climate change (Mitchell, 1989). Global energy demand is closely linked to the world population growth. Just 200 years ago the global population was less than 1 billion while in 2014 this number passed 7 billion and continues to grow up (Roser, 2015). Consequently, people put more pressure on natural resources in order to satisfy their daily needs for food, energy, water and other material goods. By 2030, global energy demand is projected to be ineluctably higher considering the rapid economic growth of the marketplaces in such countries like China and India, where industrial manufacturing is in a constant expansion (EIA, 2014). Notwithstanding that rapid economic development will continue to provide indisputably tangible life quality improvement and, of course, increase the energy demand around the world, some collective and individual actions are required in order to not only curb runaway the global energy demand but also reduce the global dependency on fossil fuel consumption. Therefore, environmentally friendly efforts such as using collective transport, reducing the global carbon footprint, switching to sustainable energy production become crucial around the world.

A very promising candidate for industrial scale production of sustainable renewable energy is biogas, produced from biomass resources. Biogas can be generated from a large variety of organic raw materials, and can be applied to various energy services such as electricity, process heat, mechanical power or vehicle fuel (Comparetti et al., 2013; Weiland, 2010). Biogas is an attractive

alternative energy supply to address the problem of switching agricultural land from food to fuel production. Biogas production derived from biomass as energy feedstock offers a large number of advantages: (i) because of economic pressures, many farmers have been forced to find alternative income sources, biogas production is subsidized in many countries giving them an additional income (Deublein & Steinhauser, 2008); (ii) production of biogas yields both energy and fertilizer, thereby reducing the need to buy mineral fertilizers; (iii) reduction of disposal costs of organic wastes; (iv) biogas production reduces gaseous emissions by preventing methane released in the atmosphere. According to Kelly & Dworjanyn (2008) the global warming potential (GWP) of methane is 25 times stronger than carbon dioxide as greenhouse gas, which means that methane will cause 25 times as much warming as an equivalent mass of carbon dioxide over a 100-year time period (Dijkstra et al., 2012; Forster et al., 2007). In addition, the biogas production process technology supports the climate protection goals that were agreed upon the negotiations for the Kyoto Protocol adopted in 1997 by the United Nations Framework Convention on Climate Change (UNFCCC), and entered into force in 2005.

In this study, we proposed to evaluate the anaerobic degradation of the marine macroalgae, *Sargassum spp.,* and its conversion to biogas. The macroalgal biofuel and biogas production project at the UPRRP Research Center for Excellence in Renewable Energy (RCERE) is providing the research, development and demonstration of macroalgal biofuels, and co-products (fertilizers), as well as cost-effective reduction of carbon dioxide emissions. Biogas production based on marine macroalgae as energy feedstock could provide an important source of renewable energy for countries with suitable coastline availability like Puerto Rico. Biofuels based on anaerobic digestion (AD) process of marine algae are an appealing opportunity. During the late 70's, various researchers around the world including US researchers had investigated the potential of different species of seaweeds as a carbon source for methane production. Their results have shown that marine macroalgae represent a good energy feedstock source for production of second-generation biofuels by the AD process due to its rapid growth rate, low land usage and high carbon dioxide absorption

and uptake rate (Dębowski et al., 2013; Hansson, 1983). Marine biomass such as macroalgae is formed by the photosynthetic capture of solar energy and stored as chemical energy. This biomass presents relative high conversion efficiencies, rapid conversion rates, and good process stability in comparison to terrestrial plants (Rajkumar, Yaakob, & Takriff, 2014). Particularly, the tissues of the brown alga such as *Sargassum spp.* are rich in carbohydrates, have a relatively low amount of cellulose in comparison to terrestrial crops, and do not contain lignin, which makes these species a potentially attractive feedstock for biogas production (Borines et al., 2013; Martone et al., 2009). Furthermore, effluents derived from the anaerobic degradation of this biomass may be a sustainable source of organic fertilizers for agriculture.

The composition of the substrate (feedstock) is a key factor to consider during the biogas production process; in addition, it plays an important role in the stability of the entire process. The growth of the microbial communities involved in the anaerobic degradation of the feedstock and its conversion into biogas is likely limited by the availability of the nutrients (Ward et al., 2014). The substrate must meet the nutritional requirements of the anaerobic microorganisms in terms of macronutrients (C, N, H, O, S, P), micronutrients (K, Ca, Mg, Na, Fe), and trace elements (Ni, Mo, Zn, Cu, Co, Mn), as well as supplying the energy required for microbial growth. In the case of degradation of organic matter during the biogas production process, the carbon to nitrogen ratio (C/N ratio) is also considered to be of great importance. It is necessary that this ratio is not too low; in other words, that there is not too much nitrogen relative to carbon. Ideal C/N ratio in anaerobic processes ranges from approximately 20:1 to 30:1 (Adekunle & Okolie, 2015). Yen & Brune (2007) reported the detrimental effect of unbalanced C/N ratios in anaerobic systems. Under excessively high C/N ratios, bacterial communities may then experience nitrogen deficiency resulting in lower gas production due to a rapid consumption of nitrogen by the methanogenic bacteria. Whereas, low C/N ratio may cause ammonia accumulation and pH values exceeding 8.5, which is toxic to methanogenic bacteria.

Numerous studies have been reported on the use of marine biomasses in AD systems operating with freshwater for biogas production (Gurung et al., 2012; Milledge et al., 2014). In this study, we propose to evaluate the anaerobic conversion of *Sargassum spp.* into biogas under high salinity conditions. This project is innovative. It is the first in Puerto Rico to demonstrate the use of seawater and marine macroalgae (large multicellular algae such as Sargassum) for biofuel production. According to scientific reports on the use of marine biomass in AD systems, the marine macroalgae-based systems should be theoretically self-sufficient in terms of nutrients composition, since the nutrients composition of sargassum should be sufficiently balanced for all the energy and nutrients required by the overall biogas production process (Bird, Chynoweth, & Jerger, 1990; Dębowski et al., 2012). Therefore, the evaluation of the chemical composition of the *Sargassum spp*. in terms of macronutrients will give us the opportunity to influence the outcome of the biogas production process, and maximize energy output.

1.2. Objectives

A detailed characterization of organic matter used as an energy feedstock in anaerobic systems proves to be an important parameter to enhance microbial community dynamics, especially, nutrient composition demand and thus maximize the output of the biogas production process. Therefore, the main objectives of our research were: (1) characterize the marine biomass *Sargassum spp.* as an energy feedstock, (2) evaluate the transformation of some of the principal macronutrients (C, H, N, S) during the anaerobic degradation of this feedstock throughout the entire biogas production process, and (3) evaluate the use of seawater in the operations of the bioreactors.

1.2.1. Specific aims

1.2.1.1. Aim (1)

In order to quantify the reduction in organic matter and its conversion to biogas, we compare the macronutrient concentrations (C, H, N, S) of the effluents from the first chamber S1 and the third chamber S3 of two 15L multi-stage bench-scale anaerobic bioreactors (MSBSABs), operating under high salinity conditions (an intermediate salinity 1.0% w/w – Control System, and a high salinity 3.5% w/w – Experimental System) while fed with *Sargassum spp.* We hypothesize that (i) due to the high salinity environment, macronutrient concentrations will be different between the control system and the experimental system, resulting in elevated levels of potentially inhibitory factors such as hydrogen sulfide and sodium in the experimental system, and (ii) the macronutrient concentrations will be dissimilar within the systems due to the differential partition of the microbial consortia in each stage of the biodigesters. Consequently, the partition of micro and macronutrients in the solids and soluble liquid phases of the bioreactor effluents must be different between the control system and the experimental system.

1.2.1.1a. Hypothesis (1)

Under anaerobic conditions during the biogas production process, sargassum as feedstock will serve as a nutritionally complete source of macronutrients for maintaining high biogas yields under steady state semi-continuous flow operating conditions even when operated in full-strength seawater without nutritional supplement. We hypothesize that the concentration of these macronutrients will be higher in the first chamber (S1) in comparison to the third chamber (S3). Specifically, we hypothesize that most of the hydrolysis of the organic polymers to monomers will occur in the first and second chambers, converting them to soluble organic matter such as organic acids.

1.2.1.2. Aim (2)

To measure the mass balance of the flows of macronutrients through the bioreactors before and after reaching steady state, and quantify the partition of macronutrients, especially carbon, nitrogen and sulfur between the biogas fraction, microbial biomass fraction (suspended effluent solids) and soluble bioreactor effluents (soluble effluent solids).

1.2.1.2a. Hypothesis (2)

Microbial inhibition activities of sulfur reducing bacteria (SRB) and other inhibitory substrates involved in AD process are known to be significant under high salinity conditions. Hence, competition between different consortia of microbial communities involved in the degradation of the energy feedstock will affect the entire biogas production process. Specifically, we hypothesize that (i) the bulk of hydrolysis of macronutrients will occur in the first chamber, resulting in the solubilization of a significant fraction of insoluble carbon from the feed; (ii) the bulk of acetogenesis and methanogenesis will occur in the third chamber, resulting in a significant reduction of total COD in S3 when compared to S1.

1.2.1.3. Aim (3)

To evaluate the use of seawater on the biogas production process of the multi-stage bioreactors while fed with the marine macroalgae, *Sargassum spp.* Due to the fact that salinity is higher in the experimental bioreactor, we hypothesize a somewhat lower biogas yield in the experimental bioreactor in comparison to the control bioreactor.

1.2.1.3a. Hypothesis (3)

We hypothesize that the biogas produced will be significantly greater in the intermediate salinity bioreactor (1.0% w/w - control system) than the high salinity bioreactor (3.5% w/w - experimental system), due to the inhibitory potential of sodium on the methanogenic community. Specifically, we hypothesize that methanogenic activities will be slower in the experimental system in comparison to the control system, resulting in a sharper reduction of available organic matter in the control bioreactor when compared to the experimental bioreactor.

This study will help us understand the anaerobic degradation efficiency of sargassum as an energy feedstock, and its conversion into biogas, microbial biomass and other soluble components such as nitrate, nitrite, carbonate, sulfate, sulfides, and phosphates. It is well documented that certain control parameters such as salinity, temperature, pH and elemental ratios like C/N, are associated with the methanisation process (Chynoweth et al., 2001). The role and importance of each of these factors are also a function of the geometry of the bioreactor, hydraulic and solids retention time as well as the richness of the feedstock used in terms of nutrients composition. Although inhibition of methanisation may result from high concentrations of substances such as phenols, heavy metals, sulfides, salts and volatile fatty acids (VFAs); nevertheless, acclimation of the microbial population to these substances seems to be a key feature of the process. Microbial communities may survive and eventually thrive under extreme conditions by long-term exposure. The slow introduction of toxic

compounds such as oxygen during feeding into the system could slowdown the biogas production process less drastically than if it was introduced suddenly in higher levels (Chen, Cheng, & Creamer, 2008; Holmer & Kristensen, 1994). Sulfur, an essential element for methanogenic fermentation, can also act as an inhibitor in high concentrations. However, the presence of sulfur is not a problem reported in the anaerobic digestion of brown algae using freshwater (Chen et al., 2008; Chynoweth et al., 2001). On the other hand, seawater itself contains significant amounts of sulfate, which may also transform into inhibitory sulfides in the reducing environment of the experimental bioreactor. Therefore, our goal is to decipher which of these well-described factors will most heavily impact the biogas production process using sargassum under full-strength seawater.

1.3. Anaerobic digestion process (AD)

The anaerobic digestion (AD) process involves the degradation and stabilization of organic raw materials in the absence of oxygen by microbial organisms and leads to the formation of biogas and microbial biomass (Kelleher et al., 2002). This technology is used to control and reduce municipal pollution from agricultural and industrial operations. According to Lettinga (1995), the AD technology is growing in importance worldwide method to manage wastewater. Chen et al. (2008) will define it as "*one of the most efficient waste and wastewater treatments of organic industrial wastes including fruit and vegetable processing wastes, packinghouse wastes, and agricultural waste*". AD systems offer numerous significant advantages when compared to aerobic wastewater treatment plants, such as low sludge production, low energy requirements and green energy recovery (Rajagopal, Massé, & Singh, 2013). This technology has a positive net energy production; in addition, the biogas produced is a sustainable alternative energy to fossil fuels.

Temperature is one of the most important factors that influence the performance of the AD process. It is important that temperature remains relatively constant because methanogens grow optimally at specific temperatures (Bouallagui et al., 2004; Ji-Shi et al., 2006; Van Lier et al., 1996).

Temperature variation in the system may cause severe disruptions during methanogenesis. Anaerobic fermentations can be maintained at psychrophilic (12-16 °C, e.g. in landfills, swamps or sediments), mesophilic (25-40 °C, e.g. in the rumen of certain mammals as well as anaerobic digesters), and thermophilic conditions (55-60 °C, e.g. in heated anaerobic digesters or geothermally heated ecosystems). Contrary to the thermophilic systems, the mesophilic AD systems appear to be the most fitting for the biogas production process from the time when at this temperature the systems may be operational even at room temperature.

To understand the AD process, the following four main microbial metabolic activities have to be considered: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The metabolic pathway of the AD process is summarized in the following figure (Fig. 1), which maps the transformation of organic feedstock into the formation of biogas and digestate as end products by methanogenic microorganisms.

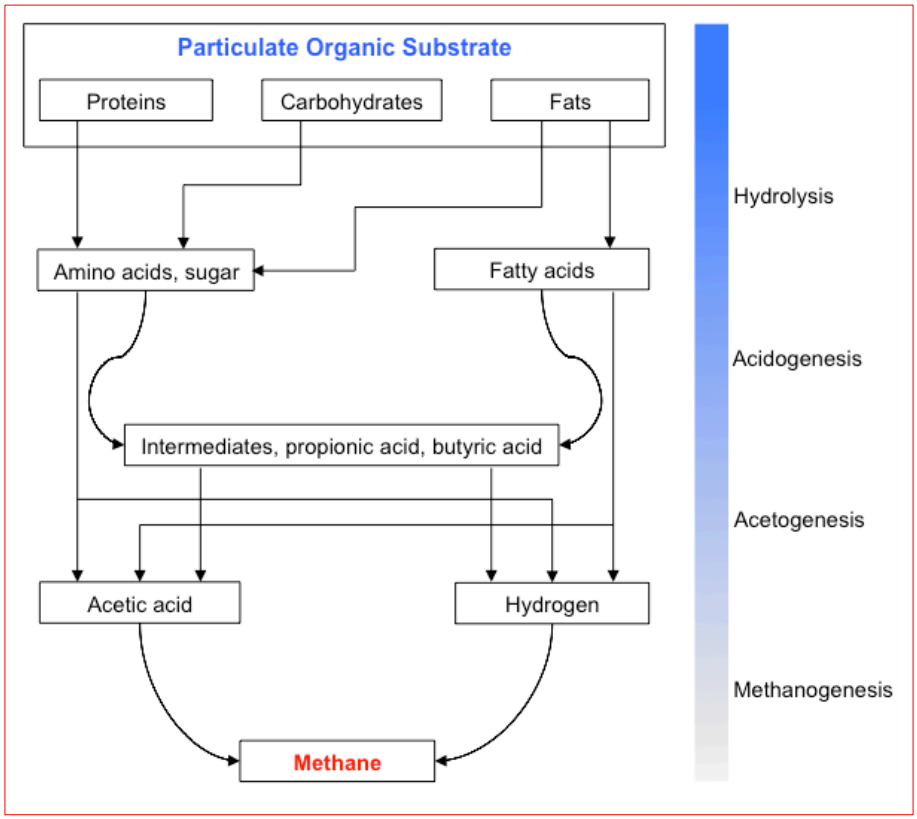

FIGURE 1: METABOLIC PATHWAY OF THE ANAEROBIC DIGESTION PROCESS

(Adapted from Diltz & Pullammanappallil, 2013)

1.3.1. Hydrolysis

Hydrolysis is the first essential step conducing to the transformation of organic matter to methane. During this phase, hydrolytic bacteria break down particulate organic substrates into liquefied monomers and polymers, which means that biopolymers such as proteins, carbohydrates and fats are transformed into amino acids, monosaccharide, and VFAs, respectively. Liu & Boone (1991) highlighted the role of diverse populations of microorganisms involved in the biodegradation of organic matter to methane and carbon dioxide. Specific types of facultative and obligate microorganisms dominate the hydrolytic phase in AD systems. These include hydrolytic genera such as *Clostridium, Peptococcus, Vibrio, Micrococcus,* and *Bacillus.* These hydrolytic microorganisms may produce extracellular hydrolytic enzymes that are capable of initiating the breakdown of complex substrates, among them are protease, lipase, cellulase, amylase, chitinase, pectinase, etc. They play a key role in the AD process because they ensure a complete degradation of the applied biomass (Lynd et al., 2002). For example, cellulolytic bacteria act in the depolymerization of the cellulose (Vavilin et al., 1996).

1.3.2. Acidogenesis or fermentation

In this phase, the derived products from the hydrolytic stage are subsequently fermented by acidogenic bacteria (also called acidogens or acid-forming bacteria), and broken down into short-chain volatile fatty acids (VFAs, e.g. acetic acid, propionic acid, butyric acid, valeric acid), and alcohols. Acetate, hydrogen, and carbon dioxide are also created and act as an initial substrate for methanogenic archaea. Acetate and hydrogen are the most important intermediates for the methanogenic phase derived from the fermentation of proteins and fats. The acidogenic metabolism depends on the environmental conditions such as hydrogen partial pressure (HPP), and others crucial factors including initial substrates. Low HPP promotes the formation of acetate, carbon dioxide, and hydrogen; meanwhile, high HPP conducts to the formation of propionate, lactate and ethanol (Conrad, 1999; Shah et al., 2014). It is mainly the obligate and facultative anaerobes that

carry out this fermentation phase. Diverse groups of bacteria act during the acidogenic phase, the majority of them are strictly anaerobic, i.e. the presence of oxidants such as oxygen or nitrate is toxic for their activity. In addition, ammonia could have an inhibitory action on acidogenesis. However, there are always facultative bacteria present in the sludge which will use traces of oxygen whenever it is available, hence protecting the obligate anaerobes from the small amount of oxygen that may enter during feeding or sampling. The participating fermentative microorganisms in the second stage of the anaerobic digestion process belong to the different genera and species, among them are *Clostridium, Bacteroides, Ruminococcus, Butyribacterium, Propionibacterium, Eubacterium, Lactobacillus, Streptococcus, Pseudomonas, Desulfobacter, Micrococcus, Bacillus,* and *Escherichia.* (Heeg et al., 2014; Krause et al., 2008).

1.3.3. Acetogenesis

In this stage, organic acids and alcohols are broken down by acetogenic bacteria into acetic acid, hydrogen and carbon dioxide, which are the only compounds that can be metabolized efficiently by the methanogens through the final step of AD. Although some acetate (20%) and hydrogen (4%) are directly produced by acidogenic fermentation of sugars, and amino acids, both products are primarily derived from acetogenesis and dehydrogenation of longer-chain VFAs (McInerney et al., 1981). In spite the fact that acetogenic bacteria are obligate hydrogen producers, hydrogen may have an inhibitory action on their metabolism through feedback inhibition when the product accumulates in the bioreactor. According to Thauer et al. (1977), the degradation of higher fatty acids depends largely on the methanogenic bacteria activity. In AD systems, molecular hydrogen is used so rapidly by the methanogens that hydrogen partial pressure can be kept low enough to ensure the active performance of hydrogen-producing acetogens (Table 1).

TABLE 1: ESTIMATED GIBBS FREE ENERGY CHANGES OF SELECTED BIOLOGICAL REACTIONS AT STP

Reaction	***ΔG°' (kJ/mol)***
Propionate → Acetate	
CH_3-CH_2COO^- + $3H_2O$ → CH_3COO^- + HCO_3^- + H^+ + $3H_2$	+ 76.1
Butyrate → Acetate	
CH_3-CH_2-CH_2COO^- + $2H_2O$ → $2CH_3COO^-$ + H^+ + $2H_2$	+ 48.1
Carbon dioxide → Acetate	
$2CO_2$ + $4H_2$ → CH_3COO^- + H^+ + $2H_2O$	-95
Acetate → Methane	
CH_3COO^- + H_2O → HCO_3^- + CH_4	- 31
Glucose → Methane	
$C_6H_{12}O_6$ + $6H_2O$ → $6CO_2$ + $6H_2O$	-26
CO_2 + $4H_2$ → CH_4 + $2H_2O$	-131

(Adapted from Mara & Horan, 2003)

The acetogenic phase consists in two operating groups of acetogenic bacteria:

(i) *Homoacetogens (or hydrogen-consuming acetogens).* The homoacetogens are strictly anaerobic microorganisms. They convert hydrogen and carbon dioxide to acetate through anaerobic respiration. This step is thermodynamically less favorable. The homoacetogenic bacteria include different genera such as *Acetobacterium, Acetoanaerobium, Acetogenium, Butribacterium, Clostridium,* and *Pelobacter.* Balch et al. (1977) isolated and identified two such homoacetogenic microorganisms: *Clostridium aceticum* and *Acetobacterium woodii.*

(ii) *Obligate hydrogen-producing acetogens, OHPA.* Also called protons-reducing acetogens, this group of microorganisms catabolizes fatty acid intermediates

(propionate and butyrate), alcohols, and other higher fatty acids (stearate, valerate, palmitate, myristate, and isovalerate) into acetate, carbon dioxide and hydrogen by acetogenic decomposers. So far, only a limited number of OHPA species have been isolated and identified, namely *Syntrophobacter wolinii* and *Syntrophomonas wolfei*, which oxidized propionate and butyrate, respectively (Balch et al., 1977; Schink, 1997). Although the first 2 two reactions are not favored thermodynamically (Table 1), the mutualistic association between syntrophic acetogens and other hydrogen-utilizing bacteria allows the formation of acetate and hydrogen (Henson et al., 1988). This is an indicator of the complex and coupled metabolic interactions occurring in anaerobic environments.

