Molecular biology approach to anaerobic digestion of marine biomass

Anaerobic digestion from marine materials. What makes a methanogen happy?

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Molecular biology approach to anaerobic digestion of marine biomass

by

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Oilthigh na Gàidhealtachd agus nan Eilean

...to my family and, to all my teachers and supervisors

past and present,

your collective influence has 'pushed' me thus far.

"*It is time for a sustainable energy practice which puts the environment, human health, consumers and peace first*"

— Dennis Kucinich

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Declaration

I, Oluwatosin Olubunmi OBATA, hereby declare that this thesis is a record of my own work and that I composed it. It has not been submitted in any previous application for a degree, in any other university. All sources of information are duly acknowledged.

Oluwatosin O. Obata

October 2015

Abstract

The current drive towards renewable energy has led to interest in the use of marine biomass, including seaweed. The presence of readily hydrolysable sugars, low amounts of cellulose and zero lignin enhances the suitability of seaweed for methane production, but this process has so far received little attention. As seaweeds have been shown to contain constituents with antimicrobial properties, understanding the microbial interactions in the system is crucial.

The aim of this study was to investigate the microbial community associated with seaweed anaerobic digestion in order to understand the intricate interaction between the microbial population and process functions. Selected seaweeds (*Laminaria digitata, Saccharina lattissima* and *Fucus serratus*) found commonly on the west coast of Scotland were subjected to 50-day anaerobic batch digestion using different inoculum sources. A number of molecular techniques including, denaturing gradient gel electrophoresis, quantitative polymerase chain reaction, cloning and sequencing were employed to study the microbial ecology of the seaweed fed reactors. Results show that marine sediment is a viable microbial source for efficient methane fermentation of *L. digitata* and *S. latissima* at seawater salinity level, and indicates that methane production from both seaweeds compares favourably with other types of biomass, including terrestrial crops.

Results obtained suggest that microbial numbers fluctuate during anaerobic digestion, potentially in response to substrates availability. Analysis of microbial community structure highlights temporal and spatial variations in microbial diversity within and across reactors, possibly as a result of process conditions and functions.

Identification of the dominant archaea and methanogens indicate that *Methanomicrobiales and Methanosarcinales*-related species could dominate sediment and sludge inoculated reactors, indicating the potential for utilisation of a diverse range of substrates. Results from the functional gene clone library, suggest that hydrogenotrophic, acetoclastic and methylotrophic methanogenesis could potentially be involved in methane production.

Overall, this study provides insights into the microbial ecology of seaweed anaerobic reactors and the microbial responses to changing conditions. Results highlight possible routes for optimisation of the anaerobic digestion process, which could help prevent system failure during large-scale seaweed anaerobic digestion.

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1. Chapter 1

"Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under one's observation in life"

- Marcus Aurelius

1. General introduction

1.1 Background

Energy is vital to the socio-economic development of man across the globe. It has become one of the most important factors in the determination of the level of wellbeing of countries and communities (Manzano-Agugliaro, et al., 2013). Global energy demand has drastically increased over the last century due largely to rapid increase in human population, changes in lifestyle and the dramatic increase in industrialisation across the globe, especially in developing countries (Baños et al., 2011; Manzano-Agugliaro et al., 2013). Moreover, most of the world's energy needs, which have increased more than twenty fold in the last century, are currently being met with the use of petroleum and other fossil fuels (Twidell and Weir, 2006). A report by Rao, et al., (2010), suggested that, of the over 13TW of energy used globally approximately 80% is obtained from burning fossil fuels.

1.2 Needs and drivers for alternative sources of energy

The extensive use of fossil fuels over the years has resulted in a drastic increase in the level of greenhouse gasses, especially CO_2 in the atmosphere (Fig. 1.1) which is known to be the major cause of global warming and the resultant climate change (Cubasch et al., 2013; Meehl et al, 2007). Apart from its contribution to climate change, other issues associated with this current energy use include: price fluctuations and growing environmental damage (Jegannathan, et al., 2009; Manzano-Agugliaro et al., 2013). According to Chynoweth et al., (2000), the eventual depletion of fossil fuels remains a long-term driver for the development of renewable and sustainable energy forms, while Adams, et al., (2011b) suggested climate change and energy security as the major drivers

for a shift from fossil fuels use to renewable energy. Baños et al., (2011) in a review, reported that drastic fluctuations in oil prices, uncertainties over its future availability and the recent realization of the need for clean and environmentally benign fuels constitute the main drivers for renewable energy production.

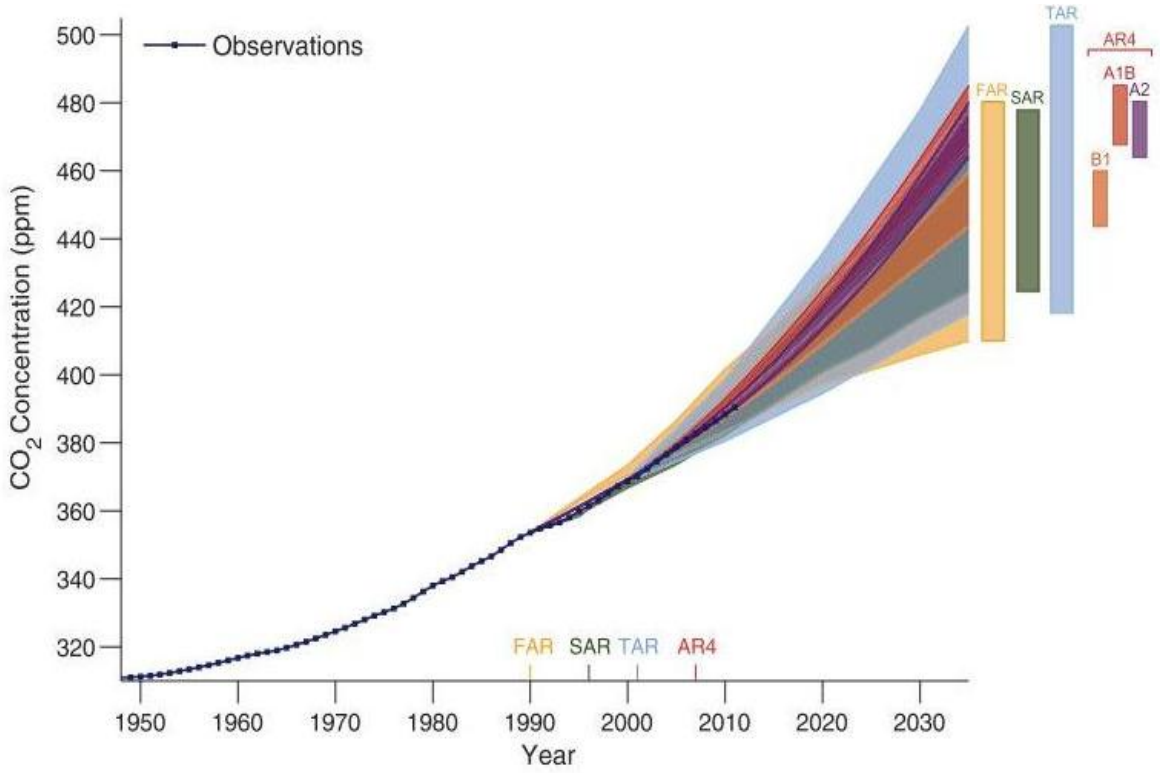

Fig. 1.1. Observed globally and annually averaged CO2 concentrations in parts per million (ppm) since 1950 compared with projections from the previous Intergovernmental panel on climate change assessments. Observed global annual CO2 concentrations are shown in dark blue. The shading shows the largest model projected range of global annual CO2 concentrations from 1950 to 2035 from FAR (IPCC, 1990); SAR (IPCC, 1996); TAR (IPCC, 2001); and from the A2, A1B and B1 scenarios presented in the AR4 (Meehl et al., 2007). The bars at the right-hand side of the graph show the full range given for 2035 for each assessment report. The publication years of the assessment reports are shown (Cubasch et al, 2013).

1.3 Sources of alternative energy

There is now growing realization that something constructive needs to be done as soon as possible to reduce greenhouse gas emissions and current dependency on the import of foreign oil, which is becoming more unreliable (Demirbas, et al., 2009; Demirbas, 2011).

Consequently, different regions and countries across the world have begun to set binding CO_2 emission reduction targets for members states. For instance, the European Union has set a target of 20% of energy use for member states to be generated from renewable sources by 2020 in order to reduce CO_2 emission (Costa, et al., 2012). In response, Spain currently generates over 25% of their electricity needs from renewable energy sources (Romero, et al., 2012), while Finland generates 20% of her electricity from biomass energy alone (Baños et al., 2011). The United States of America on the other hand is set to replace 75% of its imported oil by renewable energy by 2025 (Hahn-Hägerdal, et al., 2006), while Scotland is considering total (100%) electricity generation from renewable sources by 2020 (Scottish Government, 2012).

Due to the enormous nature of the challenge to replace fossil fuels partly or fully, with renewable and sustainable energy sources, renewable energy is now being sourced from various available materials as long as its economic viability can be achieved. A range of renewable energy sources are currently being considered including, hydropower, geothermal, solar, biomass, wind and marine energies. This range of renewable energy sources currently supply about 14% of the total world's energy demand, but is projected to reach 30-80% by 2100 (Manzano-Agugliaro et al., 2013). There are, however, peculiar challenges associated with the application of the respective sources of renewable energy. The major issues being that many are intermittent and are only suitable for the production of heat and electricity (Adams, et al., 2011a).

1.4 Biomass energy

1.1.1 Why biomass

Biomass energy is receiving increasing attention due in part, to its availability and ease of utilisation (Twidell and Weir, 2006; Chang, et al., 2010; Matsui & Koike, 2010). The level

of interest in biomass energy is clearly demonstrated by the huge number of scientific publications on biomass energy in relation to other sources of renewable energy in the last 30 years (Fig. 1.2) (Manzano-Agugliaro et al., 2013)

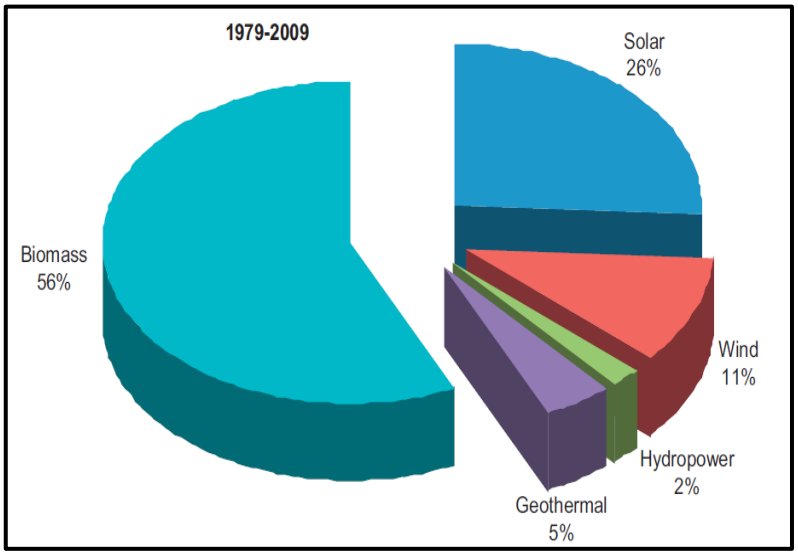

Fig. 1.2. Distribution of scientific publications (1979–2009) per renewable energy (Manzano-Agugliaro et al., 2013)

According to (Chynoweth et al., 2000) and (Ross, et al., 2008) biomass energy is not only a 'greener' source of energy, but could also mitigate atmospheric CO_2 levels as a result of its closed balanced carbon circle. Biomass is important to the renewable energy mix because it is capable of providing stored means of electricity and heat production as well as its conversion to energy fuels (Adams, et al., 2011a). As such, biomass remains one of the most promising renewable energy sources apart from the technical and economic issues, which need to be overcome for its large-scale exploitation (Baños et al., (2011). Of the main renewable energy sources such as hydro, solar and wind power; biomass energy is the most economical as it requires less capital investment and production cost (Rao et al., 2010), and due to its widespread distribution in nature, different countries across the world could exploit it to reduce their dependence on foreign oil import (Jegannathan et al., 2009; Khalid, et al, 2011).

1.4.1 Marine biomass

Although marine biomass accounts for over 50% of global primary production, little is used as a renewable energy source compared to terrestrial biomass (Adams, et al., 2011a). According to Aresta, et al., 2005) and Ross et al., (2008), marine biomass represents the most readily available resource that could be utilised for energy production on a large scale with little or no environmental costs.

The term algae (marine biomass) describe a large and diverse assembly of eukaryotic organisms that contain chlorophyll and can carry out oxygenic photosynthesis (Lee, 1999, Horn, 2000). Algae are diverse in terms of size, shape, colour and habitats with an estimated 1-10 million species worldwide (Lee, 1999; Barsanti and Gualteri, 2006). Broadly, there are two types of marine biomass or algae namely, micro- and macro algae (commonly called seaweeds).

Microalgae are microscopic unicellular plants found in both fresh and salt-water environments. They could easily be cultivated for oil production, which can be converted to energy and other applications. However, the process of harvesting microalgae is difficult and energy intensive, due to the small particle size (Demirbas, 2010; Demirbas, 2011). While microalgae are potential sources of bio-oils, seaweeds on the other hand are potential sources of biofuels (biomethane or biohydrogen) through anaerobic digestion (Adams, et al., 2011a).