Typically, syntrophic association predominates the acetogenic stage during AD in biodigesters and involves obligate hydrogen-producing acetogens, OHPA (Angelidaki et al., 2009; Shah et al., 2014). Inhibition of the process can occur by even low hydrogen partial pressure. However, methanogenic bacteria typically exist in close relationship with acetogens to form syntrophic pairs – the methanogens consume hydrogen as fast it is produced and thus prevent feedback inhibition of acetogenesis (Fig. 2).

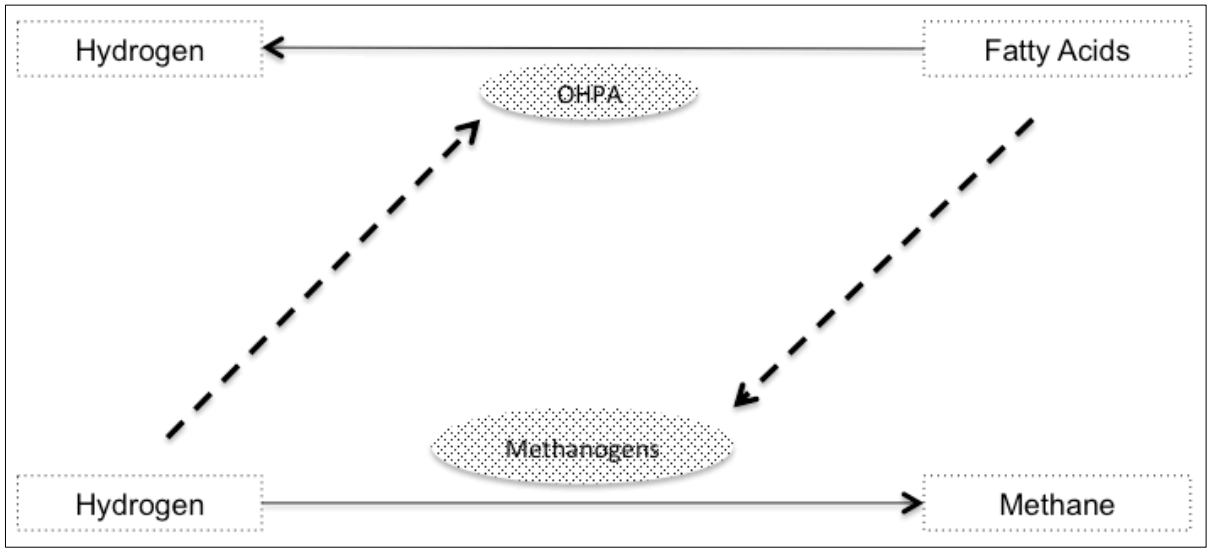

FIGURE 2: FEEDBACK INHIBITION BETWEEN OHPA AND METHANOGENIC BACTERIA

(Adapted from Anderson et al., 2003)

The conversion of acetate to methane through the acetogenic phase may be inhibited at high hydrogen partial pressure. Fortunately, the symbiotic relationship between OHPA and methanogens allows maintaining a favorable hydrogen partial pressure that is beneficial to their microbial activities (Weedermann et al., 2013). Additionally, the hydrogen syntropy prevents the conversion of acid intermediates to acetate and further consumption through methanogenesis by participating in the interspecies hydrogen transfer process, which maintains the low hydrogen concentrations required by the OHPA (Conrad, 1999). The stability of the AD process depends on the maintenance of a delicate biochemical balance between acidogenic and methanogenic microorganisms. The acetogenic step is crucial for a successful conversion of organic matter into biogas.

The schema below (Fig. 3) summarizes the relationship between OHPA and methanogenic bacteria through the AD process.

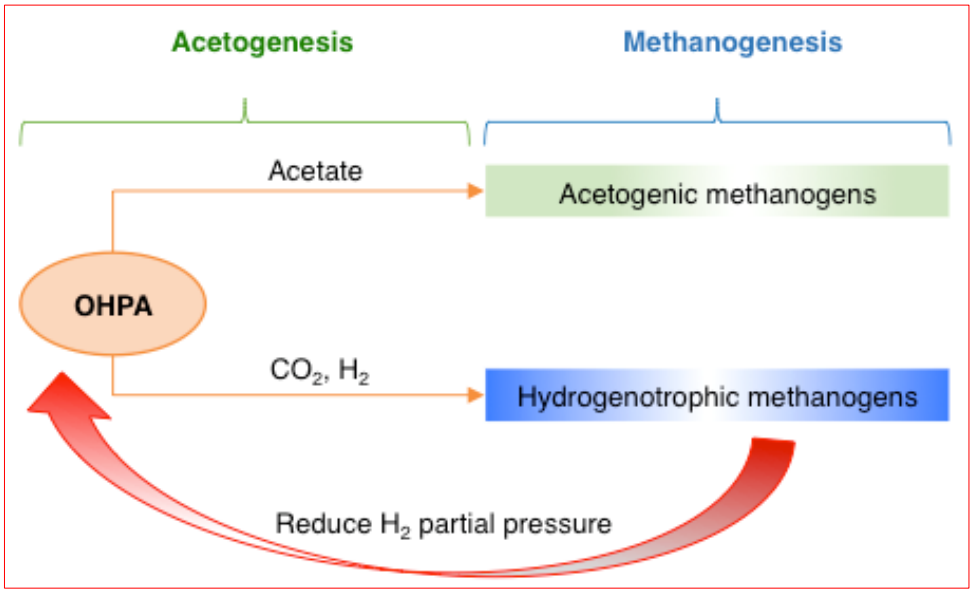

FIGURE 3: SYNTROPHIC RELATIONSHIP BETWEEN ACETOGENS AND METHANOGENS

(Adapted from Wang et al., 2013)

These first three phases (hydrolysis, acidogenesis, and acetogenesis) are often grouped together as “acid fermentation” dominated by *Eubacteria* members. It is important to note that during

the acid fermentation phase, no organic material is removed from the liquid phase but rather it is transformed into a suitable form as a substrate for the subsequent process of methanogenesis.

1.3.4. Methanogenesis

Methanogenesis is the final step of the anaerobic digestion process and takes place when the products of the acid fermentation are converted into methane and carbon dioxide by methanogenic microorganisms as end products of their metabolism. Methanogens are physiologically united as methane producers (Lessner et al., 2006). They are strict anaerobic anaerobes belonging to the kingdom *Archaea*. Their metabolism is unique due to their ability to obtain energy from selected low molecular weight carbon compounds and hydrogen with the stoichiometric production of methane. They are a large and diverse group, all of which are obligate methane-producing bacteria (MPB) that obtain all or most of their energy from methanogenic processes. Jones et al. (1987) and Yuchen et al. (2012) described the complex methanogenesis pathways. This process requires a number of unique coenzymes and membrane-bound enzyme complexes such as proteases, cellulases, xylanases, hemicellulases, among others. The methanogenic bacteria are the essential microorganisms in the methane production from acetate, hydrogen and carbon dioxide.

Three types of methanogenic pathways are recognized, based on their growth substrates:

(i) *Hydrogen-utilizing methanogens*. This group grows with hydrogen as an electron donor and carbon dioxide as an electron acceptor. The hydrogen-utilizing methanogens use methanoate (methyl formate), which is the source of both carbon dioxide and hydrogen to produce methane. They are responsible for up 30% of the total methane production in anaerobic biodigesters.

(ii) *Acetoclastic methanogens.* They cleave into a methyl and carbonyl group. Oxidation of the carbonyl group into carbon dioxide provides the potential for the reduction of a methyl

group into methane (called syntrophic acetate oxidation, SAO). The production of acetate is typically the most important precursor of methane production since it represents up to 70% of methane evolved in AD systems. The oxidation of acetate into methane involves exclusively two methanogenic genera: (a) *Methanosaeta*, a filamentous microorganism whose growth is possible only in the presence of acetate; and (b) *Methanosarcina*, an aggregate of large number of individual cells that are capable of using methanol, methyl-amines or even hydrogen and carbon oxide as growth substrates, in addition to their acetoclastic activity.

(iii) *Methylotrophic methanogens* that grow in the presence of methylated compounds such as methanol, methylamines, and methylsulfides, which act as both donor and acceptor or are reduced with hydrogen.

The cooperation of different consortia involved in the anaerobic digestion process promotes the synthesis of certain intermediates which may be used by other groups of microorganisms through the process. The following table summarizes such relationship (Table 2).

TABLE 2: MICROBIAL COOPERATION IN AD SYSTEMS

Bacteria type	***Electron donor***	***Electron acceptor***	***Product***	***Reaction type***
Fermentative	Organic carbon	Organic carbon	CO_2	Fermentation
Syntrophic	Organic carbon	Organic carbon	H_2	Acidogenesis
Acetogenic	Organic carbon/H_2	Carbon dioxide	CH_3COOH	Acetogenesis
Methanogenic	Organic carbon/H_2	Carbon dioxide	CH_4	Methanogenesis

(Adapted from Shah et al., 2014)

1.4. Toxicity

One potential limitation impeding the wider application of the biogas production process is inhibition (Rajagopal et al., 2013). AD process may be inhibited through different mechanisms including inhibition by product or substrate, inhibition by physical characteristics of the bioreactor (pH, temperature, etc.), or by inhibitory substances, both organic and inorganic, such as ammonia or reduced sulfur compounds. Solli et al. (2014) highlighted the role of the acid-forming and methane-forming bacteria (MBP) involved in the anaerobic degradation of biomass. Their studies refer to the physiology, nutritional needs, growth kinetics, and sensitivity to environmental conditions of the process. Bioreactor instability occurs when these two groups of microorganisms (acid-forming and MBP) are unbalanced due to the inhibitory substances present in the system. According to Ke et al. (2005), this failure is generally indicated by a decrease of steady state rate of methanogenesis and low bacterial growth activity, which subsequently lowers biogas production.

1.4.1. Ammonia inhibition

Ammonia is produced naturally by biological degradation of nitrogenous matter in the form of proteins, phospholipids, nitrogenous lipids and nucleic acid (Fotidis et al., 2013). Inorganic ammonia is present in two principal forms in the aqueous phase of the anaerobic digester: ammonium ion (NH_4^+) and free ammonia (NH_3). Free unionized NH_3 has been suggested to be the main cause of inhibition since it is freely membrane-permeable (De Baere et al., 1984; Fotidis et al., 2013). In addition, the hydrophilic ammonia molecule (NH_4^+) may slowly diffuse by passive transport into the cell, causing proton balance, and/or potassium. On the other hand, optimal ammonia concentration ensures sufficient buffering capacity of the aqueous medium, neutralizing the acids produced and thus increasing the stability of the digestion process. Toxicity caused by ammonia could be drastic and may be manifested by a total cessation of methanogenic activity according to Calli et al. (2005), Sung & Liu (2003) and Yenigün & Demirel (2013).

For a well-adapted process, Angelidaki & Ahring (1993) reported that the ammonia-nitrogen tolerance comes up to 3,000-4,000 mg NH_4-N/L. These observations are in accordance with those reported by Sung & Liu (2003) where they have demonstrated that higher total ammonia-nitrogen concentrations (TAN, a combination of free unionized ammonia-nitrogen and ammonium ion) greater than 4,000 mg/L have caused inhibition of methanogenesis. Whereas, Sawayama et al. (2004) observed the inhibition when TAN exceeds 6,000 mg NH_4-N/L. That is to say, a high ammonia-nitrogen concentration (< 5,000 mg/L) may cause lowered methane yield, loss of biomass (such as volatile solids), and low acetoclastic methanogenic activity. According to Zupančič & Grilc (2002), the pH also plays an important role in the case of ammonia inhibition: when the pH increases the toxicity due to ammonium increases.

1.4.2. Salt inhibition

Salt toxicity and its impacts on biogas production have been studied for several decades. Various metal ions including sodium, calcium, magnesium and potassium, are present in the effluents of anaerobic digesters. Feijoo et al. (1995) described these cations as important stimulants for microbial activities. However, they could have an inhibitory effect on the biogas production process when concentrations are higher.

1.4.2a. Calcium

Calcium is an essential cation for methanogenic bacteria. Kugelman & McCarty (1964) reported the inhibitory action of calcium and indicated that calcium ions (Ca^{2+}) had moderately inhibitory activity at a concentration of 2,500-4,500 mg/L, and strong inhibition at 8,000 mg/L. Chen et al. (2008) reported that 2,000 mg/L was the optimum Ca^{2+} concentration for methanisation of acetic acid in anaerobic conditions. Moreover, too much calcium may lead to problems with carbonate precipitation, which can lead to clogging and other solids handling problems in the system.

1.4.2b. Potassium

Osmosis of methanogenic bacteria may be negatively affected by higher potassium concentrations. Deublein & Steinhauser (2008) reported that the potassium concentrations in a range of 2,500-5,000 mg/L were inhibitory for anaerobic digesters.

1.4.2c. Magnesium

Magnesium ions have been reported as a stimulant for single cell production (Amani, Nosrati, & Sreekrishnan, 2010; Liu & Boone, 1991). However, at a concentration of 400 mg/L, bacterial growth would be affected.

1.4.2d. Sodium

Low sodium concentrations (100-200 mg/L) in mesophilic AD system are known as a vital factor for the survival of methanogenic bacteria, due to its role in the synthesis of adenosine triphosphate (ATP) and oxidation of nicotinamide adenine dinucleotide (NADH) (Feijoo et al., 1995; Kugelman & McCarty, 1964). For mesophilic systems, McCarty (1964) reported that the sodium concentrations in the range of 3,500-5,500 mg/L lead to moderate inhibition while 8,000 mg/L causes strong inhibitory effects.

Because of the particularity of our research by operating under higher salinity conditions, we expect that salt inhibition may be an important factor. However, it has been demonstrated that methanogenic populations do acclimate to high salt over time, which could permit successful functioning of an anaerobic bioreactor at concentrations that would cause perturbations at start up (Kelly & Dworjanyn, 2008). Acclimation of methanogenic bacteria to high sodium concentrations over prolonged periods of time could increase the tolerance and shorten the lag phase before methane production begins (Chen et al., 2008; De Baere et al., 1984; Feijoo et al., 1995).

1.4.3. Sulfur reducing bacteria (SRB)

Sulfate, sulfide, and organic sulfur are the common forms of sulfur in AD systems (Franke-Whittle et al., 2014; Omil et al., 1997). In fact, throughout the process sulfate is reduced to sulfide by two major groups of SRB: (1) the incomplete oxidizers, which reduce compounds such as lactate to acetate and carbon dioxide, and (2) complete oxidizers, which completely convert acetate to carbon dioxide (CO_2) and bicarbonate (HCO_3^-). By using seawater, sulfate reduction is unavoidable due to the high sulfate concentration of the seawater (2.7089 mg/L at 3.5% w/w). Chen et al. (2008) reported that the presence of high sulfate concentrations in the AD process is undesirable for biogas production. The biogas produced may contain a high level of hydrogen sulfide (H_2S), which is a toxic and corrosive gas; furthermore, its removal from biogas is very expensive. The presence of sulfate and the subsequent formation of sulfide can also induce the precipitation of non-alkali metals such as Ni, Mo, Zn, Cu, Fe, Co, and Mn in the bioreactor, consequently, reduce their availability for microorganisms. This will affect the growth of the microorganisms, which could result in a drop in biogas production (Muyzer & Stams, 2008). Additionally, hydrogen sulfide is a known inhibitor of methanogenesis as well as other anaerobes at the core of the AD process. Gunaseelan (1997) has reported that the AD process may suffer sulfate inhibition according to two separate mechanisms: first, inhibition due to the competition for common organic and inorganic substrates from SRB, and second, inhibition due to the competition between SRB and other anaerobes for available hydrogen, acetate, propionate, and butyrate.

1.4.3.1. Competition between SRB and other microorganisms

The sulfate-reducing bacteria (SRB) present different metabolic pathways. Their activities result in the complete or partial degradation of methanogenic substrates such as hydrogen, acetate, formate, pyruvate, and methanol (Bock et al., 1994), as well as propionate, succinate, fumaric acid, butyrate, short and long-chain fatty acids, malate, lactate, ethanol and higher alcohols and aromatic compounds. Based on the variety of the substrates utilized, SRB may compete with some anaerobes

such as MPB, acetogens or fermentative microorganisms for available acetate, hydrogen, propionate and butyrate (Briones et al., 2007). Competition does not occur in the hydrolysis stage; vigorous growth of SRB is not common on typical acidogenic substrates. Propionate is considered as a key intermediate, and it is a common substrate for all SRBs (Dong, Plugge, & Stams, 1994). Its degradation results in an incomplete conversion into acetate (Table 3). Contrary to the propionate-utilizing syntrophic species, propionate affinity is higher for the SRBs (Dar et al., 2008). Nevertheless, the affinity of SRBs for butyrate and ethanol is lower in comparison to non-SRBs. Acclimatization of MPB to free hydrogen sulfide (H_2S) has been reported in the literature, especially, in reactors with fixed biomass. According to Isa et al. (1986) acclimated acetotrophic and hydrogenotrophic methanogens may be slightly inhibited at concentrations above 1,000 mg/L of hydrogen sulfide.

TABLE 3: MESOPHILIC ANAEROBIC DEGRADATION OF PROPIONATE TO ACETATE

Reaction	**$\Delta G°'$ (kJ/mol)**
Propionate oxidation	
$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + 3H_2 + H^+$	+76.1
$CH_3CH_2COO^- + 2HCO_3^- \rightarrow 3HCOO^- + H^+ + CH_3COO^-$	+ 72.4
Methanogenesis	
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	- 135.6
$5HCOO^- + H+ + H_2O \rightarrow CH_4 + 3HCO_3^-$	- 130.1
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	- 31
Glucose oxidation	
$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$	-26
$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-131

(Adapted from Dong, Plugge, & Stams, 1994)

1.5. Marine biomass as energy feedstock for AD systems

Macroalgae are multicellular chlorophyllic organisms (Wiley, Campbell, & McKuin, 2011). They are classified into three major groups: brown algae (*Phaeophyceae*), green algae (*Chlorophyta*), and red algae (*Rhodophyta*). Most of them are benthic, which is to say that they live attached to the seabed between the top of the intertidal zone and the maximum depth to which adequate light can penetrate. Alternatively, they live attached to the surfaces of other organisms or grow as completely free-floating entities (Oyesiku & Egunyomi, 2014). Aside from their significant source of nutrients for marine ecosystems, marine macroalgae also soon represent an important and attractive source of carbon for bioenergy production. For centuries, marine biomass has been a commercially valuable resource for the production of agar and carrageenan (from red algae), and alginates (from brown algae). Marine biomass-based energy has been supported by various agencies including governmental and industrial groups for many years. The brown marine macroalgal biomass, *Sargassum spp.,* is known for its abundance in coastal environments, primarily in near-shore coastal waters with suitable substrates for attachment. Large amounts of *Sargassum spp.* have been located in various Puerto Rican beaches as floating forms. Floating seaweeds are considered one of the most important components of natural materials on the sea surface. The nutritional proprieties of different species of marine biomass, including Sargassum, have been reported by Narasimman & Murugaiyan (2012). Their studies emphasized on the biochemical proprieties of these species in terms of protein, carbohydrates, crude fiber, minerals, fats and ash content. Brown algae have been reported as the algal taxon to have the highest carbohydrate content (Borines et al., 2013).

1.5.1. *Sargassum spp.* in Puerto Rico

Marine biomass, including macroalgae, are proposed as valuable sources of renewable energy production such as biogas and other biofuels. Brown macroalgae *Sargassum spp.* are an unexploited marine biomass easily localized offshore along the Puerto Rican coast through the year. Their abundance and availability represent an enormous resource for exploitation as biomass energy

for renewable energy production. The brown algal species *Sargassum natans* and *Sargassum fluitans* are the most abundant species observed in Puerto Rican waters. According to Nadal, Rodríguez, & Casillas (1962), large quantities of carbohydrates were observed in *Sargassum natans,* meanwhile its nitrogen composition was low. Another Caribbean brown macroalgal species, *Sargassum polyceratium,* is also intermittently observed along Puerto Rican shore. *S. polyceratium* is commonly found firmly attached to the rocky substratum in moderately turbulent habitats from the lower intertidal zone to depths over 50 meters (Engelen et al., 2005). This species may be deposited on the shore as part of the beach wrack especially after heavy storms. The large quantity of Sargassum periodically observed on the Puerto Rican beaches may sometimes cause sulfurous odors and, therefore, limit the ecosystemic services that these beaches may afford. In fact, in the last few years, excess deposits of sargassum have forced the closing of various tourist beaches throughout the Caribbean (Higgins, 2011; Olibert, 2014). Therefore, the use of this biomass as a potential energy feedstock for biogas production could provide, from an economic point of view, not only an interesting energy alternative plus additional income for local communities but also reduce the negative impacts of sargassum on the marine ecosystem services.