Seaweeds are plant-like organisms, which generally live attached to rocks and other hard substrata in coastal environments around the world. They are fast growing, and produce more biomass per acre compared to any terrestrial energy crops due to their more efficient carbon fixation processes (Hanssen et al., 1987; Ross et al., 2008). They are considered suitable feedstock for biofuel production because of their abundance and hydrolysable

sugars content, with no lignin (Nkemka & Murto, 2010; Sung-Soo & Wakisaka, 2009). It has been estimated that the potential of municipal solid wastes and all other biomass combined (29.5EJ) for energy production is nothing compared to the potential of marine biomass alone (>100EJ) (Chynoweth et al., 2000). It is also important to know that seaweeds cultivation does not require arable lands like other energy crops and therefore would not in any way affect food production or require the use of limited freshwater resources (Adams, et al., 2011a; Hughes, Kelly, Black, & Stanley, 2012; Wegeberg & Felby, 2010). Seaweeds are classified into three groups based on pigmentation. These are *Rhodophyta* (red), *Cholophyta* (green) and *Pheophyta* (brown) algae (Ross et al., 2008).

1.4.2 Brown algae

Brown algae or *Pheophyta* are divided into 9 orders, 265 genera and over 1500 species. Their characteristic brown colour is as a result of large quantity of carotenoid-fucoxanthin in the chloroplasts (Lee 1999). Alginate is the main structural compound and the most abundant polysaccharide in brown seaweed. These polymers bind with metals to form gels, which serve as the structural units of algae (Gomez, et al., 2009; Larsen, et al., 2003; Li, et al., 2010). The intercellular matrix of the brown algae is dominated by alginate, the cell wall also contains cellulose, fucoidan and protein (Horn, 2000; Moen, et al., 1997; Rioux, et al., 2007). Laminarin, with molecular weight of ~ 5000Da is a storage carbohydrate in Laminaria (Adams, et al., 2011b; Rioux et al., 2007). Fucoidan is another storage carbohydrate present in brown algae; made up of sulphated fucan, it consists of fucose, uronic acids, galactose and sulphated fucose. Fucoidan is readily soluble in water and acid (Moen et al., 1997; Rioux et al., 2007). Mannitol on the other hand is a sugar alcohol, which corresponds to mannose, a 6-carbon sugar. It is made up 4-25% of dry weight of *Laminaria spp* (Adams, et al., 2011a; Horn, 2000). The order *Laminariales*, are present

around the west coast of Ireland and Scotland in large quantities sufficient for commercial exploitation (Ross et al., 2008; Adams, et al., 2011b). Kelps which belong to the brown algae family have the highest potential for large-scale cultivation and bioenergy production (Wegeberg & Felby, 2010).

1.5 Marine biomass in UK and Scotland

The abundance and wide distribution of seaweeds in the UK coastlines especially, the western isles of Scotland has rekindled the desire to harness its potential to contribute to the current renewable energy drive in the country (Adams, et al., 2011; Adams, et al., 2011a; Schiener et al., 2014). This coupled with the current proliferation of seaweed farming would make its utilisation for bioenergy production highly desirable especially for countries with large coastlines (Migliore et al., 2012). Seaweeds's rapid growth, estimated to be 3.3-11.3 kg dry weight m^{-2} $year^{-1}$, coupled with lack of competition for land with terrestrial energy crops could make it an ideal energy crop of the future (Horn 2000; Costa et al., 2012; Ross et al., 2008). In terms of abundance, there is currently an estimated 10 million tonnes of seaweeds on Scottish shores (Adams, et al., 2011b), and coupled with the huge prospects of cultivation, its potential for large scale utilisation is enormous. Therefore, exploiting all the various renewable energy mix across Scotland including energy generation abundant seaweeds could contribute to Scotland's ambitious quest to generate 100% of its electricity needs from renewable sources in 2020 (Scottish Government, 2012). The prospect of large scale seaweeds utilisation is particularly important for remote coastal areas of the Scottish isles; where there's a huge potential for energy generation from readily available marine materials thus, providing a sustainable energy source locally.

1.6 Methane

Different energy sources can be derived from (marine) biomass including; hydrogen, ethanol, methanol and methane, using different conversion processes. However, the selection of the type of product(s) and process to be employed depends on a number of factors; such as conversion efficiency, profitability, sustainability, product use and environmental impact of the production process (Chynoweth et al., 2000). Considering the factors listed above, methane production shows most potential. Technologies for methanol and hydrogen fuels production and use are not well developed while, ethanol production, which is currently attracting much attention, is encumbered with extensive feedstock treatment, high-energy requirement and low process efficiency (Adams, et al., 2011a). With lower environmental impact, increased use in appliances, industries, and power generation, coupled with higher process efficiency, methane production from biomass appears to be a very good option for energy production from biomass (including marine biomass) (Adams, et al., 2011a; Hughes et al., 2012; Migliore et al., 2012). Unlike fossil fuels, methane is carbon neutral, does not contribute to acid rain, ozone depletion or global warming, and as such could potentially play a vital role in attempts to reduce the environmental impacts of fossil fuel use. Utilisation of marine biomass for energy (methane) production is one of the key elements in sustainable and secure energy supply for Europe (Wegeberg and Felby 2010). Therefore, research activities geared towards making the entire process more sustainable and economically competitive would be a step in the right direction.

1.7 Anaerobic digestion (AD)

Anaerobic digestion is the process by which certain fermentative microorganisms convert biodegradable solids to methane and carbon dioxide in the absence of oxygen (Bouallagui et al., 2004; Bouallagui, et al., 2009; Bouallagui, et al, 2000; Costa et al., 2012; Zeng, et al., 2010). According to Migliore et al., (2012), anaerobic digestion is one of the most important biological processes for the conversion of biomass into energy sources. It is currently the most studied and most utilised process for the conversion of seaweed biomass into energy (Gurung, et al., 2012; Miura et al., 2014).

Anaerobic digestion is composed of a series of biochemical processes namely; hydrolysis, acidogeneis, acetogenesis and methanogenesis, carried out by diverse groups of microbes (Fig. 1.5) resulting in the production of energy rich methane and other valuable materials (Khalid et al., 2011; Demirel & Scherer, 2008). The first three stages are carried out by fast growing facultative anaerobes while the final stage (methanogenesis) is achieved by the activities of slow growing methanogens (Chang et al., 2010). The overall scheme of the process of anaerobic digestion of biomass is shown in (Fig. 1.5).