1.6. Biogas production process

A critical issue impeding wider exploitation of biogas as fuel is the question of a reliable industrial biomass supply as energy feedstock to the biogas production process. The expression "*energy feedstock*" (or biomass) refers to any carbon source available on a renewable basis for energy conversion into electricity or other forms of energy. Various forms of terrestrial and marine biomasses are routinely used as energy feedstocks in AD systems for biogas production. The mass production of first-generation liquid biofuels (produced from food crops such as cereals, sugar crops, and oil seeds) has resulted in a series of problems related to food price, land use, and carbon emissions. Meanwhile, second-generation biofuels production (produced from non-food biomass included forest residues, organic components of municipal solid wastes, vegetative grasses and

energy crops) suffers due to technological barriers, limited feedstock collection networks and cost effectiveness (Christenson & Sims, 2011; Singh & Olsen, 2013). Therefore, it would be very interesting to identify a suitable marine biomass that can meet the requirements for biomass production at industrial scale.

1.6.1. Production of biogas and biodiesel from algae

Algae, as marine biomass, represent an attractive alternative source of renewable energy to supply the dependency on fossil fuels, as fossil fuels reserves are diminishing, and the practice of burning fossil fuels to produce energy has severe negative environmental impacts. Algal biodiesel and algae-derived biogas represent an important center of interest. Due to the high photosynthetic ability of marine macroalgae, they have been investigated during several decades. Photosynthetic efficiency of algae ranges from 3-8% compared with 0.5% for typical terrestrial crops (Wiley, Campbell & McKuin, 2011). Lipids accumulated in the algal cells are used for biodiesel production. There was an important focus in this area supported by the US Department of Energy (DOE) during the last few decades through their Aquatic Species Program (ASP) (Sheenan et al., 1998). Studies on the capacity of some species of microalgae to accumulate lipids on their cells have demonstrated that lipids content may vary from 2-75% of the total mass and this number depends on the availability of nutrients (Mata, Martins, & Caetano, 2010). Contrary to algae-based biodiesel which production is centered in the lipid content of the biomass, the production of algae-derived biogas is virtually independent of lipid content.

1.6.2. Relevance of marine macroalgae for biogas production

Numerous studies on the use of marine biomass in AD systems for biogas production has been developed around the world during the last few decades. These studies suggested that marine algae appear to be an attractive and good biomass feedstock for AD to methane conversion. The conversion efficiency of a biomass is defined by its biodegradability capacity and methane yields

produced. Controlled AD systems developed for biogas production are designed biodigesters with the major objective of producing methane at low cost. Low costs derive from high methane yields (m^3 CH_4/kg feed) and high methane production rates (m^3 CH_4/m^3 bioreactor/day). Generally, high methane yields are achieved through long solids retention time (SRT) while high organic loading rates and resultant short hydraulic retention time (HRT), along with high methane yields promote high methane production rates. Bird, Chynoweth, & Jerger (1990) studied the bioconversion of some marine species to methane including various species of sargassum. They observed more bioconversion productivity for Gracilaria species with methane yields ranging from 0.28 to 0.40 m^3 *kg^{-1} VS added. Meanwhile, *Sargassum fluitans* and *Sargassum pteropleuron* were less productive with methane yields ranging from 0.12 to 0.19 m^3 *kg^{-1} VS added. Although sargassum species present less bioconversion productivity compared to other marine biomasses, some physiological parameters make them an attractive candidate to methane conversion: their tissue has no lignin, which is a structure very difficult to degrade; moreover, they are rich in carbohydrates and many of these species have nitrogen-fixing symbionts so they do not require nitrogen fertilization (Hamersley et al., 2015; Phlips, Willis, & Verchick, 1986).

PART II: METHODS AND EXPERIMENTS

2.1. Startup

This section describes the methodology for startup of operations, as well as the different tests used for monitoring the performance of the bioreactors. Our research was focused on the evaluation of the potential of the marine macroalgae, *Sargassum spp.*, to serve as an energy feedstock for biogas production under anaerobic and high salinity conditions. Various parameters are crucial for a successful biogas production process including (i) bioreactor geometry, (ii) biomass quality, (iii) hydraulic and solids retention time, (iv) pH and temperature, (v) biodegradability of the energy feedstock and (vi) volatile fatty acids (VFAs).

2.1.1. Experimental design

The AD literature describes two main modes of operation for anaerobic biodigesters: batch and continuous systems. Batch biodigesters are the simplest, with the biomass added to the reactor at the beginning and sealed for the duration of the entire process. This system presents a lot of advantages including (i) lower capital investment when compared to continuous systems, (ii) reduced risk of contamination, and (iii) higher raw material conversion levels for a controlled growth period. Nevertheless, this system may suffer odor issues, which can be a severe problem during emptying cycles. Furthermore, this technology processes only small amounts of feedstock per batch feed, resulting in lower productivity levels due to the time for inoculating, emptying and cleaning the reactor. On the other hand, in continuous systems, which are the most common mode for industrial scale operations, organic matter is constantly added to the digester and the products (effluents) are constantly removed, resulting in (i) much more constant rate of biogas produced, (ii) more reliable and more easily reproducible, (iii) higher degree of control of the biomass concentration, and (iv) suitable control of the feedstock composition for more biogas yield. The following schema (Fig. 4) summarizes the different steps of the conversion of sargassum as biomass using in our research.

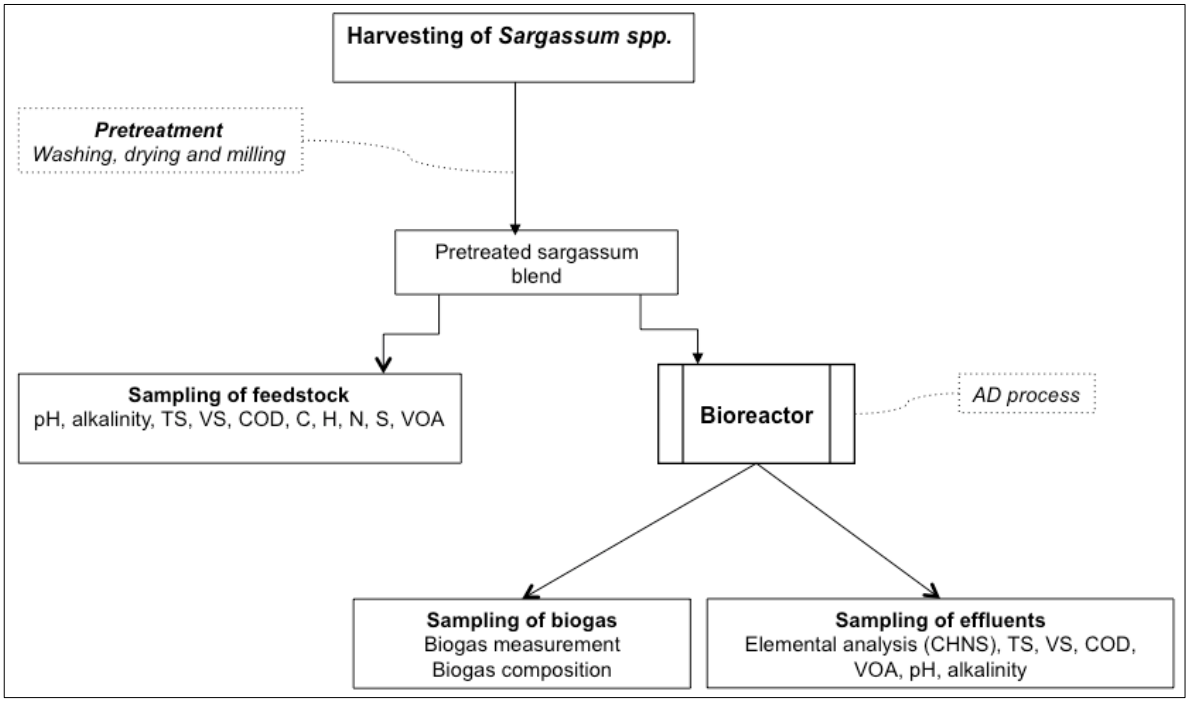

FIGURE 4: PROCESSING FEEDSTOCK TO BIOGAS

Throughout this study, two identical multi-stage bench-scale anaerobic bioreactors, MSBSAB (an intermediate salinity of 1.0% w/w as a control system, and a high salinity of 3.5% w/w as an experimental system) were constructed from acrylic tanks, polypropylene fittings, and Tygon tubing. In order to enhance the anaerobic degradation of the biomass, each bioreactor was designed with 3 chambers (S1, S2, and S3). A thin membrane of nylon mesh was applied to the walls of each chamber during construction to provide some attachment surface, leading to increased microbial retention inside the bioreactors (Fig. 5). The two MSBSABs were inoculated in November of 2012. The working liquid volume of each MSBSAB was 15 liters and operated with a hydraulic retention time (HRT) up to 30 days. The headspace of each system was connected to a floating lid gas accumulator in order to measure daily the biogas produced (Fig. 6). The water traps of the accumulators were acidified with sulfuric acid (H_2SO_4) to a pH < 2 and NaCl added to 75% saturation to minimize the escape of CO_2 and H_2S from the biogas produced according to Parajuli (2011) and Walker et al. (2009).

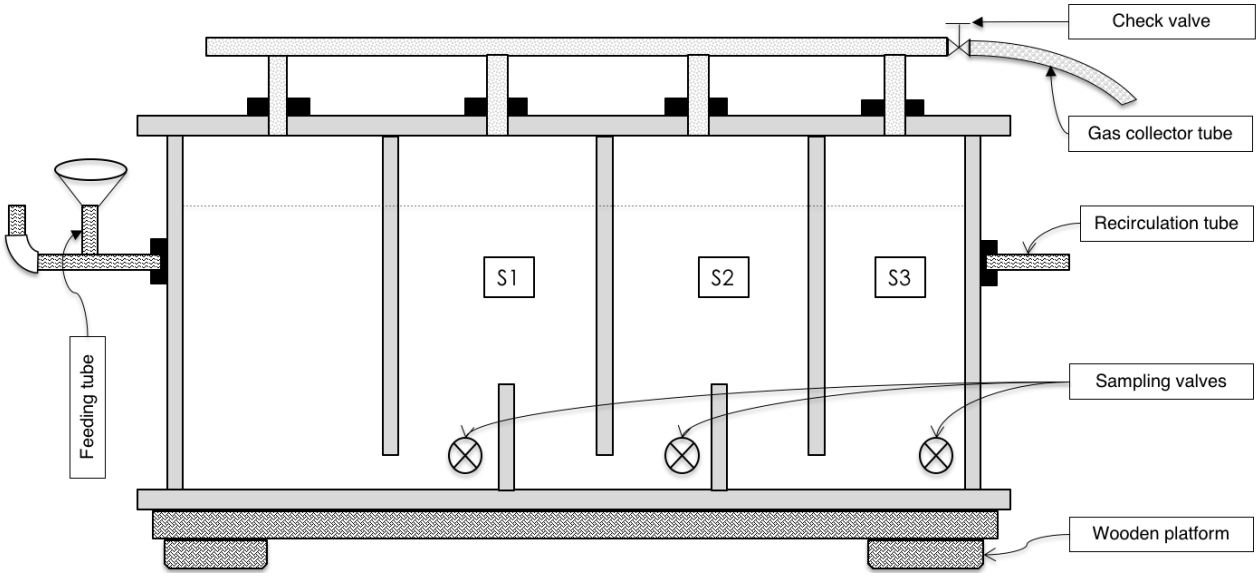

FIGURE 5: MULTI-STAGE BIOREACTOR CONDITIONED FOR BIOGAS PRODUCTION

The bioreactors were installed in a fume hood to control odor and protect personnel from H_2S exposure.

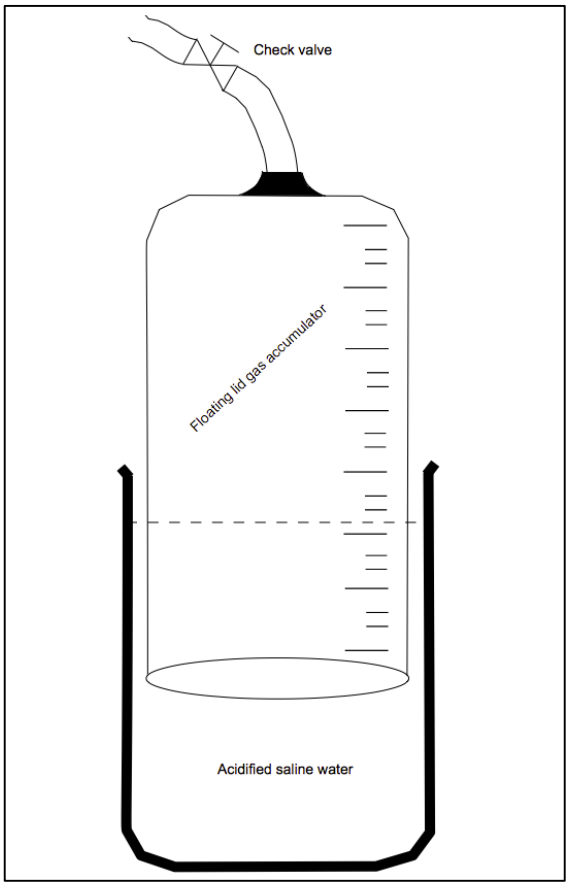

FIGURE 6: BIOGAS ACCUMULATOR

2.1.2. Inoculum

In November 2012, the two 15L MSBSABs were inoculated using anaerobic sludge taken from two preexisting 2L two-stage anaerobic digesters from the RCERE Laboratory. These AD systems (two-stage anaerobic bioreactors) had been previously operated for one year at 1.0% w/w and 3.0% w/w salinity using synthetic standard marine salts (Instant Ocean Aquarium Blend®, Table 4). These 2L anaerobic systems were initially inoculated with an anoxic sludge from the northern Puerto Rican coastal lagoon, Laguna San José (18°25'24.84"N; 66°1'31.93"W) of the San Juan Bay Estuary (SJBE). The bottom mud was drawn at 6.70 m depth, and the salinity measured was 2.5% w/w. This bottom mud sample was blended with fresh cow dung soon after it was harvested in order to increase its methanogen load.

TABLE 4: CHEMICAL COMPOSITION OF SYNTHETIC SEA SALT - INSTANT OCEAN

	Chemical composition *									
Product	*(Salinity)*	*(mmol kg^{-1})*						*(μmol kg^{-1})*		
	ppt	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**SO_4^{2-}**	**PO_4:P**	**NO_3:N**	**NH_4:N**
SW	35	470	10.2	53	10.3	550	28	0.20	0.20	0.20
IO	29.65	462	9.4	52	9.4	521	23	0.05	1.00	10.2
MW	--	23.0	39.1	24.3	40.1	35.5	32.1	31.0	14.0	14.0

(*): Atkinson & Bingman (1998)
SW: Seawater
IO: Instant Ocean
MW: Molecular weight ($gmol^{-1}$)

2.1.3. Bioreactor inoculation

The MSBSAB (control system) was firstly inoculated on November 7, 2012, and the MSBSAB (experimental system) two weeks after (November 27, 2012). The 15L MSBSABs were inoculated

using the existing 2L anaerobic biodigesters previously operated under 1.0% w/w and 3.0% w/w salinity, respectively, as described in the section above. Prior to using the inoculum was tested for salinity, dissolved oxygen (DO), pH, and temperature. The 15L biodigesters were filled with appropriate salinity synthetic seawater, and the oxygen was stripped from the bioreactors with a nitrogen gas purge. Each multi-stage bioreactor was connected in series to its corresponding 2L two-stage anaerobic system (1.0% w/w and 3.0% w/w, respectively) thru a peristaltic pump to assure that all the chambers receive a homogenous and similar inoculum. A total of at least 20 L of liquid was pumped through each 15L system and re-circulated to its source 2L system. Air penetration into the bioreactors was minimized during inoculation in order to protect the methanogens from oxygen toxicity. Salinity was maintained using synthetic marine salts, Instant Ocean Aquarium Blend®.

2.1.4. Hydraulic retention time (HRT)

The hydraulic retention time (HRT) is a crucial operational variable to be considered when designing AD systems. It refers to the average time that a given volume of water spends in the bioreactor. It is reported as the ratio of bioreactor volume to the volume of feedstock added per day to the system. The efficiency of the system with regard to biogas production is closely linked to the hydraulic retention time, as well as the operating temperature, the shape of the bioreactor and the biodegradation capacity of the energy feedstock. Throughout this research, the bioreactors were operated at mesophilic conditions (25 °C controlled room temperature) with an HRT of up to 30 days.

2.2. Characterization of *Sargassum spp.* as energy feedstock for biogas production

2.2.1. Harvesting, pretreatment and storage

According to the literature, as a general rule, the brown algae decompose more easily than the green algae, and the green algae degrade more easily than the red algae (Kelly & Dworjanyn,

2008). Our research was focused on the use of a mixture of three common Puerto Rican species of brown algae: *Sargassum fluitans*, *Sargassum natans,* and *Sargassum polyceratium*, in order to evaluate their potential as energy biomass under anaerobic and salt conditions. Harvesting the algae was a challenge throughout this study. We used a blend of all three common local species because they were found on the beach in tightly intertwined bundles, and separating them would be virtually impossible. This biomass was collected dry off the beach (beach wrack dried by the sun), which may have reduced some of the available organic carbon. The principal components of the blend were *Sargassum fluitans* and *Sargassum natans* (Fig. 7) – two benthic species deposited by wave action along the beaches.

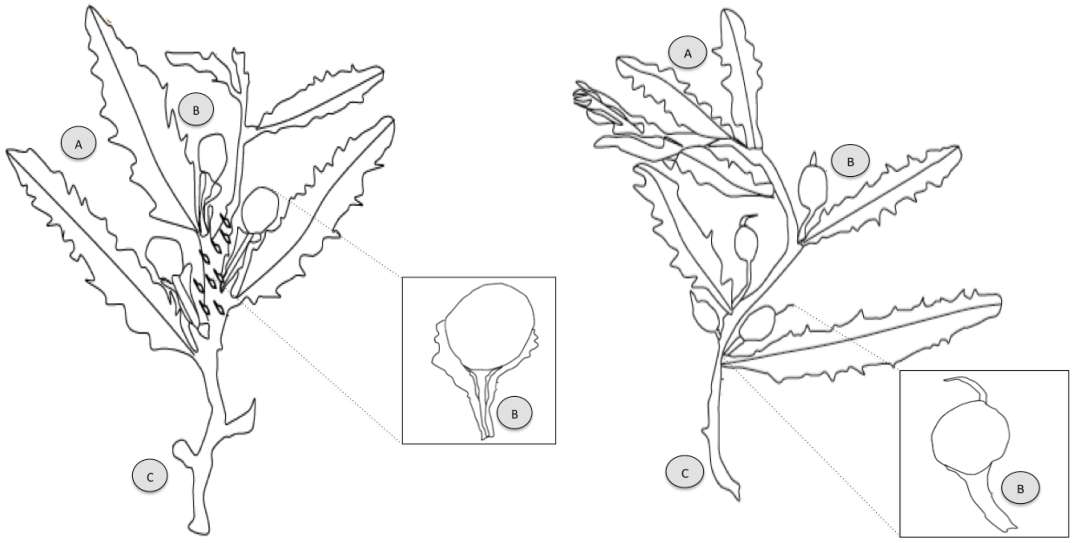

FIGURE 7: MORPHOLOGICAL STRUCTURE OF *S. FLUITANS* AND *S. NATANS*

Left: *Sargassum fluitans*
Right: *Sargassum natans*
(A)Blades; (B) Gas bladder; (C) Lateral branch.
(Adapted from Oyesiku & Egunyomi, 2014)

Sargassum species present a complex morphology with blades, bladders, and stipes, which makes them easier to cultivate as a floating crop. The most remarkable morphological differences between the two principal species used in this study reside in their bladder structure and their lateral

branches: a rigid spine is observed on the bladder of *S. natans*, while a winged tissue around the bladder petiole characterizes the bladder of the *S. fluitans* (de Széchy et al., 2012). However, both species present similar blade shapes. Their lateral branches are also morphologically distinct with the present of developed spines in *S. fluitans*. *Sargassum polyceratium* is a common genus of the eastern coasts of America and extensively distributed in Florida and Bahamas (Kilar & Hanisak, 1988). It is a pseudo-perennial genus, and the maximal growth has been reported from mid-fall to mid-winter. Its presence was observed intermittently and probably deposited after storms. Depending on the season the structure of this species may vary: smooth or spiny stems and large or small blades.

These algal species were collected during the experimental phase in three different beaches of the San Juan Bay Estuary (SJBE), Puerto Rico (Fig. 8). The SJBE is a tropical estuarine system located on the north coast of the island of Puerto Rico and composed of eight water bodies and others systems including cemented sand dunes, mudflats, marshes, coral communities, and sand beaches. These beaches included *Escambrón Beach* (18°27'57.50''N, 66°5'13.60''W), *Condado Beach* (18°27'35.80''N, 66°4'40.60''W) and *Ventana al Mar* (18°27'29.77''N, 66°4'26. 90''W). The algal collection included material deposited by waves along the shores and recovered dry on the beaches. Once collected, the organic raw material was transferred to collection bags for transport to the lab for pretreatment and storage. In order to obtain a feedstock with more consistent proprieties, the algae were washed with tap water to remove the excess of sand and salt. Drying was performed at 65 °C for a maximum of 48-72 hours using a Fisher Scientific™ Isotemp™ Microbiological Incubator so as to remove the excess of water generated during washing. Once dried, the feedstock was mechanically milled to fine particles (0.1-1 mm) using a Manual Grain Grinder, then placed into a desiccator where the sargassum powder may be stored until used in the bioreactors.

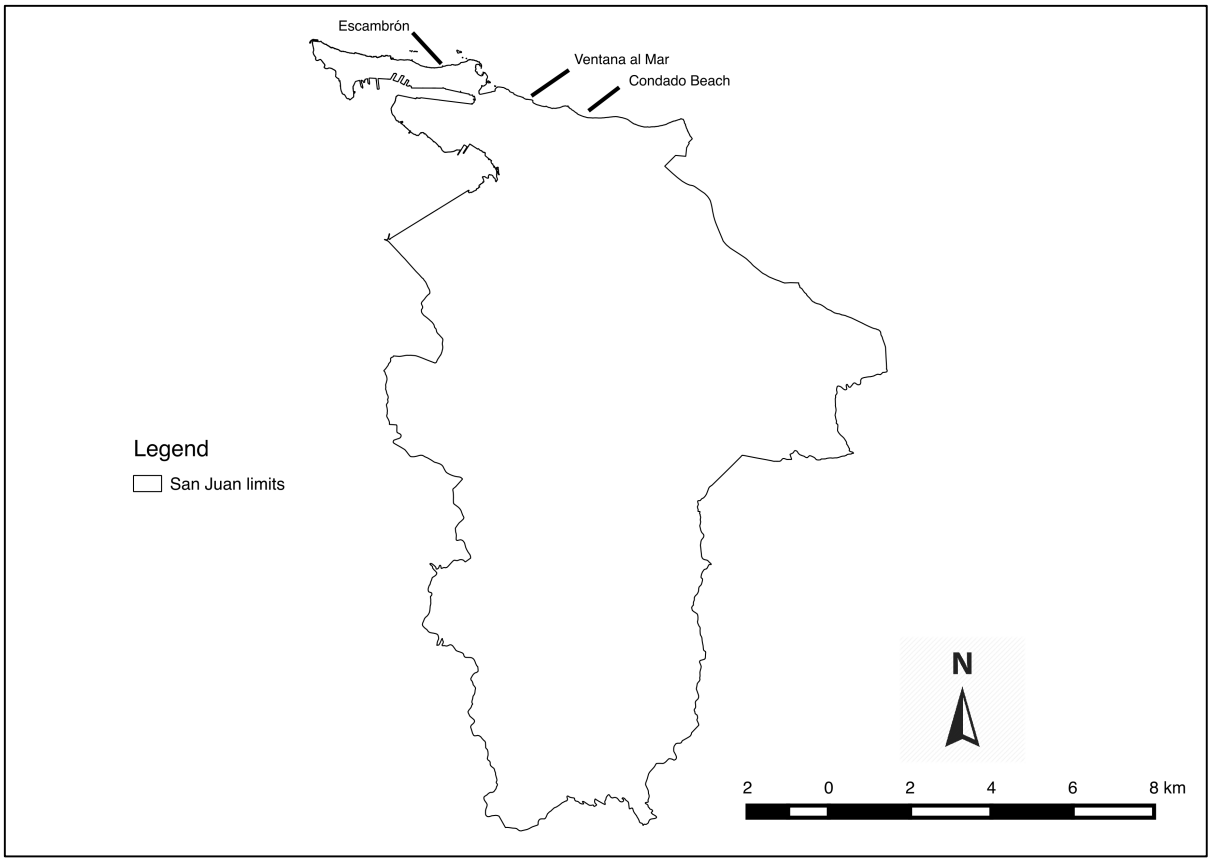

FIGURE 8: HARVESTING LOCATION

2.2.2. Physical and chemical analysis

The chemical composition in terms of macronutrients of the sargassum blend used in this research was evaluated in order to estimate its mass balance in the biogas and effluents produced. Different routine analyzes were used including volatile solids (VS), organic volatile acids (VOAs), total solids (TS), chemical oxygen demand (COD) and the elemental composition of the principal macronutrients (carbon, hydrogen, nitrogen and sulfur) present in the feedstock.

2.2.2.1. Total and volatile solids content (TS & VS)

Total solids content, as a control parameter, is very important when designing AD systems. Typically, there are 3 ranges of solid content defined in AD systems: low solid (LS) with less than

10% of TS, medium solid (MS) with 15-20% of TS, and high solid (HS) with 22-40% of TS. Results of the chemical analyzes for biomass samples are typically reported on a 105 °C dry weight basis. The TS content of the sargassum collected for this project were evaluated according to the standard methods (APHA, AWWA & WEF, 2005). 2-5 g of milled dry sargassum blend, previously dried at 65 °C, were placed in pre-weighed oven-dried ceramic crucibles and dried at 105 °C for a maximum of 48 hours until complete evaporation of the moisture. They were subsequently stored in a desiccator at room temperature prior to reweighing. Once reweighed, the dry material were reweighed then burned in a muffle oven at 550 °C for 24-48 hours, and then the VS and TS content was evaluated according to Sluiter et al. (2008) and APHA, AWWA & WEF (2005). The method of using the same ceramic crucibles to analyze the TS and VS content of the dry sargassum blend successively was to minimize the variability of the measurements.

2.2.2.2. Volatile organic acids (VOAs)

Volatile organic acids were performed by colorimetric titration of steam-distilled samples, a modified protocol of the standard methods for the examination of water & wastewaters, (APHA, AWWA & WEF, 2005). A volume of 50 ml of each sample previously acidified to a pH < 4 was placed into a distillation flask, and 8 ml of 6N H_2SO_4 were added to completely protonate all of the VOAs present in the sample. The first 10 ml of the condensate were discarded as it contained primarily carbonic acid. Then 100 ml of the condensate was collected and collected for colorimetric titration. 50 ml of collected condensate were titrated with standardized 0.05N NaOH to a phenolphthalein (pKa = 9.5) endpoint. Then the concentration of VOAs of the original samples was estimated using the volume of titrant and reported as acetic acid equivalents (ppm HOAce Eq).

2.2.2.3. Alkalinity and pH

Four times a week, a volume of 100 ml of effluents was sampled from each bioreactor, alternately between S1 and S3, for further analyzes. Subsequently, the samples were processed for

pH and alkalinity. A Fisher Digital pH meter, previously calibrated with a pH 7 and a pH 4 calibration standards for a two-point calibration, was used to evaluate the samples alkalinity. Each sample was titrated with a standard 1N H_2SO4 solution to a final pH endpoint less than 4 so as to prevent potential microbial activities during storage. Afterward, the acidified sample was diluted to a final volume of 200 ml (dilution factor 1:4), and stored at 4 °C for subsequent analysis. The alkalinity of the samples was reported as milligram per liter of Calcium Carbonate equivalents (mg/L $CaCO_3$ Eq) in the bioreactors (APHA, AWWA & WEF, 2005).

2.2.2.4. Chemical oxygen demand (COD) as estimate of organic matter content

The chemical oxygen demand (COD) tests have been the most widely used to estimate the organic matter content of anaerobic systems (García-Morales, Nebot, Romero, & Sales, 2001). As a measure of organic matter content, the COD test was essential to evaluate the bioreactors' performance. Total and soluble COD (tCOD and sCOD) of the sargassum blend have been measured in a Hach DR5000 Spectrophotometer using the standard high range COD kit test (Digestion solution for COD, 20-1500 mg/L range, Cat. 2125915). The high range test kit of Hach measures the formation of reduced manganese when heated in the presence of organic matter in a strong acid solution. The absorbance (optical density) of the reaction tubes is measured at fixed wavelength of 620 nm. Traditionally, the sCOD test is performed by filtration of the sample prior the measurement. Due to the extremely high-suspended solids content of the bioreactor samples, we have elected to use a modified method to estimate the sCOD of the samples. Small samples of approximately 1 ml were placed in 1.5 ml Eppendorf polypropylene microcentrifuge tubes. After being centrifuged for 3 minutes at 3,000 RPM equivalent to a G-Force = 1207; 0.5 ml of supernatant was appropriately diluted in distilled water, mixed with the Hach digestion solution, heated for two hours at 120 °C using a Hach DRB200 Digital Reactor Block, and subsequently, analyzed in the Hach DR5000 spectrophotometer.

2.2.2.5. Elemental analysis (C, H, N, S)

These chemical elements are the principal macronutrients central to all biological processes. Therefore, the dry sargassum blend was tested for carbon, nitrogen, hydrogen and sulfur content. After being pretreated and milled to fine particles, the sargassum blend was dried at 105 °C for 48 hours according to Vergara-Fernández et al. (2008); subsequently, the concentrations of the four principal macronutrients (C, H, N, S) were determined by pyrolysis using the PerkinElmer Elemental Analyzer EA 2400 Series II. The operation of the EA 2400 Series II is based on the classical Pregl-Dumas method, where samples are burned in a pure oxygen environment (975 °C), with the resultant combustion gasses measured in an automated fashion following frontal chromatography through a capillary column that allows more reliable and accurate determination of the combustion gasses than standard gas chromatography. We used the "*CHNS Mode*" to simultaneously determine carbon, hydrogen, nitrogen and sulfur in the sargassum samples. Accurate weighing is a prerequisite for organic elemental analysis since results are presented on a weight percent basis. For more accuracy, a Denver Instrument Ultra Microbalance was used. A total of 2-3 mg of dry sargassum blend from each lot of the feedstock involved in this project were weighed in a standard tin capsule then processed on EA 2400 Series II. The instrument was validated and calibrated using acetanilide, sulfamic acid, and cysteine as per the manufacturer's instructions.

2.3. Bioreactor monitoring and operation

Four times a week, 100 ml of samples (effluents) were drawn (alternately between S1 and S3) from each multi-stage bioreactor for routine chemical and physical analysis. To improve the homogeneity of the samples, the bioreactors were agitated using two systems: (i) a *wooden platform* was used to rock the entire bioreactor and mix all three chambers several times per day, and (ii) a *manual-powered peristaltic pump* was connected to the chamber to be sampled and several hundred millimeters of liquid from the bioreactor were pulsed through the chamber prior to sampling. Each sample was acidified using 2N H_2SO_4 to a pH less than 4 in order to inhibit microbial activities post-

sampling, and the alkalinity was calculated according to APHA, AWWA & WEF (2005). Subsequently, samples were diluted to a final volume of 200 ml, placed in polyethylene bottles, then stored at 4 °C until further analysis: volatile organic acids (VOAs), chemical oxygen demand (COD), total solids (TS), volatile solids (VS) and elemental analysis.

During the AD process, digestate recirculation from the methanogenic phase to the hydrolytic phase plays an important role since it provides optimal conditions for improved retention of hydrolytic bacteria in the system, as well as maintaining the pH value and the alkalinity (Aslanzadeh et al., 2013; Milledge et al., 2014). Therefore, a total of 500 ml of supernatant (around 3.3% of total volume of the bioreactor) was recirculated daily from S3 to S1.

2.3.1. Bioreactor tests run

According to Chynoweth et al. (2001), bioreactors are designed with the major objective of keeping the costs low. Low costs require high methane yields (volume of methane/kg feed) and high production rates (volume of methane/L*day). Starting the day after the inoculation, the daily biogas volume produced was measured in each gas accumulator. As indicated above in section 2.1.3, each bioreactor was maintained at its target salinity (1.0% w/w and 3.5% w/w) using synthetic marine salts. The bioreactors were fed daily with an aqueous suspension of a finely ground blend of sargassum as a carbon source. No additional supplements were provided other than the nutrients already present in the macroalgae feed suspension. The total solids, VS, and COD were monitored throughout the start-up phase, and continued as the system approached the steady state phase of operations (1.5 g VS/L*day feed). The VS measurements were used as a surrogate of organic load in both feedstock and bioreactor effluents. Although bioreactor performance has been assayed based on the changes in VS concentration and biogas production, tCOD, and sCOD measurements have been used to validate the VS content as a basis for estimating organic matter content in the bioreactors.

2.3.2. Analysis of the effluent load

To evaluate the macronutrient composition of the effluents and the degradation of the feedstock throughout the two multi-stage systems (1.0%w/w intermediate salinity and 3.5% w/w high salinity), representative samples have been collected, acidified and stored for routine and specific analyses: elemental analysis, VOAs, VS, TS, COD, Alkalinity, pH and ammonia content.

2.3.2.1. Total and volatile solids content (TS & VS)

The percentage of total solids present in the bioreactor effluents was calculated by drying 100 ml of acidified effluents (pH < 4.0) in an oven at 105 °C for a minimum period of 48 hours. Furthermore, the VS have been determined by: (i) burning 200-500 mg samples of the previously dried total solids samples in a muffle oven at 550 °C for a period of 24 hours in a ceramic crucible, and (ii) weighing the residual ash according to APHA, AWWA & WEF (2005).

2.3.2.2. Chemical oxygen demand (COD)

Total and soluble COD of representative samples from each chamber (S1 and S3) have been measured periodically using the standard test kit of Hach Company (Digestion solution for COD, 20-1500 mg/L range, Cat. 2125915). These tests were performed as described in the section 2.2.2.4. In addition, the total and soluble COD of the resuspended feed was monitored periodically for comparison.

2.3.2.3. Volatile organic acids (VOAs)

The VOAs of the effluents load from S1 and S3 was performed four times a week. 100 ml of effluent were acidified using a solution of 2N H_2SO_4 to a pH less than 4, then stored at 4 °C for further analysis. A volume of 50 ml of acidified sample was steam distilled and titrated with standard 0.05N NaOH and phenolphthalein indicator (pka = 9.5) to estimate the VOAs concentration. This procedure

is a modification of the published protocol of APHA, AWWA & WEF (2005), as described in the section 2.2.2.2.

2.3.2.4. Elemental analysis

Elemental analyses of the bioreactor effluents for total carbon, sulfur, hydrogen, and nitrogen were performed by the classical Pregl-Dumas method using the PerkinElmer Elemental Analyzer 2400 Series II, and may be summarized in 6 steps: sampling, acidification, drying at 105 °C, weighing, pyrolysis and data analysis (Fig. 9). Representative samples of each bioreactor from S1 and S3 have been used to estimate the CHNS content of each bioreactor effluents during the months of December 2013 to March 2014 (approaching steady state of operations). A total of 2-3 mg of TS (routinely tested in triplicate) was used for the CHNS content. In addition, oven-dried samples of the algal feedstock were periodically analyzed for macronutrients using the same instrument for comparison purposes.

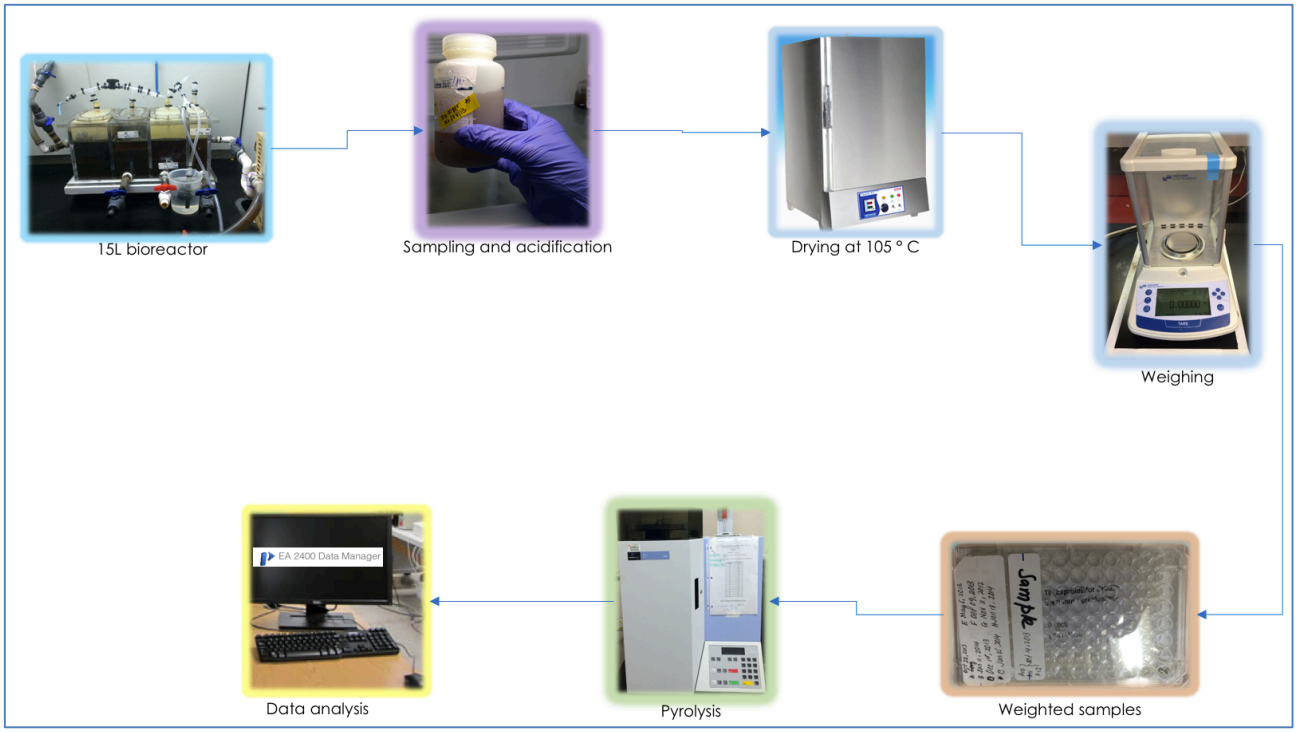

FIGURE 9: ELEMENTAL ANALYSIS FRAMEWORK

2.3.3. Biogas production measurement and composition

The volumetric biogas produced per gram of volatile solids reaching in the AD system per day is a critical parameter that provides information about the health of the system. We evaluated the efficiency of our bioreactors based on their biogas production as the end product in relation to the total solids mass of the energy feedstock used. The volumetric biogas production was daily measured as milliliter of biogas per gram of VS fed per day. The biogas accumulators were sampled and stored in Fluorinated Ethylene Propylene (FEP) gas sampling bag, then analyzed for two principal compounds, methane (CH_4) and carbon dioxide (CO_2).

Quantitative analysis of the biogas produced was carried out according to Fourier-Transform Infrared Spectroscopy (FTIR) using a Thermo Nicolet NEXUS 470 FTIR. A standard calibration blend from Linde Gas Inc., composed of 10% carbon dioxide, 10% methane, 5% hydrogen sulfide and 75% nitrogen was used to calibrate the instrument prior the analyzes. A new calibration curve was generated every time we operated the instrument, and, at least, six different volumes (from 500 μl to 3000 μl of standard calibration blend) were used. Biogas samples of the control and experimental systems were processed in triplicate to assure the accuracy of the results. A volume of 500 μl of each biogas sample was drawn using a 1cc syringe, then injected into a 72 ml CaF2 gas cell with a 100 mm path length from PIKE Technologies, following a nitrogen purge of the cell, and the final infrared spectra were then preserved for interpretation and any further manipulation. The following schema summarizes the process of the characterization of the biogas with regard to chemical composition (Fig. 10).

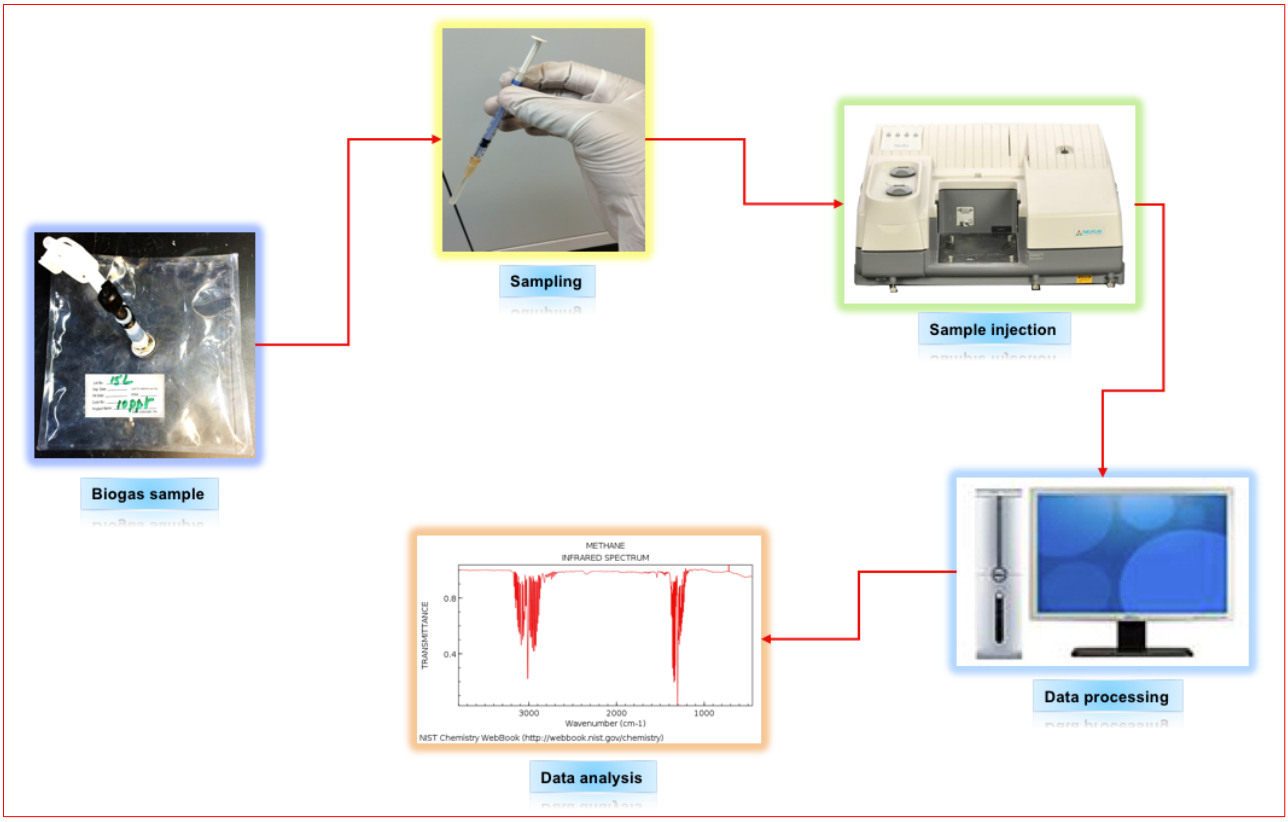

FIGURE 10: BIOGAS CHARACTERIZATION FRAMEWORK

PART III: RESULTS

This section depicts the results of different physical and chemical tests performed, including the total solids (TS), volatile solids (VS), mass balance, volatile organic acids (VOAs), elemental composition, ash content, pH, alkalinity and biogas production, and composition.