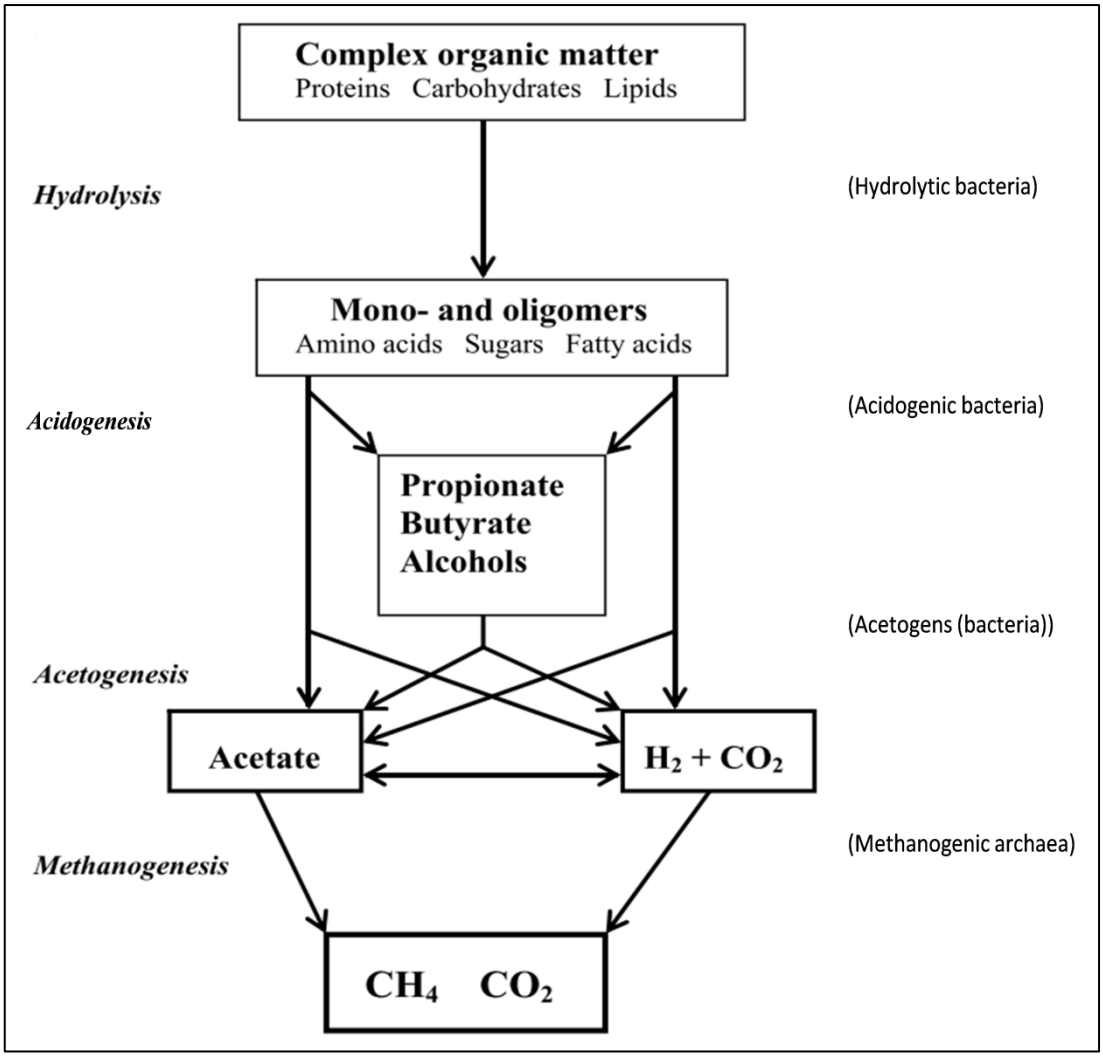

Fig. 1.3. Schematic representation of stages involved anaerobic digestion process Adapted from (Demirel & Scherer, 2008; Horn, 2000). Stages of the process in italics (left) while the type of microorganisms involved is in parenthesis (right).

1.7.1 Overview of anaerobic digestion process

1.7.1.1 Hydrolysis

The first stage of anaerobic digestion (hydrolysis) is carried out by a group of bacteria, which hydrolyse particulate complex polymers such as carbohydrates, and proteins into their respective monomers. These organisms also consume the resident O_2 left in the digester thereby creating anaerobic conditions required for methane production during the subsequent processes (Chanakya, et al., 2006; Horn, 2000; Khalid et al., 2011; Raposo, et

al., 2012; Sundberg et al., 2013). At this stage of the process, hydrolytic bacteria secrete extracellular enzymes such as cellulases, amylases, lipases and proteases for the degradation of organic polymers producing soluble compounds (Chanakya, et al., 2006; Chynoweth et al., 2000; Horn, 2000; Jegannathan et al., 2009). Hydrolysis of organic polymers can be affected by particle size of the substrates and operational temperature (Raposo, et al., 2012).

1.7.1.2 Acidogenesis

At this stage of the process, products of hydrolysis are fermented to acetic acid, hydrogen and carbon dioxide as shown in the reactions below (Gray, 2005; Chanakya, et al., 2006; Horn, 2000; Jegannathan et al., 2009; Khalid et al., 2011).

Other products includes lower weight volatile acids such as propionic and butyric acid and simple alcohols which are later converted to acetic acid in a process called acetogenesis. Formation of acetic acid is very important, as the bulk of methane production (up to 70%) from anaerobic digestion results from acetic acid reduction.

1. Acidogenic fermentation of glucose:

$C_6H_{12}O_6 \longrightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2$ (Eq.1.1)

$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3COOH + 2H_2O$ (Eq.1.2)

$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 4H_2 + 2CO_2$ (Eq.1.3)

2. Acetogenic oxidation reaction:

$CH_3(CH_2)_2COOH + 2H_2O \longrightarrow CH_3COOH + CO_2 + 3H_2$ (Eq.1.4)

$CH_3CH_2COOH + 2H_2O \longrightarrow CH_3COOH + CO_2 + 3H_2$ (Eq.1.5)

Chanakya, et al., (2006)

1.7.1.3 Methanogenesis

The final step in the process leading to methane formation is termed methanogenesis. It is carried out by two types of methanogens; acetoclastic methanogens belonging mainly to the order *Methanosarcinales* (Cho, et al., 2013; Ferry, 2010; Smith & Ingram-Smith, 2007; Song, et al., 2010; Williams et al., 2013), which produce methane by cleaving acetate into a methyl and a carbozyl group. The latter is oxidised into CO_2, to provide the reducing potential needed to reduce the methyl group into methane. The other group, hydrogenotrophic methanogens utilise hydrogen H_2 as the electron donor and carbon dioxide CO_2 as the electron acceptor to produce methane. Some of these methanogens are also able to use formate; a source of both CO_2 and H_2 (Ferry, 2010).

Overview of methane production reactions in acetoclastic (Eq.1.6) and hydrogenotrophic (Eq. 1.7) methanogenesis.

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \qquad \text{(Eq.1.6)}$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \text{(Eq.1.7)}$$

(Ferry, 2010)

Apart from these two main routes to methane production, methane can be produced by methanogens during the reduction of methylated substrates such as methanol, methylamines and methyl sulphides, a process referred to as methylotrophic methanogenesis. All methanogens are thought to be highly sensitive to oxygen concentration in the digester and operate optimally at pH range of 6.5-7.5 (Chanakya, et al., 2006; Jegannathan et al., 2009; Rincón, et al., 2008).

Since the production of methane depends mostly on acid production, and the acidogenic step could limit the supply of substrates to methanogens, it has been proposed to be the

rate-limiting step in AD. However in practice, because methanogens are slow growing and are very vulnerable to inhibition, the availability of volatile fatty acids is not necessarily the limiting factor for methane generation, and so the conditions affecting methanogens may be considered the rate-limiting step of the overall reaction (Gray, 2005; Montero, et al., 2008; Vergara-Fernández, et al., 2008).

While in theory, hydrogen should be the preferred route to methane formation as a result of it being energetically favoured, within AD systems acetate utilisation dominates methane formation (Morris et al., 2014), and therefore *Methanosarcinales* are considered to be the dominating archaea responsible for methane production in AD. *Mathanosarcinales* are the only groups of methanogens able to utilise acetate as well as hydrogen and CO_2 for methane formation. However, while investigating methanogenic population dynamics in an upflow AD sludge blanket treating swine wastewater Song, et al., (2010), reported the dominance of methanogens belonging to the order *Methanobacteriales*, suggesting hydrogenotrophic methanogenesis as the main route for methane production in that system, supporting the idea that the dominance of specific groups of microbes in AD may depend on a number of factors including substrates and other process parameters (Amani, et al. 2011; Beckmann et al., 2011; Cook, et al., 2010; Dhaked, et al., 2010; Song et al., 2010; Wilkins, et al., 2015; Zhang et al., 2012)

1.8 Anaerobic digestion of seaweeds

The accumulation of *Ulva sp.* on the beaches and bays around Japan and many parts of Europe (Matsui & Koike, 2010), results in stench odour and unsightly scenes as the seaweeds decompose around the beaches (Nkemka & Murto, 2010; 2012) and eventually pose a big threat to shellfish production near the shallow waters as well as recreational activities. Similar scenarios of huge seaweeds cast around the beaches have been reported

in Sweden (Nkemka and Murto 2012), UK (Adams, et al., 2011a), France and Italy (Matsui & Koike, 2010).

Furthermore, seaweed is currently being cultivated in Japan primarily to protect fisheries against ocean surges due to its ability to absorb nutrients from the sea and provide some remediation effects. *Laminaria* species of seaweeds have been effectively used in that project and harvested periodically. This has resulted in the creation of another problem- which is what to do with the harvested seaweeds (Matsui and Koike, 2010). These situations have led to the creation a huge stream of unwanted seaweeds, which is thought, could be used as a source of energy.

Moreover, apart from the use of 'nuisance' seaweeds for energy production, they could also be cultivated and harvested for the sole purpose of energy production, just like other energy crops. It has been suggested that the potential energy yield from anaerobic digestion of seaweeds compares favourably with that of terrestrial crops due to its high biodegradability (Hughes et al., 2012). Wegeberg & Felby, (2010), argued that methane production from seaweeds by methanogenic archaea is the most suited technology for utilising seaweeds as a source of renewable energy.

Some studies have looked at the possibility of generating renewable methane from seaweeds, but have been focused mainly on the amount of methane produced. Adams, et al., (2011b), in an experiment to determine the effect of time of harvest on methane production from *Laminaria digitata* found that the highest amount of methane (254 cm^3/gVS added) was produced from July harvest while the lowest amount of methane (197 cm^3/gVS added) was produced from the seaweeds harvested in March. Although only *Laminaria digitata* was used in that research, the same variation is likely to be observed in other seaweeds especially brown seaweeds. This knowledge would help in planning

seaweeds harvest for large scale anaerobic digestion in order to obtain optimum methane production. Raposo et al., 2011 reported methane production of 260-280 ml/gVS from *Laminaria digitata* using a particle size of 0.8mm. Furthermore, during a laboratory test of washed *Laminaria sp* and *Ulva sp*, and co-digesting with milk, using a laboratory test apparatus and porous ceramic material for fixing bacteria, Matsui and Koike (2010) reported methane production of 220 mL/g for *Laminaria sp* and 170 mL/g for *Ulva sp*. during a 100 day anaerobic digestion process. Again, a batch anaerobic digestion of *Saccharina latissima*, inoculated with bovine slurry, carried out by Vanegas & Bartlett, (2013), reported methane production of 565 ml/g VS after 109days. This is the highest methane production rate reported for seaweeds within the currently available literature.

1.9 Factors affecting anaerobic digestion of Seaweeds

For efficient methane production during anaerobic digestion, interactions between the various microorganisms involved in the process and the substrates must be in equilibrium. However, several factors affect the process and hamper the attainment of this equilibrium. In their review articles, Khalid et al., (2011) and Raposo et al., (2012), reported that methane production during anaerobic digestion of organic solid is a direct function of the type and composition of substrates, microbial composition, temperature, moisture and bioreactor designs. Some of the factors that affect anaerobic digestion of seaweeds are discussed below.

1.9.1 Temperature

Temperature remains one of the most critical parameters that affect anaerobic digestion of biomass, due mainly to its far-reaching effects on the microbial community structure. However, opinions are divided on the exact impact of temperature on methane production

during the process. For instance, Lianhua et al., (2010), carried out a batch assay to examine the effects of temperature on anaerobic digestion of rice straw and found that higher methane was produced at mesophilic conditions (35-37^{o}C), compared to thermophilic conditions (55^{o}C). Reports suggest that at thermophilic range, the rate of biodegradation occurs more rapidly, but at that temperature the process become very susceptible to environmental changes (Ferrer, et al., 2010; Lu, et al., 2007 Ortega, et al., 2008). According to Gray, (2005), the rate of anaerobic digestion and gas production depends on temperature, with optimum gas production at the higher temperature ranges (50-55^{o}C). Thermophilic temperatures have also been reported to reduce the retention time of anaerobic digestion process and enhance pathogen destruction during anaerobic digestion processes (Rubio-Loza and Noyola, 2010). Although increase in temperature is said to increase the rate of reaction during the process (Ortega et al., 2008; Ponsá, et al., 2008), research has shown that thermophilic condition does not enhance methane production (Ferrer et al., 2010). Unlike the thermophilic anaerobic digestion process, mesophilic condition is more amenable to commercial production because of better stability, low cost of heating and its ability to adjust to different environmental changes (Kim, et al., 2006). Raposo et al., (2012), concluded in their review that anaerobic digestion is very efficient at mesophilic temperature around 35^{o}C and that any increase in temperature brings about little or no gain in the process considering the associated cost of heat supply.

1.9.2 pH

pH remains one of the critical factors affecting methane production during anaerobic digestion (Chanakya et al., 2006). It is a measure of acidity or alkalinity of the liquid content of the reactor. Unlike other factors, the pH requirement of the different organisms

involved in the process varies considerably. For instance, most methanogenic microorganisms have optimum pH of between 6.8 and 7.8, while the acid-forming bacteria often require lower pH of between 4.5 and 6.8 (Chanakya et al., 2006; Raposo et al., 2012). More so, pH is currently used as one of the parameters for determining the progress and stability of the anaerobic digestion process (Raposo et al., 2012), because when the pH of the reactor is outside of the optimum range, inhibition of the process could occur. The issues relating to pH remain the main driver for the use of a 2-stage anaerobic system due to differences in optimum pH requirement of the two main phases of the process (acid production and methane production) (Demirel & Scherer, 2008; Khalid et al., 2011; Mata-Alvarez, et al., 2000).