3.1. Chemical and physical parameters

3.1.1. Characterization of the feedstock

This section contains results for total and volatile solids content and elemental composition (carbon, hydrogen, nitrogen and sulfur) as chemical parameters of the energy feedstock.

3.1.1a. Total and volatile solids content (TS & VS)

The total and volatile solids content of the dry sargassum blend used throughout this research was evaluated. The averaged mean rate of TS of the dry energy feedstock was about 90.13± 2.69% after being processed at 105 °C, and the averaged mean of ash content was about 19.71± 9.45% (Fig. 11). Note that this represents the residual humidity remaining in the feedstock after drying at 65 °C.

The averaged mean of VS content measured for the collections of dry energy feedstock used during our study, by burning the samples at 550 °C for approximately 24 hours, was about 80.02± 2.35%. These values were statistically tested in order to determine any variability between the collections of sargassum. At a 95% confidence interval, the independent one-way ANOVA statistic test confirmed that the VS content were significantly different between the collections of sargassum (F-stat = 43.46 largely superior to F_{crit} = 2.51 with P-value = 0.000001). These results presented a significant difference in terms of organic material of the different collections of sargassum used in this research.

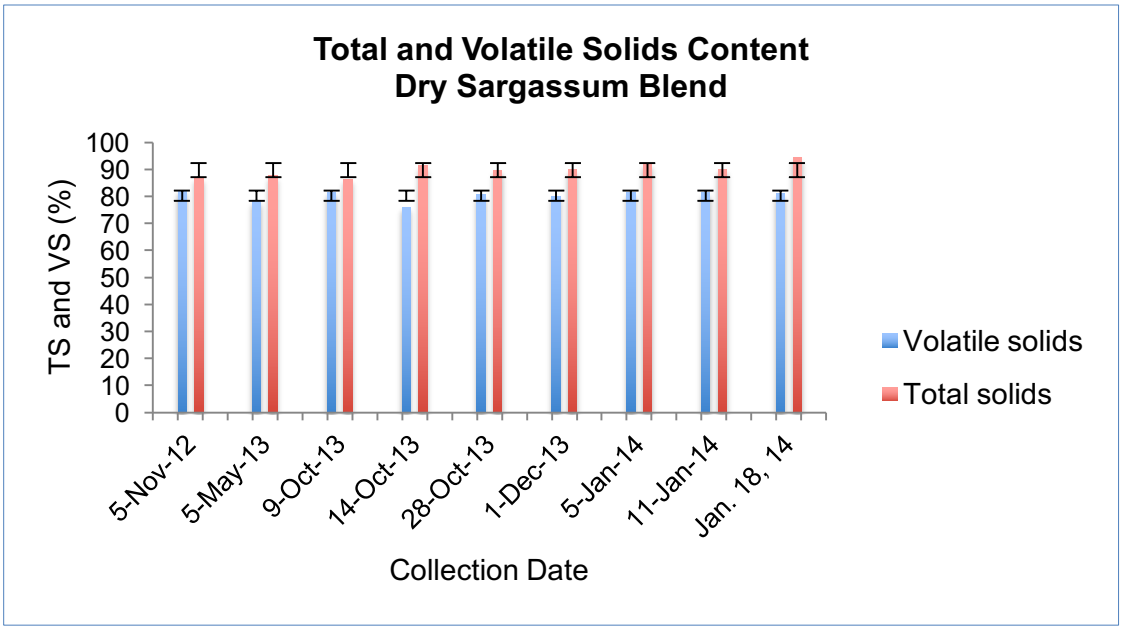

FIGURE 11: VS AND TS OF SARGASSUM BLEND COLLECTIONS OVER TIME

TABLE 5: TS AND VS OF THE SARGASSUM BLEND COLLECTIONS

Harvesting	TS ± STD (%)	VS ± STD (%)	Ash ± STD (%)
November 5, 2012	86.73± 0.03	82.25± 0.20	17.75± 0.93
May 5, 2013	87.75± 0.05	78.15± 0.16	21.85± 0.57
October 9, 2013	86.24± 0.25	81.44± 0.08	18.56± 0.35
October 14, 2013	91.26± 0.13	76.14± 0.29	23.86± 0.92
October 28, 2013	89.51± 0.34	80.51± 0.20	19.49± 0.84
December 1st, 2013	89.83± 0.63	80.13± 0.13	19.87± 0.53
January 5, 2014	92.04± 0.78	81.56± 0.15	18.44± 0.67
January 11, 2014	90.01± 0.24	81.22± 0.13	18.78± 0.56
January 18, 2014	94.44± 0.17	81.93± 0.14	18.07± 0.64

(The sargassum samples were previously dried at 105 °C)

3.1.1b. Elemental composition (C, H, N, S)

The elemental composition of the dry energy feedstock, *Sargassum spp.,* used during this study is illustrated in Table 6. This biomass presented a higher level of nitrogen (lower C/N) when compared to other organic matter sources reported in the literature. The carbon/nitrogen ratio was somewhat low, considering that the optimal ratio for AD systems is about 20:1 to 30:1 (Adekunle & Okolie, 2015) while the estimated values of the carbon/nitrogen ratio in the dry energy feedstock were about 5.8:1 to 6.2:1.

The carbon values of the energy feedstock blend represented 20-38% of the total dry mass. The carbon concentration did not vary significantly between samples of sargassum obtained on different dates and at different locations since at a 95% confidence interval, P-value = 0.061, F-stat = 2.375 was inferior to F_{crit} = 2.51.

The concentration of hydrogen in the sargassum was between 5-6% of total mass of dry solids. Within the 95% confidence interval (P-value = 0.23), the hydrogen concentration of the collection did not vary significantly between harvests, since F-stat = 1.48 < F_{crit} = 2.51.

Nitrogen concentration was about 5-7% of total mass of dry solids. Within the 95% confidence interval (P-value = 0.043), the collections of Sargassum showed significant differences in terms of nitrogen concentration between harvests, since F-stat = 2.60 > F_{crit} =2.51.

The energy feedstock collections had a sulfur concentration which varied from 0.5 to 1.3%. However, based on the statistic test with a P-value = 0.804, the sulfur concentration did not differ significantly between the various collections of sargassum, since F-stat = 0.55 was inferior to F_{crit} = 2.51.

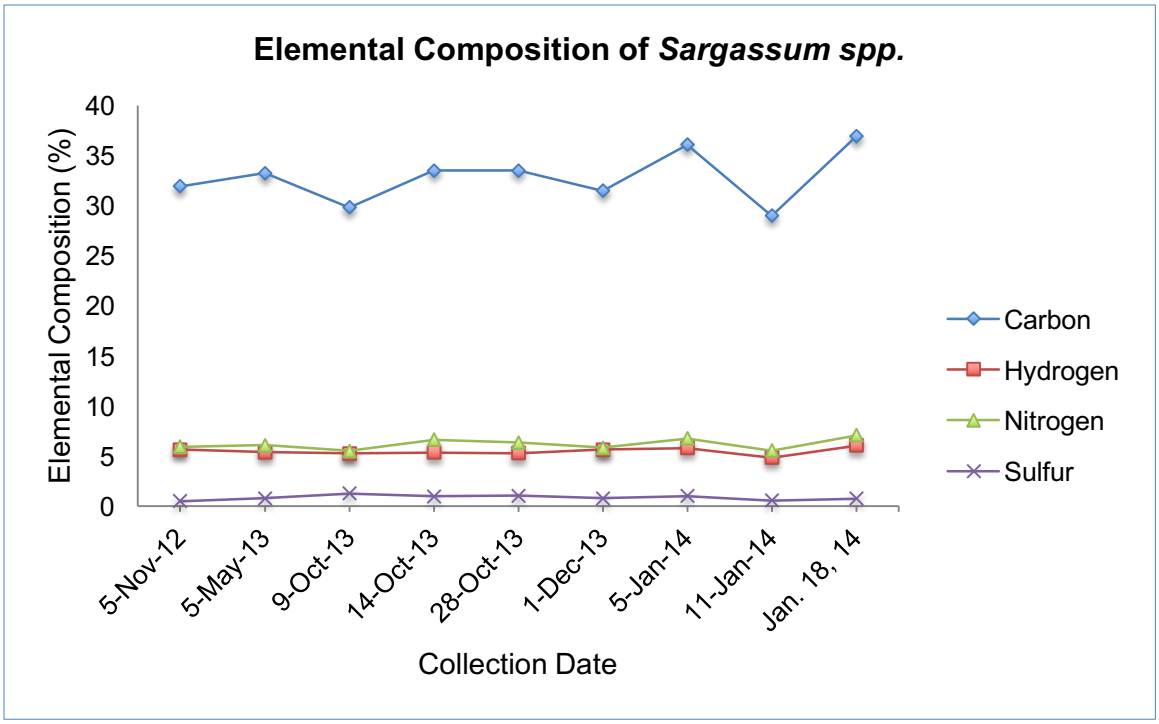

FIGURE 12: ELEMENTAL COMPOSITION OF SARGASSUM BLEND COLLECTIONS

TABLE 6: ELEMENTAL COMPOSITION OF DIFFERENT COLLECTIONS OF *SARGASSUM* BLEND

	Element ± STD (%)*								
Harvesting	05/11/12	05/05/13	09/10/13	14/10/13	28/10/13	01/12/13	05/01/14	11/01/14	18/01/14
Carbon	31.94 ±1.34	33.26 ±0.63	29.83 ±0.74	33.51 ±1.13	33.50 ±1.58	31.51 ±2.07	36.10 ±1.40	28.98 ±8.03	36.96 ±1.12
Hydrogen	5.64 ±0.25	5.40 ±0.16	5.27 ±0.17	5.35 ±0.25	5.30 ±0.24	5.64 ±0.18	5.80 ±0.09	4.83 ±1.43	6.08 ±0.08
Nitrogen	5.92 ±0.44	6.10 ±0.13	5.52 ±0.11	6.63 ±0.19	6.35 ±0.31	5.83 ±0.35	6.75 ±0.38	5.56 ±1.55	7.08 ±0.20
Sulfur	0.49 ±1.05	0.78 ±0.11	1.27 ±1.16	0.97 ±0.38	1.04 ±0.27	0.79 ±0.10	1.02 ±0.40	0.57 ±0.31	0.75 ±0.10
C/N	5.4	5.5	5.4	5.1	5.3	5.4	5.3	5.2	5.2
C/S	65.2	42.6	23.5	34.5	32.2	39.9	35.4	50.8	49.9
N/S	12.1	7.8	4.3	6.8	6.1	7.4	6.6	9.7	9.6

(*): Based on the running tests performed in the PerkinElmer Series II 2400 Elemental Analyzer.

3.1.2. Bioreactor test runs

In this section, we report the results of the different analytical tests used as control parameters of the bioreactors in order to evaluate their efficiency and performance. These analyzes include the pH, alkalinity, total and volatile solids content of the effluents, elemental composition, biogas production, biogas composition, volatile organic acids and organic matter content. Our research has been conducted over two years of operation. For experimental purposes, we decided to focus our analysis of COD during a specific experimental period of 18 weeks of operation with an organic loading rate (OLR) of 1.5 g/L of VS fed per day. During this period, the bioreactors' activities seemed to be more stable regarding the reduction of organic carbon, compared to the performance at higher OLR. Only the first chamber S1 and the third chamber S3 of both bioreactors (control and experimental) were used for sampling. This was based on the geometry of the bioreactors and the hypothesis that microbial activity would partition so that hydrolysis would primarily occur in S1 while methanogenesis would primarily occur in S3. Most of the digestible organic carbon would be available in the latest stage S3 for the methanogens, resulting in a significant reduction of organic carbon fraction.

3.1.2a. Alkalinity and pH

Effluent pH and alkalinity of the MSBSAB were monitored over 2 years of operation (Fig. 13 & Fig. 14, control & experimental systems, respectively). Throughout the course of the 18-week experimental period of operation, the pH values of the control system varied between 6.49 to 7.69 in the first chamber S1 and 6.70 to 7.94 in the third chamber S3. Meanwhile, in the experimental system the pH values varied from 6.48 to 7.96 in S1 and 6.64 to 7.91 in S3. Both bioreactors showed a high stability of pH.

Alkalinity was somewhat higher during the first 4 months of operation but rapidly stabilized through the remaining period of the study. During the 18-week experimental period of operation, the experimental bioreactor seemed to be more alkaline than the control system, and the values were

between 2.32 to 5.94 g/L $CaCO_3$ Eq in the first chamber S1 and 2.69 to 4.52 g/L $CaCO_3$ Eq in the third chamber S3. Meanwhile, the alkalinity values of the experimental bioreactor varied from 4.47 to 7.17 g/L $CaCO_3$ Eq in S1, and 3.43 to 6.19 g/L $CaCO_3$ Eq in S3. The greater values of alkalinity observed in the experimental bioreactor can be explained by the fact that the seawater itself represents a good source of magnesium and calcium carbonate. These two compounds are the main source of alkalinity of the system.

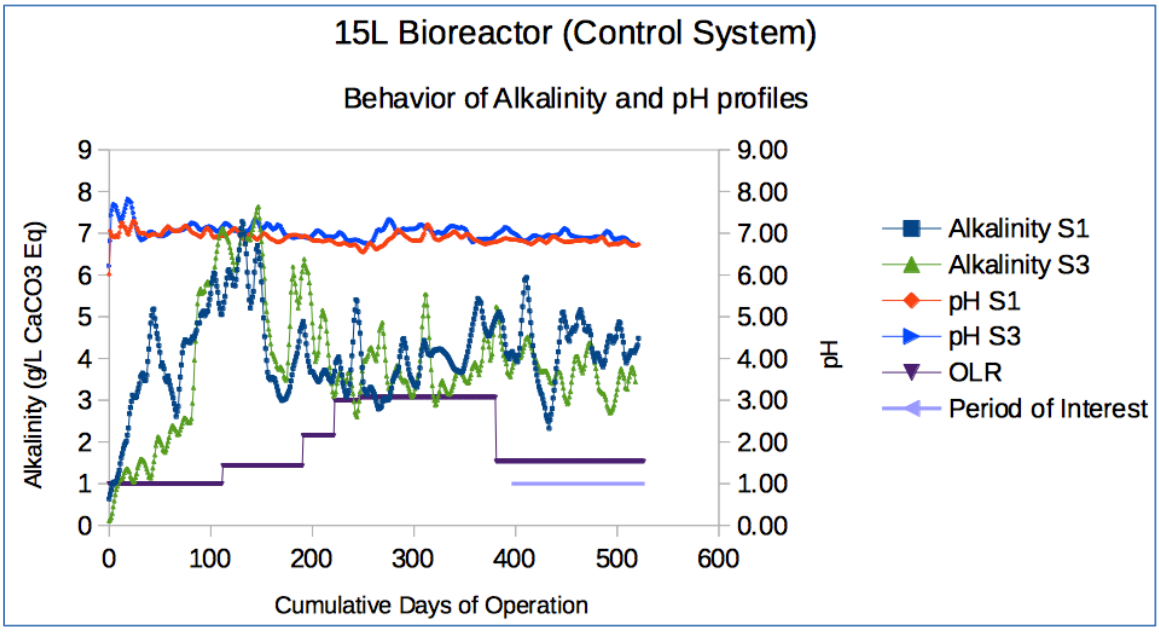

FIGURE 13: VARIATION OF PH AND ALKALINITY OF THE CONTROL SYSTEM

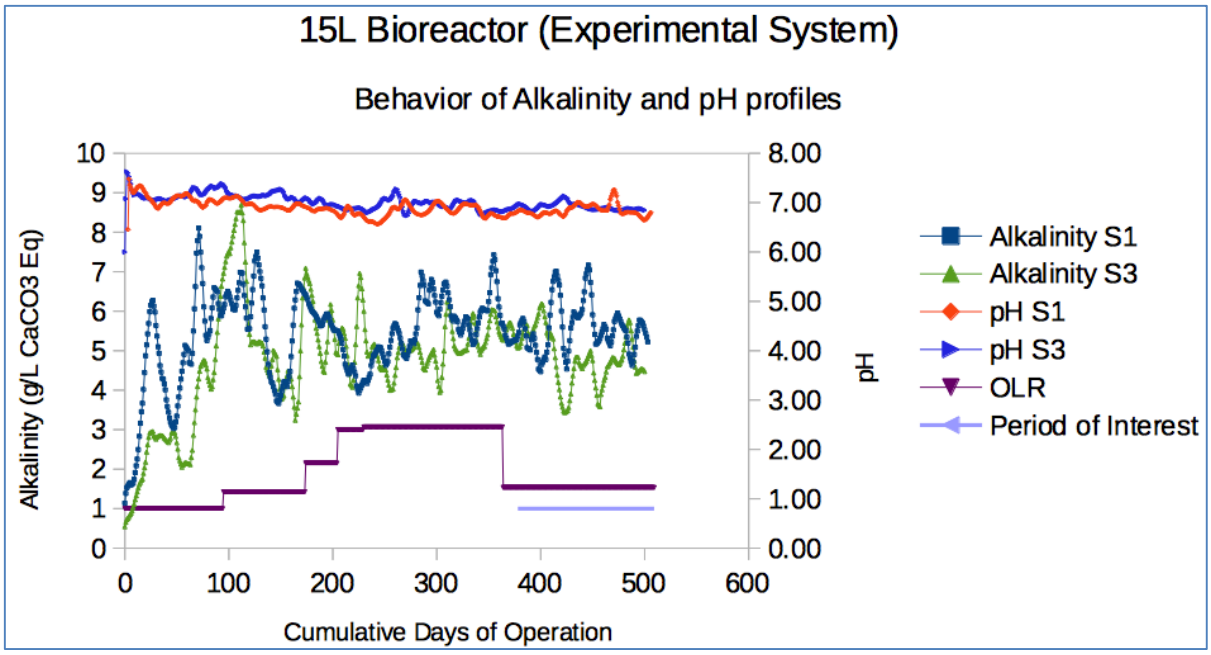

FIGURE 14: VARIATION OF PH AND ALKALINITY OF THE EXPERIMENTAL SYSTEM

3.1.2b. Total and volatile solids content (TS &VS)

Seven-day running averages of the total and volatile solids content were used to estimate the efficiency of the bioreactors over the 18-week experimental period of operation (Fig. 15 & Fig. 16). The TS content of the control bioreactor was significantly higher in the first chamber S1 (13.17± 3.39 g/100ml) when compared to the third chamber S3 (9.35± 2.39 g/100ml), with a 95% confidence interval (P-value < 0.0001). Similar observations have been noted in the experimental bioreactor: the TS content was greater in S1 (17.92± 3.94 g/100ml) when compared to S3 (13.46± 2.25 g/100ml) with a P-value less than 0.0001. The TS values were typically approximately 30% higher in the experimental system when compared to the control system.

The results of volatile solids content calculated in the bioreactors' effluents over the 18-week experimental period of operation are illustrated in the figures 16 and 17. Statistical analysis confirmed that, for a 95% confidence interval (P-value < 0.0001), VS content of the control system was significantly higher in S1 (9.03± 2.83 g/100ml) when compared to S3 (6.30± 1.79 g/100ml). Similarly, in the experimental system the VS content was statistically greater in S1 (10.25± 2.65 g/100ml) than S3 (6.90± 2.39 g/100ml), with a P-value < 0.0001. As expected, both systems (control &

experimental) presented a similar pattern displaying a notable reduction of organic matter in the third chamber S3 when compared to the first chamber S1. Between systems, the comparison showed that the VS reduction rate was greater in the control bioreactor than the experimental bioreactor at a 95% confidence interval (P-Value = 0.0225, t = 2.295).

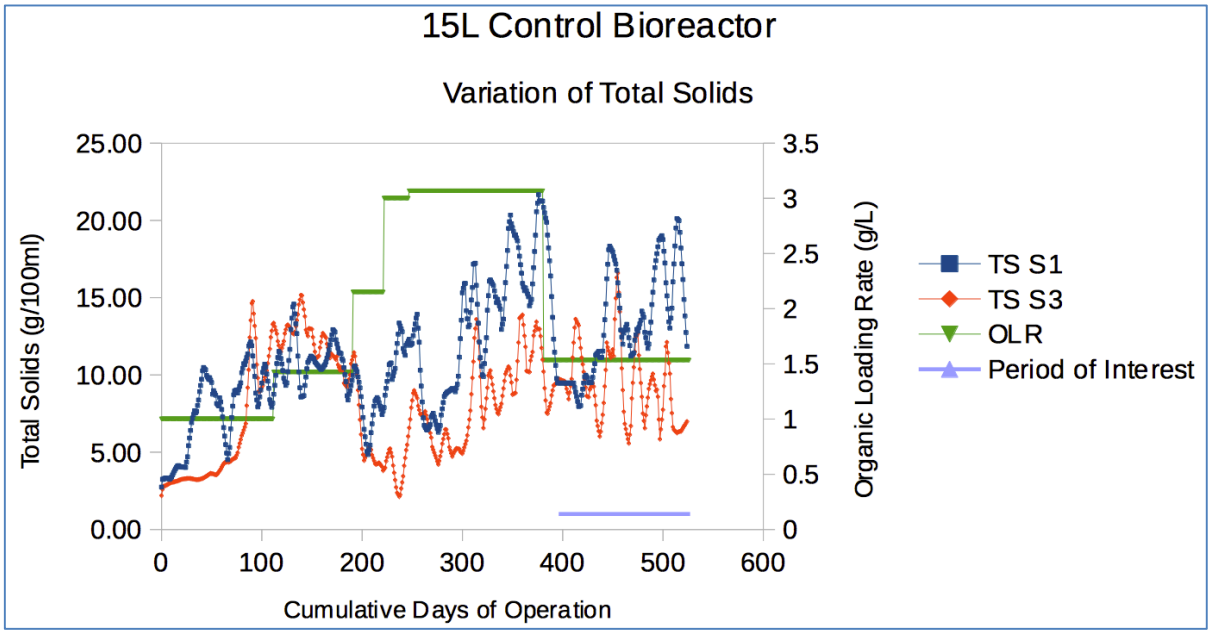

FIGURE 15: VARIATION OF TOTAL SOLIDS OF THE CONTROL SYSTEM

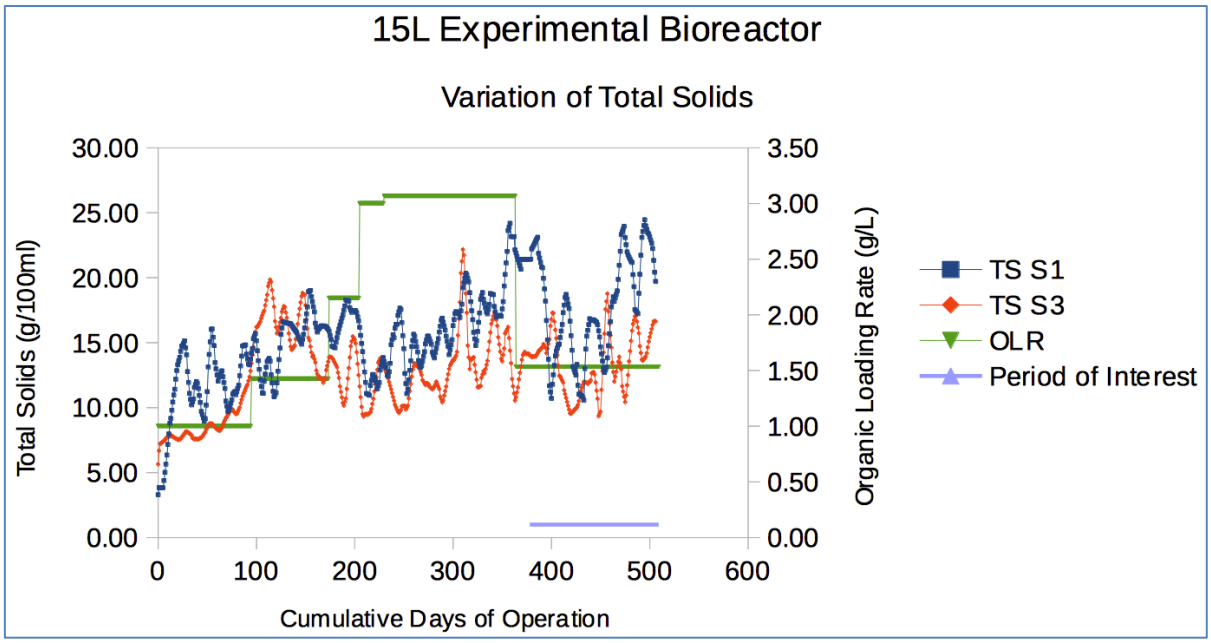

FIGURE 16: VARIATION OF TOTAL SOLIDS OF THE EXPERIMENTAL SYSTEM

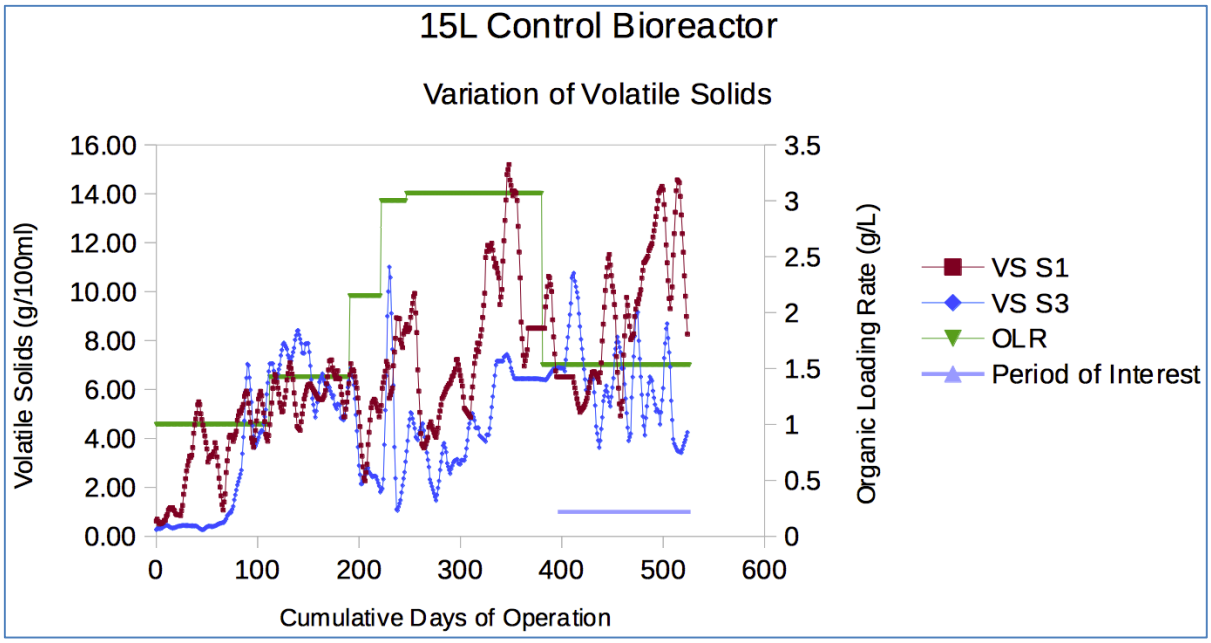

FIGURE 17: VARIATION OF VOLATILE SOLIDS OF THE CONTROL SYSTEM

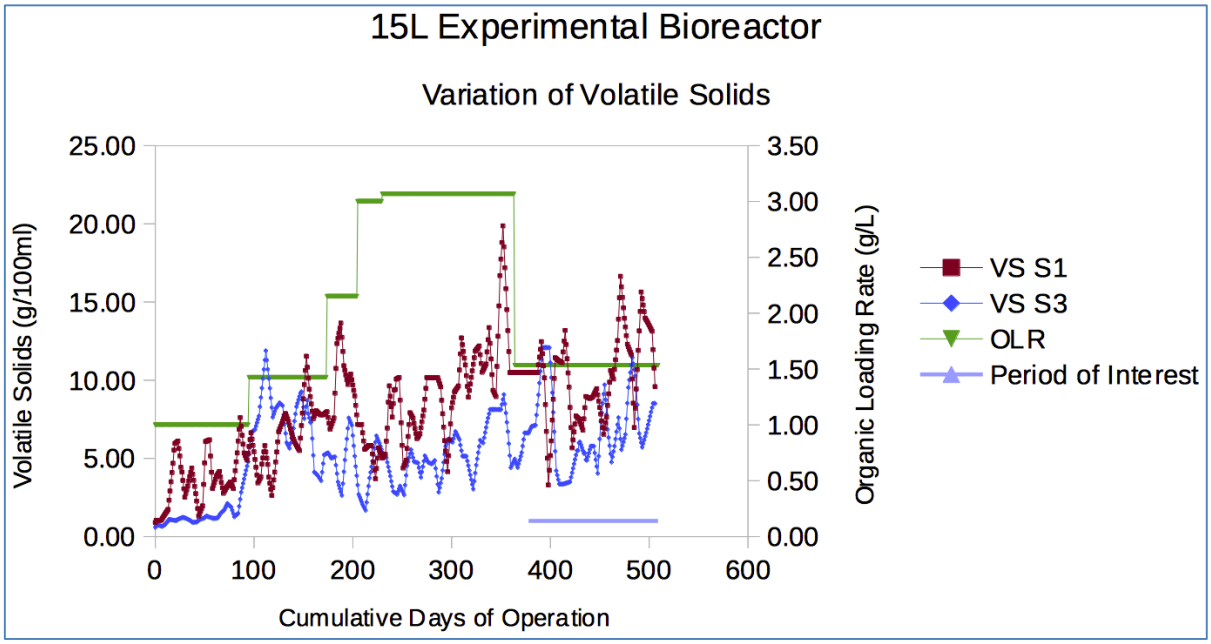

FIGURE 18: VARIATION OF VOLATILE SOLIDS OF THE EXPERIMENTAL SYSTEM

3.1.2c. Elemental composition (C, H, N, S)

The elemental composition of the bioreactor effluents from the first and third chambers (S1 and S3 respectively) of each bioreactor (control system and experimental system) were measured during the experimental period when the organic loading rate was held at a constant 1.5 g/l of VS per day, by pyrolysis using the PerkinElmer Elemental Analyzer Series II 2400 (Table 7). The effluent samples used for the elemental analyzes were previously tested for total solids by drying them at 105 °C for a period of time of 24-72 hours. These analyzes were performed on the bioreactor effluents collected over 18 weeks out of a total of 550 days' period of operation. The figures below (19, 20, 21 & 22) illustrate the elemental composition of effluents from each chamber in terms of carbon, hydrogen, nitrogen and sulfur during the experimental period of operation.

TABLE 7: ELEMENTAL COMPOSITION WITHIN THE BIOREACTOR STAGES

Elemental composition ± STD (%)*				
Elements	***Control bioreactor***		***Experimental bioreactor***	
	S1	**S3**	**S1**	**S3**
C	33.9± 3.20	28.1± 6.85	24.9± 4.63	22.6± 4.32
H	4.7± 0.45	3.9± 0.90	3.4± 0.54	3.3± 0.53
N	6.5± 0.64	5.5± 1.21	5.8± 2.22	5.3± 2.17
S	1.8± 1.07	2.8± 1.31	1.7± 0.57	2.1± 0.70

(*): These results of the elemental composition are reported as percent of oven-dried total solids at 105 °C. The elemental composition is a combination of the feedstock fraction plus the fraction of the synthetic seawater, Instant Ocean.

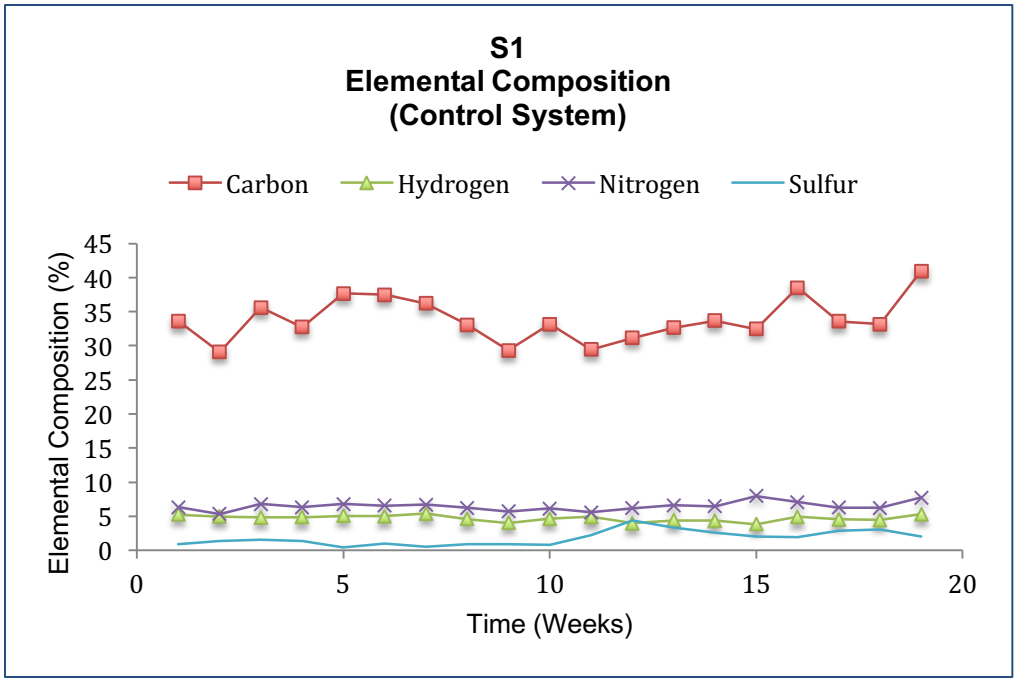

FIGURE 19: ELEMENTAL COMPOSITION OF S1 (CONTROL SYSTEM)

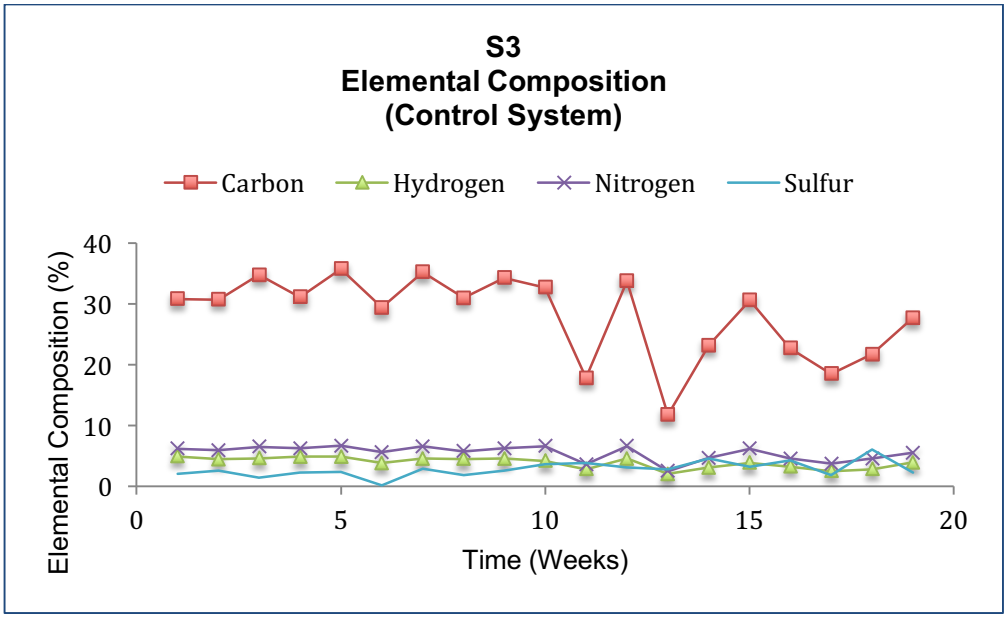

FIGURE 20: ELEMENTAL COMPOSITION OF S3 (CONTROL SYSTEM)

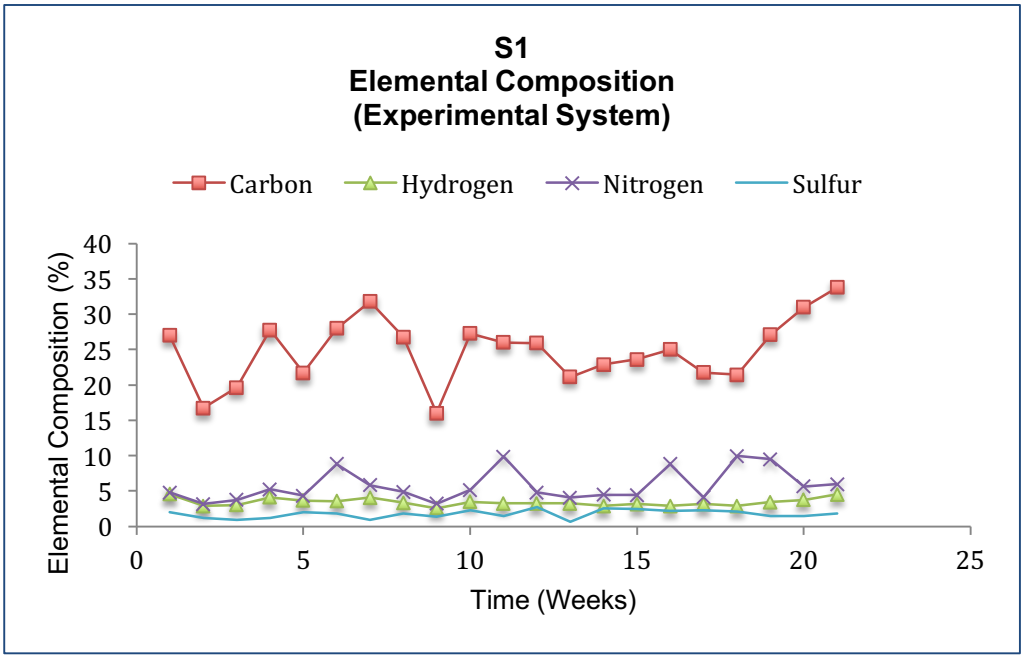

FIGURE 21: ELEMENTAL COMPOSITION OF S1 (EXPERIMENTAL SYSTEM)

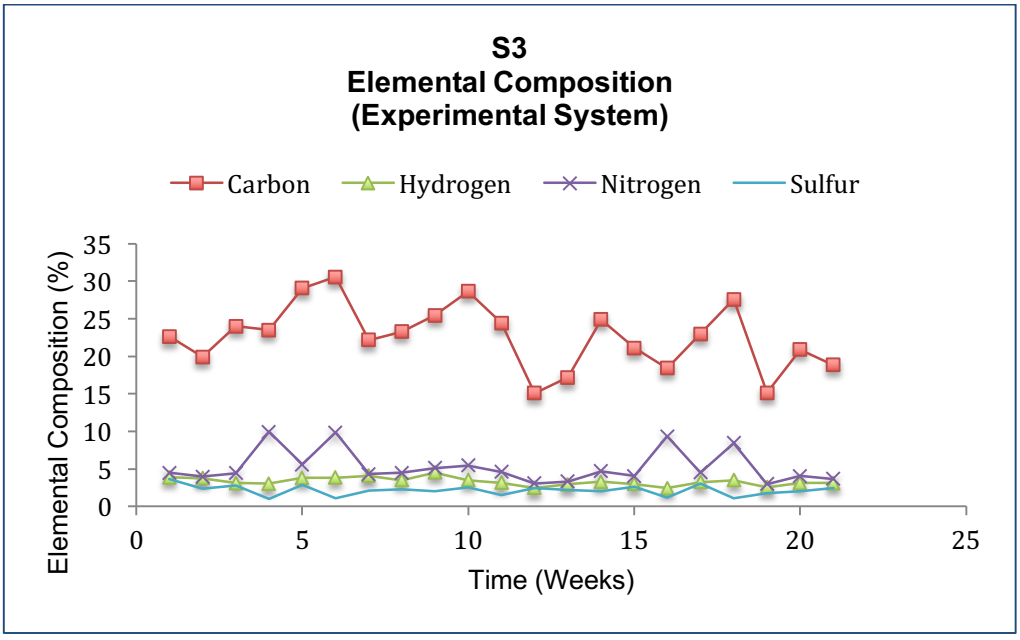

FIGURE 22: ELEMENTAL COMPOSITION OF S3 (EXPERIMENTAL SYSTEM)

3.1.2c.1. Comparison between S1 and S3 (Control system)

The reduction of organic matter content during the anaerobic degradation of the *Sargassum spp*. in the bioreactors was evaluated throughout the experimental period of operation. This trial period covers 18 weeks of operation in which the two multi-stage bioreactors were operated under an organic loading rate of 1.5 g/l of VS per day. The samples collected during this period were tested for TS then analyzed for elemental composition. The elemental composition of the bioreactor effluents was performed by pyrolysis using a PerkinElmer 2400 Series II Elemental Analyzer, and the resulting data were statistically evaluated in order to compare the reduction rate of the organic matter from the first chamber S1 to the third chamber S3 of each bioreactor.

The average mass fraction of carbon measured in the first chamber S1 and the third chamber S3 of the control bioreactor was about (33.88± 3.20)% and (28.10± 6.85)% of total mass, respectively. The percent reduction of carbon across the system was equal to 17.1% between S1 & S3. As expected, the Student's t-test confirmed that there was a significant reduction of organic carbon content in S3 when compared to S1, with P-value = 0.0020 within a 95% confidence interval (t = 3.32).

A similar pattern was observed in terms of reduction of nitrogen on a percent dry solids basis. The average nitrogen mass fraction was (6.48± 0.64)% and (5.48± 1.21)% of total mass from S1 and S3, respectively. The percent reduction of nitrogen was equal to 15.4%. this decrease is statistically significant at a 95% confidence interval (P-value = 0.0031, t = 3.17).

The average mass fraction of hydrogen measured in S1 was (4.70± 0.45)% while in S3 this fraction was about (3.90± 0.90)% on a percent dry basis. The reduction in hydrogen mass fraction equal to 17% between S1 and S3 was significant at a 95% confidence interval (P-value = 0.0013, t = 3.48).