1.9.3 Biomass composition

The overall performance of any AD methane production process depends mainly on the nature and type of biomass being digested (Akunna, et al., 2007), as the composition of the substrate provide both the raw materials for conversion and the necessary nutritional materials for the microorganisms involved in the process (Bouallagui et al., (2009). Therefore, any inhibitory or refractory materials present could affect the levels of activity of the microorganisms, and as such the quality and quantity of methane produced (Chen, et al., 2008; Hunik, et al., 1990). For instance, the amount of laminarin in *L. digitata* reaches the peak in July and it's at its lowest in March (Fig. 1.4) (Adams, et al., 2011b), the same result was reported for methane production. Some of the (peculiar) components of seaweeds that can also affect anaerobic digestion process include heavy metals, polyphenols and high salt content.

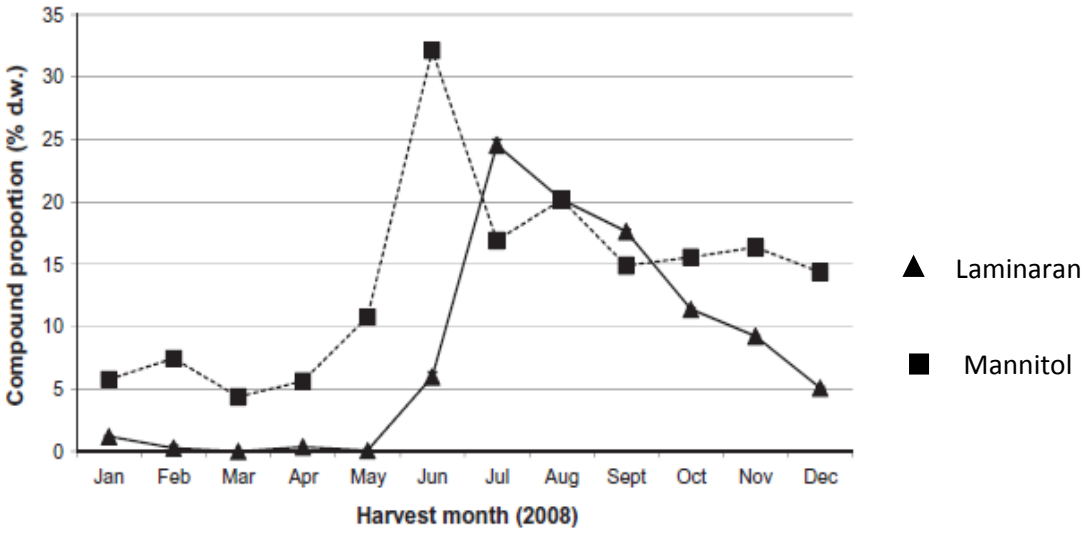

Fig. 1.4. Seasonal variation in carbohydrate composition (% dry weight) of *Laminaria digitata* (Adams, et al., 2011b).

1.9.4 Retention time

Retention time is the length of time during which the substrate is in contact with the microorganisms during anaerobic digestion. It is another very important parameter during the anaerobic digestion process (Ponsá, et al., 2008). The length of retention time affects or determines methane production. Generally, longer retention times produce higher methane yields (Rubio-Loza & Noyola, 2010). According to Yang, et al., (2004), a long retention time is required for the slow growing methanogenic archaea to produce desirable amounts of gas. However, retention time in turn depends on the operating temperature of the system because thermophilic processes proceed faster, while mesophilic processes take longer to complete (Ferrer, et al., 2010). Depending on the feedstock used, mesophilic digestion may take between 15-30 days while thermophilic digestion would be completed within 14 days (Kelly & Dworjanyn, 2008). A shorter retention time has also been reported to favour volatile fatty acids (VFAs) production (Rincón, et al., 2008).

1.9.5 Salt content

High salt concentration has been reported to inhibit biological processes due to induction of osmotic stress (Riffat & Krongthamchat, 2006). Kelly & Dworjanyn, (2008), reported that one of the limiting factors involved in the conversion of seaweeds to methane is the inhibition of microorganisms by the high concentration of salt in the substrate. So desalting has been attempted to reduce salt from the feedstock before anaerobic digestion. However, the process of desalting often lead to loss of degradable materials leading to low methane production (Horn, 2000; Kelly & Dworjanyn, 2008). The use of freshwater for seaweed washing will also undermine the sustainability of the process.

1.9.6 Type of inoculums and concentration of substrates

The type of inoculums utilised during anaerobic digestion of biomass goes a long way to determine the productivity of the process. Different sources could lead to different degrees of degradation as a result of differences in microbial population (Khalid et al., 2011; Raposo et al., 2012). Zeng et al., (2010), showed in a batch experiment that maximum methane production decreases as the inoculum/substrate ratio decreases from 2.0 to 0.5 during anaerobic digestion of algae (*Microcystis spp*). Therefore, a balance in the concentration of inoculum in relation to substrate concentration is needed to obtain optimum methane production.

Inoculum source and type remains one of the critical parts of batch anaerobic digestion tests. This aspect of digestion process is the most varied of all the parameters involved in anaerobic batch digestion. According to (Raposo et al., 2012), even if all other experimental parameters could be harmonised in a batch digester, variability persists because of the biological nature of the system. It is therefore important to characterize inoculums prior to use in anaerobic batch digestion because the characteristics of microbes

to be used as inoculums vary significantly between sources and seasons (Raposo et al., 2012; Zeng et al., 2010). This characterization can be carried out using two parameters; Volatile suspended solids (VSS) or volatile solids. However, due to the inaccuracy of the determination of the volatile suspended solids as a measure of the characteristics of the inoculum, volatile solids determination is used as the alternative measure of microorganism content. Nevertheless, this approach too does not distinguish between microorganisms and other organic matter nor does it differentiate between living or dead microorganisms (Raposo et al., 2012).

Different sources of inoculums have been reported in the literature such as sewage sludge, anoxic sediment, animal rumen contents and animal manures, and waste activated sludge, however, sewage sludge has been the most frequently used partly because of its availability across the world (Migliore et al., 2012; Raposo et al., 2012; Williams, et al., 2013).

In order to obtain sustainable methane production from seaweeds through anaerobic digestion, a great deal more work is required to study the activities of microorganisms involved in the process and the way they respond to the changes that occur in AD reactors. This knowledge will help to better harness the degradation abilities of the microbial consortia associated with the process for better and sustainable methane production especially on a large scale.

1.10 Microorganisms in Anaerobic digestion of organic solids (including seaweeds)

Anaerobic digestion process is carried out by a mixed population of different groups of microorganisms and the cooperation among these microorganisms in anaerobic digesters makes methane production possible (Ali Shah, et al., 2014). These include fermentative, synthrophic and acetogenic bacteria as well as methanogens belonging to the domain

archaea (Table 1) (Ali Shah et al., 2014). The bulk of the current study is dedicated to the studies of methanogenic archaea based on their susceptibility to inhibition (Ali Shah et al., 2014) and the prospect of methanogenesis being the rate limiting step of the process (Traversi, et al., 2012; Zhang et al., 2011).