The average mass fraction of sulfur values were (1.81± 1.07)% in S1 and (2.83± 1.31)% in S3 on a percent of dry solids basis. The fraction of sulfur was significantly greater in third chamber S3 when compared to the first chamber S1 at a 95% confidence interval (P-value = 0.0124 and t = 2.63).

the fact that the sulfur concentration increased by 56% between S1 and S3 suggests that the system had not yet a true steady state during the 18 weeks of the experimental period, although the high concentration of inorganic sulfates in seawater (28 mmol/kg) complicates the interpretation.

3.1.2c.2. Comparison between S1 and S3 (Experimental system)

The elemental composition of the effluents of the experimental bioreactor was also examined. As expected, and similar to the results reported above for the control system, the weight fractions of CHNS were lower in the third chamber S3 of the bioreactor during the 18-week trial period in comparison to the first chamber S1. These results are consistent with the hypothesis that through the anaerobic degradation of the sargassum feed more of the organic carbon was available in the third chamber for the methanogens because the hydrolytic bacteria in the first two chambers slowly transformed the energy feedstock into a digestible form. As a result, the mass fraction of CHNS fell and the mass fraction of ash (mineral) rose between S1 & S3, which confirms the changes detected in volatile solids across the system.

The average values of the carbon mass fraction in S1 and S3 were (24.88± 4.63)% and (22.64± 4.32)%, respectively. Although a reduction of the carbon fraction equal to 9% from S1 to S3 was observed, this reduction was not statistically significant at the 5% level (t-test = 1.616, P-value = 0.1139).

The average mass fraction of nitrogen was (5.75± 2.22)% and (5.25± 2.17)% of total mass from S1 and S3, respectively. At the 5% level, the observed reduction of nitrogen fraction equal to 8.7% was not statistically significant between the first chamber and the third chamber (P-value = 0.4667 and t = 0.734).

The average mass fraction of hydrogen values were (3.42± 0.54)% and (3.33± 0.53)% of total mass from S1 and S3, respectively. At the 5% level, the observed reduction of the hydrogen mass fraction equal to 2.6% from the first chamber to the third chamber of the experimental bioreactor was not significantly different (P-value = 0.5799 and t = 0.558).

The average mass fraction of sulfur values were (1.75± 0.57)% and (2.15± 0.70)% of total mass from S1 to S3. At the 5% level, the sulfur fraction was significantly higher in the third chamber S3 when compared to the first chamber S1 (P-value = 0.0489 and t = 2.0316). The increase in the sulfur mass fraction between S1 and S3, equal to 22.8%, suggests that the bioreactor had not yet achieved true steady state during the 18-week experimental run, although the high concentration of inorganic sulfates (28 mmol/kg) in seawater complicates the interpretation.

3.1.2c.3. Mass balance of carbon

The elemental analysis carried out through the Perkin Elmer Series II 2400 Elemental Analyzer provided relevant information regarding the mass balance over the 18-week experimental period throughout the AD process and included the fraction of CHNS before and after digestion. These results are depicted in table 8 and 9. The mass balance of the systems may be defined based on the following equations:

(a). Mass balance Equation:

$$\frac{mass\ (g)}{Day} Fed = \frac{mass\ (L)}{Day} Biogas + \frac{mass\ (g)}{Day} Effluents\ (s) + \frac{mass\ (g)}{Day} Effluents\ (l)$$

$$\frac{mass\ (g)}{Day} Fed = \frac{mass\ (g)}{mass\ of\ 1\ mole \frac{(g)}{Day}} Biogas + \frac{mass\ (g)}{Day} Effluents\ (s) + \frac{mass\ (g)}{Day} Effluents\ (l)$$

Using the ideal gas law (equation b), the number of moles (*n*) of each gas component was calculated. Subsequently, the molar amounts were converted to mass equivalents in grams.

(b). Ideal Gas Equation:

$$pV = nRT$$

Where:
n: Number of moles (mol)

p: Atmospheric pressure at STP (atm)

V: Volume measured (L)

R: Universal gas constant (8.3144 $JK^{-1}mol^{-1}$)

T: Temperature (K)

TABLE 8: MASS BALANCE OF CARBON OF THE CONTROL SYSTEM

Element	Elemental Composition (%)			
	Feed (g)	Biogas ($CH_4 + CO_2$)	Effluents (Liquid)*	Effluents (Solid)
C	100	19.76± 0.01	--	85.56± 6.85
H	16.7	4.94± 0.01	--	11.87± 0.90
N	18.8	Nd	--	16.68± 1.21
S	2.6	Nq	--	8.61± 1.31

(Nd): Not detected
(Nq): Not quantified
(*): Not Analyzed

TABLE 9: MASS BALANCE OF CARBON OF THE EXPERIMENTAL SYSTEM

Element	Elemental Composition (%)			
	Feed (g)	Biogas ($CH_4 + CO_2$)	Effluents (Liquid)*	Effluents (Solid)
C	100	11.87± 0.01	--	68.94± 4.32
H	16.7	2.96± 0.02	--	10.14± 0.53
N	18.8	Nd	--	15.98± 2.17
S	2.6	Nq	--	6.54± 0.70

(Nd): Not detected
(Nq): Not quantified
(*): Not Analyzed
[These results are based on the repartition of the elemental composition measured in the energy feedstock, biogas produced and the total solids content of the bioreactor effluents].

As observed in tables 8 & 9, the biodegradability of the sargassum blend was very low. Only a small fraction of the organic carbon of the feed was converted into methane: 9.8% of the carbon from the feed was converted into methane for the control system, compared to 6.0% for the experimental system. This may have resulted on the harvesting process of the energy feedstock since it was collected directly from the beach as sun-dried beach wrack. The feedstock lost a significant amount of easily digestible carbon under these conditions. Unfortunately, due the high concentration of salt concentration and intense color of the aqueous samples of the effluents, we were unable to process the liquid phase of the bioreactor effluents for key chemical parameters such as sulfate, nitrate, ammonia and nitrite, using the test kits we had available in our laboratory. Furthermore, because the elemental analysis requires that the samples be completely dry, only the solid phase of the bioreactor effluents was processed through the Elemental Analyzer Series II 2400.

3.1.2d. Volatile organic acids (VOAs)

During the 18-week experimental period, the concentration of volatile organic acids in the control system varied between 404 to 775 mg/L HOAce Eq for the first chamber S1, 245 to 696 mg/L HOAce Eq for the third chamber S3 (Fig. 23). Meanwhile, for the experimental system the concentration of VOAs ranged between 301 to 864 mg/L HOAce Eq in S1 and 182 to 896 mg/L HOAce Eq in S3 (Fig. 24). As observed throughout the study, for each incremental increase of OLR in the systems, the concentration of VOAs spiked and then settled down. This phenomenon is very common in anaerobic systems. The VOAs concentration increases with the increasing of OLR (Chaisri et al., 2007). As the hydrolytic bacteria proliferate faster than the methanogens, this induces a spike in VOAs when increasing the organic loading rate.

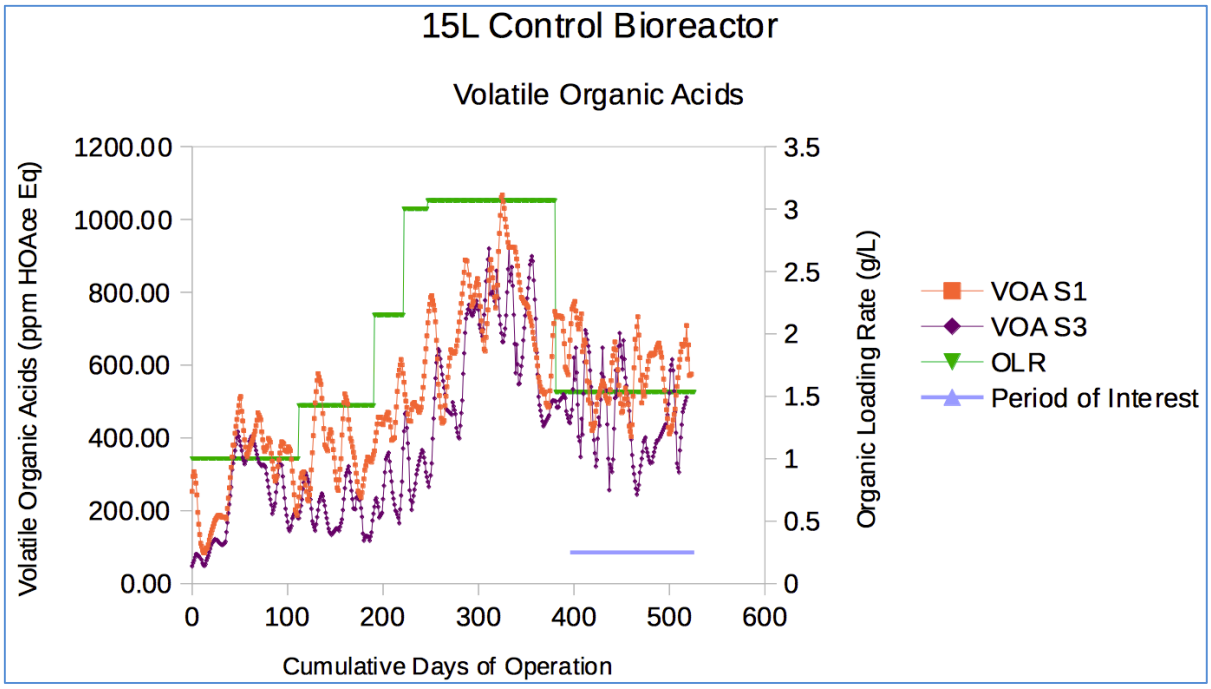

FIGURE 23: VOLATILE ORGANIC ACIDS CONTENT OF THE CONTROL SYSTEM

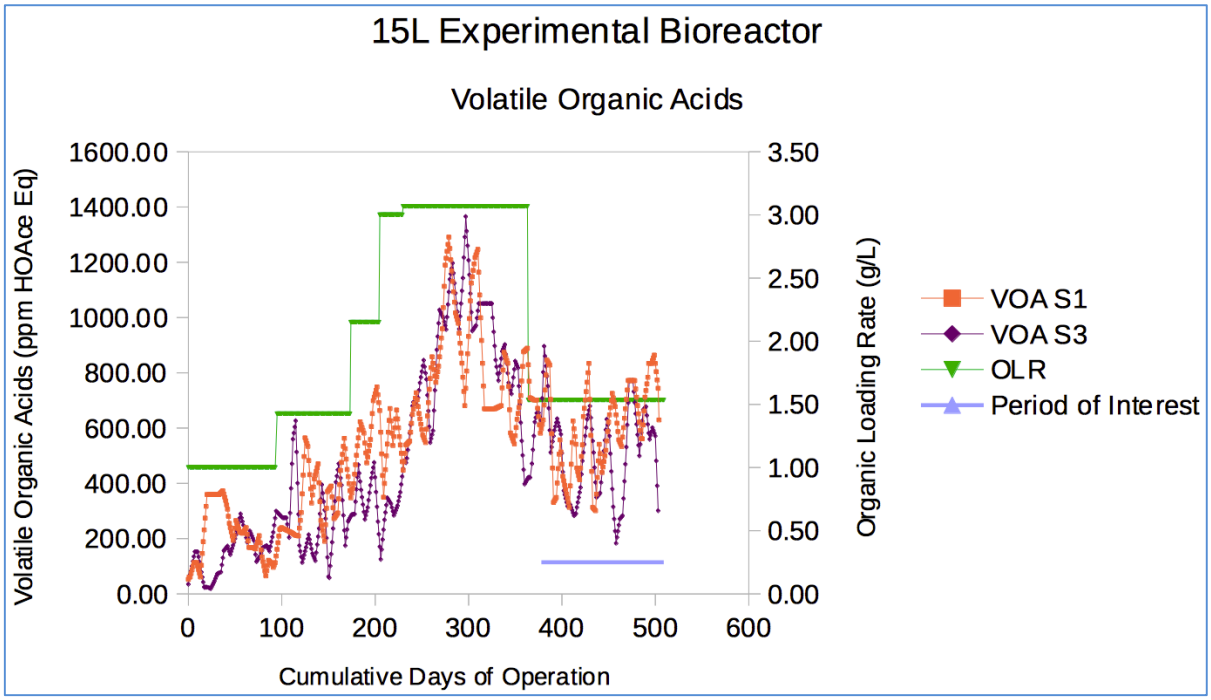

FIGURE 24: VOLATILE ORGANIC ACIDS CONTENT OF THE EXPERIMENTAL SYSTEM

3.1.2e. Biogas measurement

The volumetric biogas production from the first day of operation to the present has been logged. The daily rate of both systems (control & experimental) is shown in figure 25. For the purpose of our experiments, the biogas production rate over the course of the 18-week experimental period was evaluated in order to estimate the biodegradation capacity of the feedstock and the efficiency of our systems. The production rate was different in the control system compared the experimental system. For the control bioreactor, the volumetric production rate was 2972.42± 13.42% ml of biogas/day, while the production rate of the experimental system was 2048.73± 27.50% ml of biogas/day. The Student's t-test was highly significant (P-value less than 0.0001, t= 15.37) and demonstrated that the volumetric production rate of the control bioreactor was significantly higher when compared to the experimental bioreactor. Our results suggest that the bioreactor operating in full-strength seawater (3.5% w/w) was approximately one-third less efficient when compared to biogas production achieved in the control bioreactor operating in intermediate salinity (1.0% w/w). These observations suggest a normalized rate of 91 ml of biogas per gram of VS fed per day in the control bioreactor to a 132 ml of biogas per gram of VS fed per day in the experimental bioreactor. As expected, the control system produced approximately 30% more biogas than the experimental system. However, we need to consider the fact that we were operating with seawater, accordingly the cost may be acceptable in some jurisdictions where freshwater is scarce.

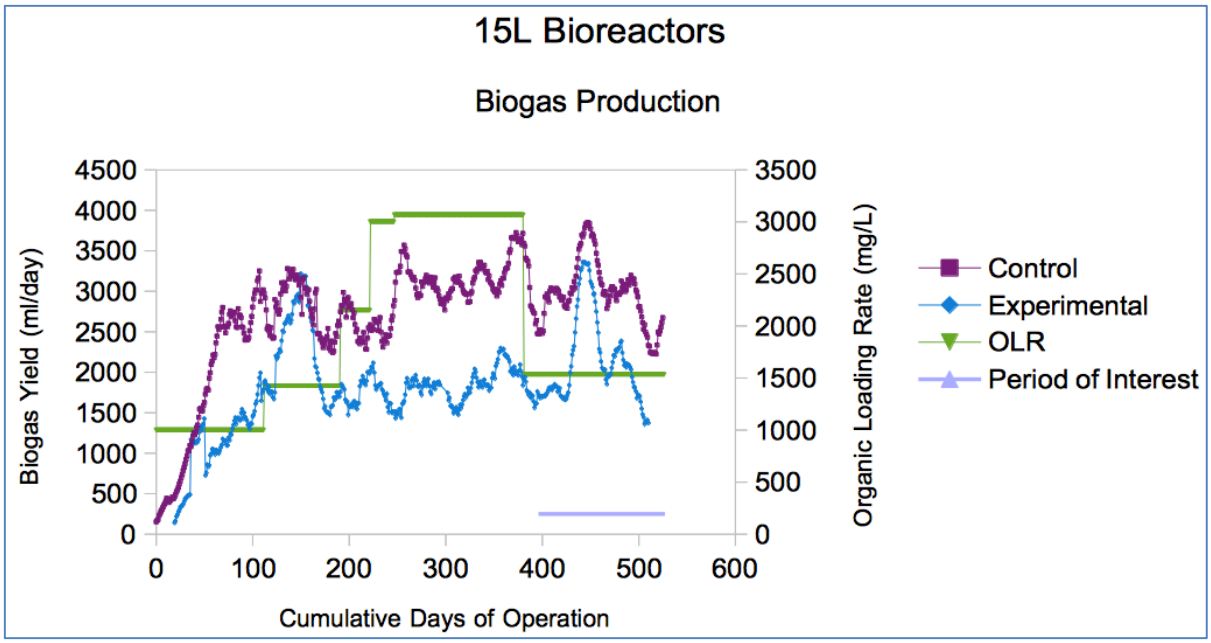

FIGURE 25: BIOGAS PRODUCTION

3.1.3. Biogas composition

The biogas composition of both control system (1.0% w/w) and experimental system (3.5% w/w) was evaluated during the study by Fourier Transform Infrared Spectroscopy (FTIR); using a Thermo Nicolet NEXUS 470 FTIR (Figures 26 & 27). The composition of the biogas samples was calculated based on the calibration curve of the instrument for both methane and carbon dioxide. The instrument was calibrated using a calibration standard of known composition. The regression line of the calibration curves of peak area versus partial volume of each principal component gave excellent linear correlations for both compounds (R^2 = 0.982 for methane; R^2 = 0.989 for carbon dioxide).

The control bioreactor presented a biogas composed of (53.82± 5.10)% CH_4 and (38.53± 4.31)% CO_2, meanwhile, the composition of the experimental system was (61.28± 1.70)% CH_4 and (34.64± 6.25)% CO_2. We focused on methane and carbon dioxide because they represent the two main components of the biogas. However, some others gaseous compounds, especially hydrogen sulfide, and water vapor, as well as other trace gasses must be present in the biogas samples.

Unfortunately, we did not have access to functional FTIR during the 18-week study period, so these tests were carried out months later. Considering that the composition of the biogas produced was extremely important for monitoring our bioreactor performance, we processed the biogas samples as soon as the instrument was made available, but unfortunately, there is a 15-month lag between the end of the study period (18-week experimental run) and the period during which the biogas composition was analyzed.

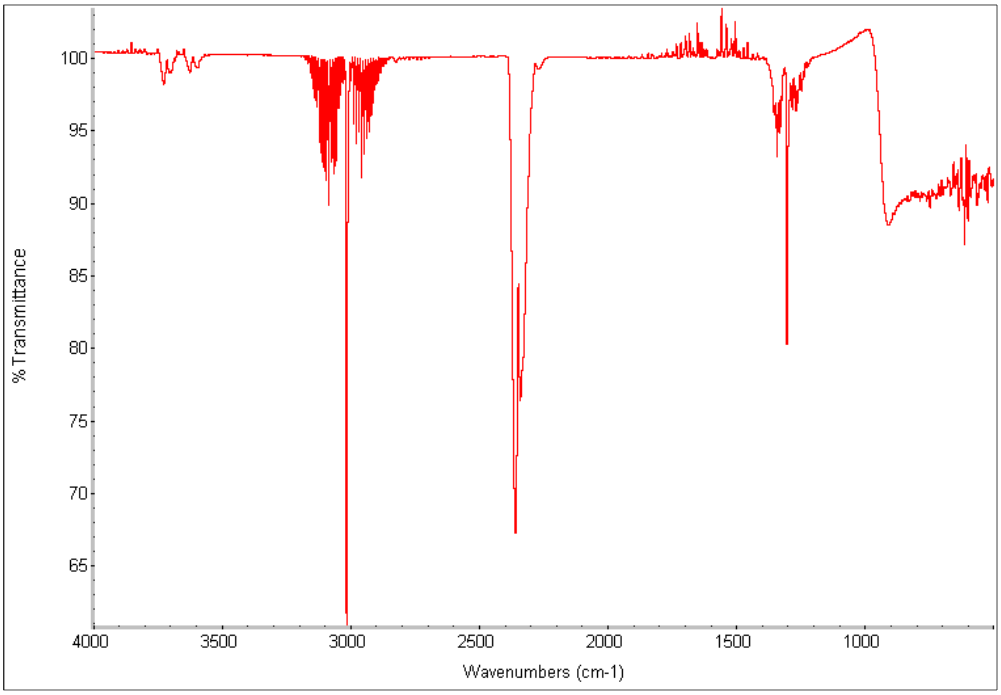

FIGURE 26: FOURIER TRANSFORM INFRARED SPECTRUM OF A REPRESENTATIVE BIOGAS SAMPLE (CONTROL SYSTEM)

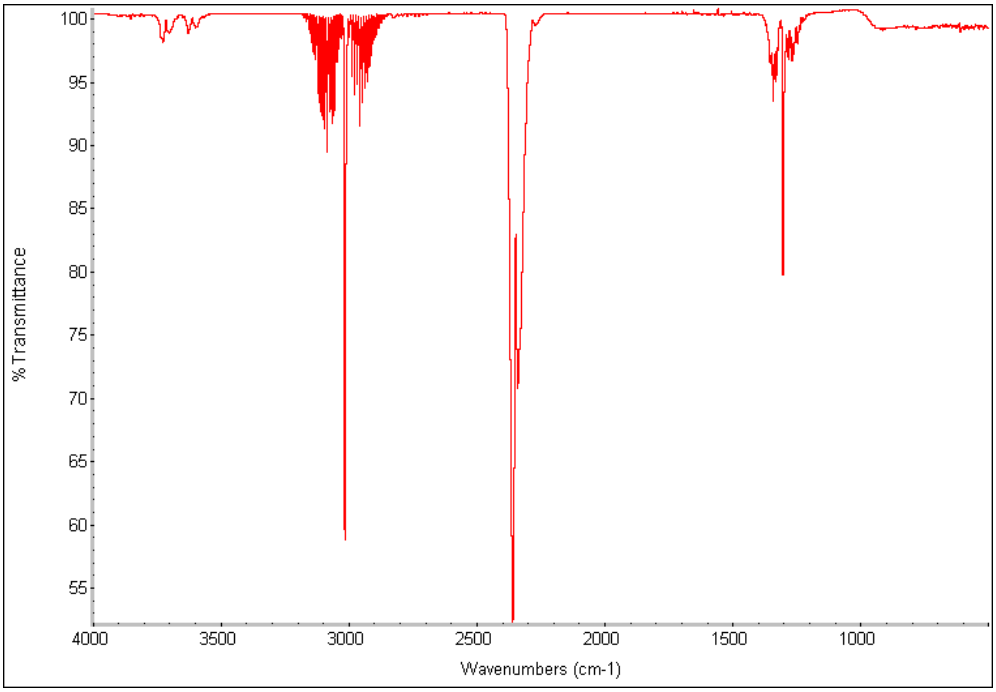

FIGURE 27: FOURIER TRANSFORM INFRARED SPECTRUM OF A REPRESENTATIVE BIOGAS SAMPLE (EXPERIMENTAL SYSTEM)

Each peak depends on the absorbance capacity of the given compound.
CH_4: 3,000 - 2,850 cm^{-1} and CO_2: 2,300 - 2,500 cm^{-1}

The following table summarizes the performance of the bioreactors based on the biogas composition measured throughout the 18-week experimental period (Table 10).