Table 1.1: Microbial cooperation in organic matter degradation (Ali Shah, et al., 2014)

Microorganisms	Electron donor	Electron acceptor	Product	Reaction type
Fermentative bacteria	Organic carbon	Organic carbon	CO_2	Fermentation
Syntrophic bacteria	Organic carbon	Organic carbon	H_2	Acidogenesis
Acetogenic bacteria	Organic carbon/H_2	CO_2	CH_3COOH	Acetogenesis
Methanogenic archaea	Organic carbon/H_2	CO_2	CH_4	Methanogenesis

1.10.1 Archaea

Archaea are groups of microorganisms described as the 'third domain of life', composed of predominantly methane producing organisms which thrive under extreme environmental conditions (Huang, 2012; Woese & Fox, 1977). Year 2015 marks the 38th anniversary of the discovery of archaea reported in the proceedings of the national academy of science in 1997. Using rRNA sequence characterisation, living organisms were delineated along 3 aboriginal lines; the typical bacteria called *Eubacteria*, *Eukaryotes* for organisms with cytoplasmic cells and then *Archaeabacteria* for archaea (and methanogens) which lacked typical peptidoglycan which is a characteristic of all bacteria (Woese & Fox, 1977). Most archaea are characterized as extremophiles as they thrive under environmental extreme of temperature, pH, salinity and oxygen availability and also possess well defined physiological capabilities (Swan, et al., 2010).

1.10.2 Methanogens

Methanogens; the methane producing microorganisms belong to the domain archaea (Huang, 2012). Methanogens produce methane by utilising acetate, or carbon dioxide and hydrogen, formate, methanol or methylamine for energy (methane) production. Methanogenic archaea are found in anaerobic environments such as rice fields (Watanabe, et al., 2004), anaerobic digesters (Jin, et al., 2011; Keyser, et al., 2006; Steinberg & Regan, 2008; Traversi et al., 2012; Zhang et al., 2012; Zhang et al., 2011), freshwater and marine sediments (Ditchfield et al., 2012) as well as the digestive tracts of different organisms, including humans and ruminants (Jin, et al., 2011). They are also found in geothermal environments such as hot springs (Barns, et al., 1994), seafloor (Biddle, 2006) and hydrothermal vents (Breuker, et al., 2011; Nercessian, et al., 2005; Takai & Horikoshi, 1999). In all cases, they act as the major components of the final stages of the degradation of organic matters in the absence of oxygen. Methane produced by these methanogenic archaea is a potent greenhouse gas which if properly harnessed would be a rich source of renewable energy through the process of anaerobic digestion (Banning et al., 2005).

Although they are commonly found in anoxic conditions or environments such as landfills, paddy fields and ruminant guts, methanogens play important roles in the global carbon cycle (Liu, et al., 2012). Methanogens are obligate anaerobes classified into five orders in the archaea domain namely: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanopyrales* (Yu, et al., 2005). Methanogens are known to possess a narrow range of substrate utilization, and in fact, much more limited are those substrates required for methane production (Yu et al., 2005).

1.10.2.1 Order Methanosarcinales

Methanosarcinales carry out the terminal step in the degradation of organic matter in anoxic environments where light and electron acceptors other than CO_2 are limiting (Beckmann et al., 2011; Kendall & Boone, 2006; Steinberg & Regan, 2008; Zeleke et al., 2013). Amongst methanogens, *Methanosarcinales* have the widest substrate range, with many of the species generating energy for growth by reducing CO_2 with H_2, others by dismutating methyl compounds and some by splitting acetate. While some species can only utilise one of the catabolic path, others can use all the three routes (Kendall & Boone, 2006).

The order *Methanosarcinales* is made up of two families namely: *Methanosarcinaceae* and *Methanosaetaceae*. One genus from the family *Methanosataceae* i.e. *Methanosaeta* depend solely on acetate utilisation for their energy source leading to methane production (Kendall & Boone, 2006).

Methanosarcinales can be found in various harsh and difficult anaerobic environments, however in low salt environment, acetate and H_2 are the important substrates for methanogenesis. Although many *Methanosarcinales* can utilise H_2 and CO_2, they are however outcompeted by other methanogens. However members of *Methanosarcinales* are responsible for all methane production from acetate utilisation (Beckmann et al., 2011; Juottonen, 2008; Kendall & Boone, 2006; Kim, et al., 2013; Munk, et al., 2010; Nayak, et al., 2009).

Members of the genus *Methanosaeta* are found in anaerobic digesters as well as in sediments. Genus *Methanosarcina*, which are halotolerant and halophilic are also commonly, found in anaerobic digesters (example Fig. 1.5) (Ditchfield et al., 2012; Kendall & Boone, 2006).

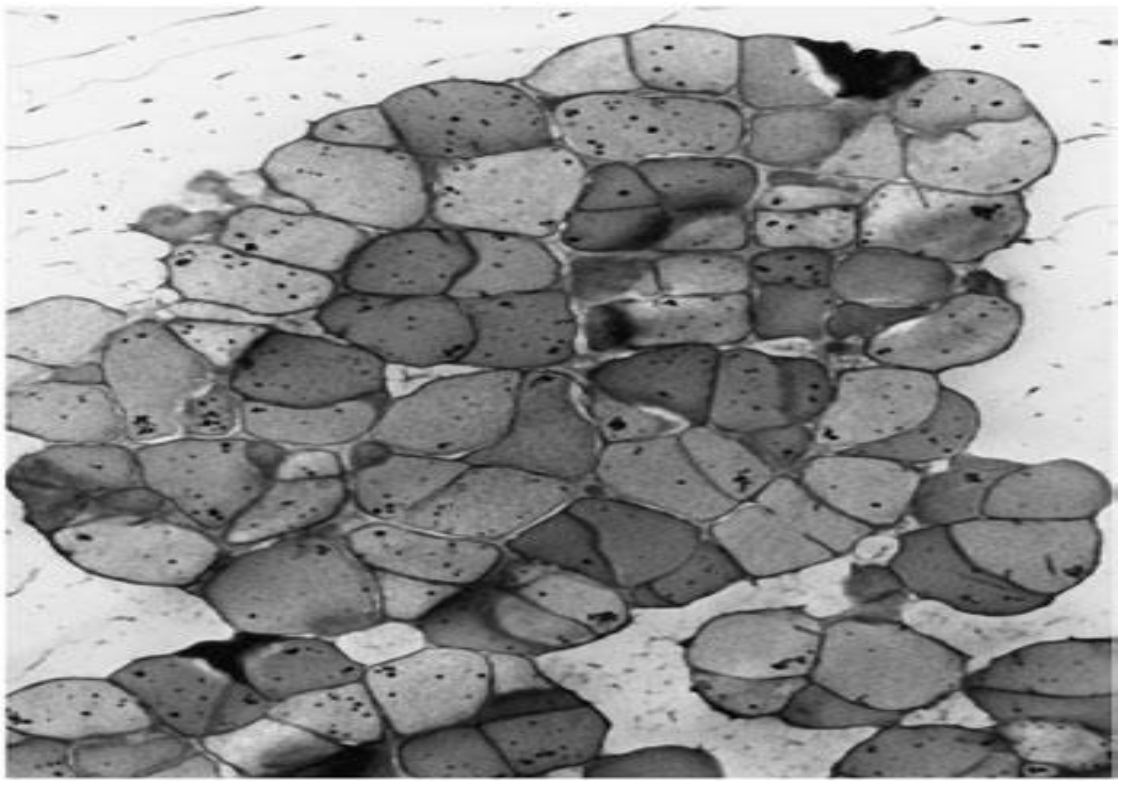

Fig. 1.5. Thin section of aggregates of *Methanosarcina barkerii*. Courtesy of Henry Aldrich, Department of Microbiology and Cell Science, University of Florida, Gainesville, FL (Kendall & Boone, 2006).