TABLE 10: PRODUCTIVITY OF THE BIOREACTORS

Bioreactors	Experimental values*					
	Salinity	***OLR***	***Biogas yield***	***Biogas composition***		***CH_4/CO_2***
	(% w/w)	***(g/L)***	***(ml/g VS fed)***	***(%)***		***Ratio***
				(CH_4)	*(CO_2)*	
Control	1.0	1.5	132.42	53.82± 5.10	38.53± 4.31	1.4
Experimental	3.5	1.5	91.05	61.28± 1.70	34.63± 6.25	2.5

(*): These analyses were performed using a Thermo Nicolet NEXUS 470 FTIR.

The following table summarizes the production rate of selected marine biomass for biogas production.

TABLE 11: ENERGY BIOMASS, PRODUCTIVITY AND ELEMENTAL COMPOSITION

Biomass	**Harvesting**	**Elements (% dry weight)**				**Methane yield**	
(Marine)	**(***Condition***)**	*C*	*N*	*H*	*S*	*(ml g^{-1} VS added)*	
***Sargassum fluitans* Δ**	Fresh	46.7	1.7	6.3	Nq	150-200	
***Sargassum pteropleuron* Δ**	Fresh	45.6	1.3	5.8	Nq	410-450	
***Gracilaria* Δ**	Fresh	44.3	6.1	6.8	Nq	280-400	
Sargassum spp.*	Sun-dried	32.8	6.2	5.5	0.9	***Control***	***Experimental***
(Beach wrack)						67.5-75.5	47.3-57.4

Nq: not quantified
(*): Experimental values
(**Δ**): Bird et al. (1990)

3.1.4. Calorific values

Seaweeds species suitable for biogas production should display high productivity with regard to biomass yield. According to Alburo et al. (2010), the calorific value of a material is defined as the amount of heat generated by this material during combustion. Table 12 summarizes the calorific values of some common fuels and the estimated calorific values of our biogas. The energy value of biogas depends primarily on the methane content and the methane to carbon dioxide ratio. In addition, the biogas content is closely linked to the feedstock composition, the production process, and others biochemical factors such as temperature, pH, salinity, etc. As the main component of biogas, methane (CH_4) is considered to be a good cooking fuel since it is colorless, odorless and flammable gas. H_2S, while also a fuel, is toxic and has a very disagreeable odor.

TABLE 12: CALORIFIC VALUES OF COMMON FUELS

Fuel type	Calorific values			
	Pure methane (*)	***Biogas from AD (τ)***		***Natural gas***
		Control	***Experimental***	
% Methane	100%	53.82%	61.28%	
Calorific values (kJ/m^3)	35,815	19,276	21,949	29,870

(*): Salunkhe, Rai, & Borkar (2006)
(τ): Calculated values

PART IV: DISCUSSION

4.1. Bioreactor performance and monitoring

Throughout the course of the 18-week period of interest during which the operation was quantified, the two multi-stage bioreactors were operated under an OLR of 1.5 g/L VS of fed per day. Different control parameters were gathered four times per week, including total solids (TS), volatile solids (VS), pH, and alkalinity. In addition, the elemental composition, biogas production, and composition were analyzed in order to assess the performance of the bioreactors. The *Sargassum spp.* beach wrack blend was also characterized to better understand its potential as energy feedstock and its anaerobic degradation throughout the study.

4.1.2. Alkalinity and pH

As reported in the AD literature, anaerobic microorganisms, principally methanogenic bacteria, demonstrate a characteristic sensitivity to extremes of pH. The best pH range appears to be around neutrality while the range between 6.5 and 7.8 is considered to be optimal for AD systems (Adekunle & Okolie, 2015; Rajagopal et al., 2013). Because the hydrogen ion concentration may affect the biochemistry, and hence the microbial activities of the system, maintaining a suitable and stable pH within the bioreactor turns out to be a crucial priority to enhance and ensure the efficiency of the methanogenic community. Throughout the 18-week period of operation, both bioreactors were very stable regarding the pH, and they have shown healthy pH values which held a range of 6.5 and 8.0 (Fig. 13 & 14). One of the serious issues facing operators of anaerobic systems such as municipal WWTPs is the accumulation of VFAs which acidify the bioreactor and that leads to the inhibition of the methanogenic bacteria, and in some cases, to the complete cessation of the microbial activities within the systems (Lettinga, 1995; Poggi-Varaldo et al., 1997). Our data confirm that seawater offers a huge buffering capacity due to the fact it is rich in calcium and magnesium carbonates, which help

to neutralize the volatile organic acids produced during the hydrolytic phase, thus eliminating the need to implement pH control systems in the bioreactors.

The experimental system was found more alkaline than the control system during the study. As expected, the higher concentration of alkalinity in the experimental bioreactor was most likely due to the seawater composition itself that is rich in calcium and magnesium forming alkaline carbonate salts.

4.1.3. Mixing and recirculation

The performance of the anaerobic digestion process can be improved by mixing, which consequently allows a better interaction between the organic matter and the bacteria communities. Naturally, this process occurs in AD systems because of continuous rising gas bubbles within the bioreactor. This natural process might be sufficient at very low levels of OLR for laboratory-scale studies; however, at higher levels of organic loading rate, this natural mixing procedure is considered to be rate limiting to ensure a stable digestion process and reactor performance (Stafford, 1982). On the other hand, according to Smith et al. (1996), excessive mixing may affect the growth rate and distribution of the bacteria within the sludge, the substrate availability, and subsequent biogas production rate. Therefore, it is necessary that a mixing procedure has to be fitted to create a more homogeneous aqueous environment in the bioreactor so that the system volume can be fully exploited during the process. Thus, for future seawater bioreactor designs, a well-controlled mixing system should be incorporated in order to enhance a better partitioning of the microbial communities throughout the system. During the 18-week experimental period, the recirculation rate of each bioreactor was a total of 500 ml of sludge per day, that represents around 3.3% of the total working liquid volume as suggested by Milledge et al. (2014). As observed during our research, sedimentation was the principal issue impeding a homogenous mixing of the chambers since we only used intermittent procedures to periodically enhance the contact of microorganisms with substrate (manual powered peristaltic pump, wooden rocking platform, recirculation). The lack of satisfactory mixing led

to some bias in sample composition for samples drawn from the bioreactors – they were enriched for sediment compared to the samples drawn from the algae feedstock, which were more nearly homogeneous. This technical problem leads to an underestimation of bioreactor performance. Future designs with improved agitation system will eliminate this problem.

4.1.4. Total and volatile solids (TS & VS)

The total and volatile solids content are critical control parameters for wastewater treatment plants (WWTPs), and may affect the biogas production efficiency when unbalanced (APHA, AWWA & WEF, 2005). Total and volatile solids of different collections of the energy feedstock, *Sargassum spp.*, were evaluated. The sargassum blends used throughout this project have been collected over two years of operation (from November of 2012 to January of 2014). Based on the data that we have processed; significant differences were observed in VS between the collections of sargassum blend. This situation resulted in the fact that the feedstock was harvested in different periods of time and various locations under different environmental conditions. The harvesting was performed directly from the beaches (beach wrack), we expected that these stocks would be depleted in an easily digestible organic matter and would, therefore, be more difficult to degrade in anaerobic reactors – and these expectations were confirmed by our data. However, the differences observed in the VS values between the collections may be due to the method used to carry out the VS analysis. By using the same ceramic crucible to determine the TS and VS content successively, this procedure may underestimate the variability of the measurements, consequently, overestimate the significance of the difference in measurement.

As expected, the accumulation of TS in the effluents was greater in the first chamber S1 of both bioreactors (control & experimental), when compared to the third chamber S3. The control bioreactor was more efficient in terms of VS removal rate than the experimental bioreactor. However, because seawater was used during bioreactor operation, the volatile solids test measures a combination of organic matter plus calcium and magnesium carbonates, and so the correlation

between the VS values and the organic load is weaker than would be the case in a traditional anaerobic wastewater treatment plants (EPA, 2006).

4.1.5. Volatile organic acids (VOAs)

As observed throughout the study, for each incremental increase of OLR in the systems, the concentration of VOAs induced a spike followed by a settling down period and subsequently a slight drop of the pH. This tendency is a common particularity of AD systems. The concentration of volatile organic acids (VOAs) in AD processes is a key factor that provides information about when the steady state of operation has been being reached. However, throughout our research, we never reached a true steady state of operation. This is one of the limitations of our study that may lead to the underestimation of the performance of our bioreactors. Although the VOAs concentration was higher in the S1 stage of both bioreactors, as observed in figures 23 & 24, the variability in the data was quite large. The noisy data were probably due to the technical limitation of the system used for agitating the bioreactors. The system relied on manually powered peristaltic pumps for re-suspending the solids once per day. This intermittent mixing may have added to the variability inherent in the AD process.

4.1.6. Elemental analysis and mass balance of carbon

The chemical composition of the feedstock in terms of carbon, hydrogen, nitrogen and sulfur is very useful in the evaluation process of digester performance as well as the characterization of the organic matter as a carbon source for AD systems. The partition of valuable macronutrients throughout the anaerobic digestion process is a major feature to understand and may help improve the performance of the bioreactors. The efficiency of AD systems depends on their capacity to transform organic matter into microbial biomass plus biogas as end products. This is conditioned by various factors including temperature, salinity, feedstock biodegradability, etc. As observed during our study, only a small fraction of the organic matter was converted into methane. The data showed that

only 9.8% of the total carbon of the feed was transformed to methane for the control bioreactor, compared to 6% for the experimental system (Table 8 & 9). These numbers confirmed the poor biodegradability of our substrate observed by Díaz-Vázquez (2015) (Personal communication). As noted in section 2.2.1, the energy feedstock was harvested onshore (beach wrack), and dried by the sun, which suggests that a significant part of the available digestible nutrients of the *Sargassum spp.* (the energy feedstock) may have lost.

One of the major advantages of multi-stage anaerobic digestion systems is that they are designed to enhance the optimization of the AD process and improve the specific conditions under which the process takes place. Ideally, microbial activities naturally partition in the different stages allowing close to optimal conditions for each of the metabolic processes (sulfur reduction, hydrolysis, acidogenesis, acetogenesis, and methanogenesis). Our multi-stage bench-scale anaerobic bioreactors (MSBSABs) have been designed with the idea that it would allow a significant reduction of sulfur early in the process, and thus, protect the methanogens in S3 from exposure to excess hydrogen sulfide. However, our data showed an accumulation of total sulfur in the solids in samples drawn from the third chamber of both bioreactors. Those observations suggest that the bioreactors had not yet achieved true steady state during the 18-week experimental period, and then the competition between methane-producing bacteria and sulfate reducing bacteria was predominant. The sulfur must be released by the SRBs in the form of hydrogen sulfide (H_2S) or sulfide (HS^-). This may be expressed by the following equation:

$$SO_4^{2-} + 4H_2 \rightarrow S^{2-} + 4H_2O$$

4.2. Biogas production

The productivity and efficiency of AD systems depend on various physical and biochemical factors including digester design, temperature, harvesting condition, feedstock biodegradability, mixing and substrate availability, among others. During the 18-week experimental period, a higher biogas production was registered in the control bioreactor as expected (Fig. 25). The control system

produced approximately 30% more biogas than the experimental system. However, we need to consider the fact that we were operating with seawater, accordingly the cost may be acceptable. Similar results have been observed by Marty et al. (2014) (Personal communication) using fresh *Sargassum spp*. to produce biogas under high salt conditions. Their study revealed similar tendencies in their 1.0% w/w anaerobic digester, which production was 30% greater than their 3.0% w/w system. Their anaerobic systems were fed with fresh *Sargassum spp.* that was immediately washed, oven-dried and milled after being harvested from the sea. Contrary to the freshly raw materials used in their study, our feedstock was collected off the beach and already dried by the sun (beach wrack), which must have reduced available organic carbon and loss of valuable nutrients that might promote the microbial activities within the bioreactors. Despite these differences, similar results were obtained from both studies in terms of the relative yield of biogas in high salt versus intermediate salt bioreactors.

The methane yields were lower using dry *Sargassum spp.* (beach wrack blend), with a compositional chemistry relatively lower in comparison to other fresh marine species used in AD systems by Bird et al. (1990). Our data suggest that the biodegradability of the energy biomass (beach wrack blend) may have been impacted by the harvesting process, and also by the potential inhibitory capacity of seawater (Table 11). We already knew that by operating with seawater rather than freshwater we would sacrifice a fraction of the methane production capability of our digesters. However, this price may be acceptable when considering the advantage of eliminating the requirement of freshwater for AD operation, especially in island jurisdictions where freshwater is scarce. The economic viability of operating in full-strength seawater has to be addressed at a larger scale, but for some locations, it may be interesting to consider operating a biogas digester in seawater.

4.2.1. Biogas composition

The characterization of the biogas produced through this study was crucial to quantify the efficiency of our bioreactors. Unfortunately, due to technical issues beyond our control, the biogas composition study was carried out 15 months after the 18-week trial period. The FTIR analysis of the biogas samples from both digesters (intermediate salinity 1.0% w/w and high salinity 3.5% w/w) showed the methane composition in Table 10. The methane and carbon dioxide percentages for the control system were about (53.83± 4.31)% and (38.53± 5.10)% respectively, while the biogas composition of the experimental system was about (61.28± 1.70)% methane and (34.64± 6.25)% carbon dioxide. The fraction of methane in both bioreactors was quite similar or better than those reported for more traditional AD process. Although there was a time lag between the 18-week trial period and the 15 months when biogas composition was analyzed by FTIR, there is nothing in the operational procedures of the bioreactors that would suggest a major shift in biogas composition between the two study periods.

4.4. Concluding statement and recommendations

The following conclusions emphasize the potential of the marine macroalgae, *Sargassum spp.,* to serve as a potential energy feedstock for biogas production in anaerobic digesters operating under full-strength seawater. This study was based on monitoring two multi-stage bench-scale anaerobic bioreactors (MSBSABs) with an operating volume of 15 liters. The study was conducted over the course of an 18-week trial period at laboratory-scale and involved sun-dried sargassum blend collected onshore from three northern Puerto Rican beaches. Data from the experimental period of operation supports the conclusion that our bioreactors present similar behaviors to traditional anaerobic freshwater systems. AD systems can play a major role in the production of renewable energy and also provide tools for suitable management and recycling of organic nutrients as fertilizer. However, before full implementation of a commercial algae-based energy system, some additional questions will have to be addressed including (i) the economic viability of this technique at industrial scale, (ii) mixing of the bioreactors, (iii) harvesting and processing sargassum at an industrial scale, and (iv) many other questions related to scaling up a laboratory system, including the economic feasibility of operating AD systems using seawater and biomass availability.

1. As expected, the concentration of volatile organic acids (VOAs) was consistently higher in the first chamber S1 than the third chamber S3 of both bioreactors (control system and experimental system). Previous studies related similar scenarios in multi-stage freshwater AD systems. Typically, the multi-stage AD process provides a certain degree of improved control of the rate of the hydrolysis and the methanogenic phases by permitting some natural partitioning of microbial populations and metabolic activities. As the breakdown of polymers occurs early during the AD process, especially, during the hydrolytic phase, then the bulk of methanogenesis occurs in the later stages providing improved yield when compared to single-stage bioreactors.

2. The biogas yield, expressed as liter per gram of VS added per day (volume biogas/g VS fed per day), was found to be significantly higher in the intermediate salinity bioreactor compared to the high salt experimental reactor. This higher performance was expected, and may be due to the inhibitory potential of sulfate and high sodium concentrations in the experimental bioreactor, and the competition that may exist between the sulfate-reducing bacteria (SRB) and methane forming bacteria in the system for carbon. We observed that by operating in full-strength seawater, we sacrifice approximatively 1/3 of biogas production compared to the intermediate salinity system.

3. Previous studies have reported that *Sargassum spp.* has a very low methane production potential. As related by Díaz-Vázquez (2015) (Personal Communication), the hydrolysis of our feedstock is so low that even after having crossed all the stages of digestion, elevated polymers concentration was found in the effluents of the digesters. That suggests that the efficiency and performance of our bioreactors could be better if we used other marine biomass with better biodegradability. The use of the marine macroalgae, *Sargassum spp.,* as energy feedstock in this research was based on the fact that (i) they are easily found along the Puerto Rican coasts, (ii) offer significant advantages when compared to terrestrial biomass including their relatively fast growth rates, ease of harvesting and low production cost (Guo et al., 2012; Rajkumar et al., 2014). Furthermore, many species of Sargassum have nitrogen-fixing symbionts so nitrogen fertilization is not required for their growth (Hamersley et al., 2015; Phlips et al., 1986).

4. After over two years of operation, we have achieved a good understanding of the behavior of our bioreactors. The primary goal of the project to demonstrate that marine biomass can serve as feedstock for operating AD system in full-strength seawater was achieved. While operating under these conditions (high salinity and high sulfur environment), the bioreactors never entered a steady state of operation. Reliable steady state operation is crucial for the

evaluation of the AD process, so more research is needed. The failure to achieve steady state may be due to the low biodegradability of the sun-dried Sargassum combined with the accumulation of excess solids inside the digesters due to the poor mixing, which led to excess variability in the data.

5. As mentioned in the section 3.1.3, the analysis of the biogas samples was accomplished 15 months after the trial period. Nevertheless, the data showed no significant differences in terms of composition between the biogas samples from the two bioreactors (control system and experimental system). In spite of the delay to analyze the biogas, the biogas sampling was carried out under the similar operating conditions. Assuming that the biochemical conditions of the bioreactors were not changed drastically between the experimental period and the time where the analysis of the biogas composition was performed, the composition of the biogas should have been similar between the two periods of monitoring. However, it might be ideal processing these analyzes at the same experimental period, which may avoid (i) bias in the data and (ii) underestimation of our bioreactor performance.

6. In spite of the lower productivity of the anaerobic bioreactor operating in full-strength seawater (experimental system) when comparing to the intermediate salinity anaerobic bioreactor (control system), the biogas composition of the high salinity system (61% CH_4, 35% CO_2) was of better quality than the control bioreactor (54% CH_4, 39% CO_2). The higher methane concentration observed in the experimental system may be due to the fact that the seawater has a lot of calcium and magnesium ions, which may allow a much higher rate of carbonate precipitation in the bioreactor, providing an alternative sink for carbon dioxide and reducing the amount released in the biogas. On the other hand, by operating with seawater our experimental bioreactor, we sacrifice around 1/3 of its production capacity compared to the control system. This reduction might be acceptable considering the costs and scarceness of freshwater. The exploitation of AD technology using seawater could be very interesting in

some Caribbean islands facing the problem of algal blooms, above all in those locations where access to basic services such as energy and freshwater are very limited. This research provides us a better understanding of the AD process running in high salinity. Our data suggests that some parameters should be addressed in the future to optimize the efficiency of the AD dynamics within the bioreactors: (i) an upgraded mixing system, which would improve the contact between the substrate and the microbial consortia, (ii) we recommend the use of fresh marine biomass in lieu of dried biomass, which would enhance the biodegradability of the energy feedstock, hence increase the biogas yield, (iii) we recommend the implementation of an aerobic system to manage the effluents generated during the AD operation – we propose the aerobic treatment of AD bioreactor effluents to reduce organic load of the waste produced from the system prior to discharge, and also to transform the aerobic sludge into fertilizer for algae production to close the nutrient cycle between biomass (macroalgae) production and biomass conversion to biogas.

7. Other studies need to be done in order to assess the economic and technical viability of a commercial scale AD system fed with marine macroalgae as energy biomass. Therefore, a much larger-scale pilot plant is needed. Considering the refractile nature of the feedstock (sun-dried beach wrack), the results are promising and merit further research to optimize performance.

8. More studies regarding the microbial groups involved in the bioreactors need to be done in order to better understand the dynamics of the consortia activities involved in the anaerobic degradation process within the bioreactors while working under high salinity conditions. For the experimental purpose of this research, only the first chamber S, and the third chamber S3 were used during sampling. Previous studies regarding the microbial diversity within the bioreactors conducted by Dérilus (2014) have shown a predominance of *Bacteroidetes* and *Firmicutes* groups in both 15L systems (1.0% w/w control bioreactor, and 3.5% w/w

experimental bioreactor). The relative fraction of the operational taxonomic units (OTU's) total of these hydrolytic bacteria in S1 and S3 was greater than other anaerobic and facultative taxa involved in the AD of *Sargassum spp*. Therefore, we recommend the microbial characterization of the intermediate chamber S2, which would provide valuable information regarding the microbial dynamics within the bioreactors; particularly the microbial partition of different bacterial groups involved in the AD process.

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