1.10.2.2 Order Methanobacteriales

Members of this order are different from all other methanogens in that they have a limited range of catabolic substrates as well as morphology and lipid metabolism. They are generally hydrogenotrophic, and use H_2 to reduce CO_2 to produce CH_4, although some members can utilise formate, CO and secondary alcohols as electron donors for CO_2 reduction (Bauer, et al., 2008; Bonin & Boone, 2006; Cho et al., 2013; John Parkes et al., 2012; Song et al., 2010; Steinberg & Regan, 2008; Watanabe et al., 2004; Zeleke et al., 2013)**.** There is however one genus called *Methanosphaera* which can utilise H_2 to reduce methanol to methane. The order *Methanobacteriales* is divided into two families *Methanobacteriaceae* and *Methanothermaceae*, while the former utilise H_2, CO_2, formate

and CO as substrates for methanogenesis, the latter group is only able to utilise H_2 and CO_2 for methane production (Bonin & Boone, 2006).

All genera in this order can grow using hydrogen and CO_2 but with the exception of species within the genus, *Methanosphaera* (of the family *Methanobacteriaceae*) which require H_2 and methanol for growth. This unique characteristic distinguishes *Methanosphaera* from all other methanogens. Methanogens belonging to the order *Methanobacteriales* occur widely in nature, but rarely exist in any systems that exceed 70^{o}C. The family *Methanobacteriaceae* is composed of four genera namely: *Methanobacterium, Methanobrevibacter, Methanosphaera* and *Methanothermobacter* (Bonin & Boone, 2006).

1.10.2.3 Order Methanococcales

Methanococcales utilise CO_2 as the terminal electron acceptor to reduce H_2 and sometimes formate. Members of this order cannot utilise acetate, methanol or ethanol as substrates for methanogenesis (Banning et al., 2005; Ellis, et al., 2012; Franke-whittle, et al., 2014; Rastogi et al., 2008; Whitman & Jeanthon, 2006). Although genetically diverse, members of *Methanococcales* are phenotypically similar to one another- made up of irregular cocci of 1-3µm. They are commonly and mostly found in the marine environment and require a level of salinity for optimal growth and development. The optimal growing temperature varies considerably- from mesophilic to hyperthermophilic temperature ranges. Under these conditions, *Methanococcales* are among the fastest growing methanogens ever known. For instance members of this order have generation time of 2 hours at 37^{o}C and less than 30 minutes at 85^{o}C (Whitman & Jeanthon, 2006).

Methanococcales is divided into two main families and four genera based largely on the optimum temperature for growth. There are two hyperthermophilic genera-

Methanocaldococcus and *Methanotorris* belonging to the family *Methanocaldococcaceae*. The family *Methanococcaceae,* is made of one mesophilic genus *Methanococcus* and one thermophilic genus *Methanothermococcus* (Whitman & Jeanthon, 2006)

1.10.2.4 Order Methanomicrobiales

Methanogens belonging to this order are morphologically very diverse, but they can be distinguished from other methanogens by their growth properties and cell wall composition (Garcia, et al., 2006). All *Methanomicrobiales* can metabolise H_2+CO_2 as substrates for methanogenesis, while most can also utilise formate and some alcohols. They are unable to utilise acetate and methylated carbon-1 compounds such as methanol and methylamines as substrates (Cho et al., 2013; Siriwongrungson, et al., 2007; Steinberg & Regan, 2008; Zhang et al., 2012; Zhu, et al., 2011). However, some members require acetate as a source of carbon. This feature distinguishes them from the order *Methanosarcinales* (Garcia et al., 2006).

Methanomicrobiales have been divided into three families and nine genera based on the phylogenetic analyses of the 16S rRNA genes with 24 species described within this order so far. Eight of the 24 known species of the order *Methanomicrobiales* have been isolated from anaerobic digesters or sewage sludge. In addition, six species belonging to the genera *Methanolacinia, Methanogenium* and *Methanoculleus* have been recovered from marine sediments (Garcia et al., 2006). Some members of this order also inhabit sub terrestrial habitats such as oil reservoirs and groundwater (Bergmann & Naturwissenschaften, 2012; Tabatabaei et al., 2010). Some members of this order are responsible for the large amount of methane produced in animal rumen. For instance, some species of the genus *Methanobrevibacter*, order *Methanobacteriales* are the predominant methanogens in

bovine and caprine rumen whereas *Methanomicrobiales* are predominant in ovine rumen (Chaudhary, et al, 2012; Garcia et al., 2006; Zhu et al., 2011).

Physiologically, there are two main groups of methanogens namely; hydrogenotrophic and acetoclastic methanogens (Song et al., 2010; Yu et al., 2005). The two groups of methanogens are thought to be simultaneously involved in methane production during anaerobic digestion of biomass. However, a shift from acetoclastic to hydrogenotrophic methanogenesis has been observed as the operational temperature goes from mesophilic (30-37^{o}C) to psychrophilic (5-18^{o}C) (Zhang et al., 2012). Yu et al., (2005), reported that acetoclastic methanogenesis is the main route to methane production during anaerobic digestion of biomass since acetate accounts for more than 70% of methane formation. However, Song et al., (2010) suggested that hydrogenotrophic methanogenesis involving syntrophic oxidation of volatile fatty acids is the major route to methane formation during anaerobic digestion of swine wastewater in a phenomenon attributed to its high ammonium concentration. Nonetheless, acetoclastic methanogens are thought to determine the quantity and quality of methane produced during anaerobic digestion of biomass (Yu et al., 2005).

All acetoclastic methanogens belong to the order *Methanosarcinales* which, comprise of two families namely; *Methanosarcinaceae* and *Methanosaetaceae*. *Methanosarcinaceae* has six genera- one of which is *Methanosarcina*, a very versatile group. The family *Methanosarcinaceae* has a very high affinity for acetate but possesses a low growth rate while *Methanosaetaceae* has a lower growth affinity for acetate but possesses higher growth rates (Yu et al., 2005).

1.11 Why study methanogens

Methanogenesis has often been identified as the rate limiting step during anaerobic digestion (Ali Shah et al., 2014; Izumi et al., 2010; Shin, et al., 2008; Traversi et al., 2012; Velmurugan & Ramanujam, 2011; Wilkins, et al., 2015; Zhang & Fang, 2006), as such, efficient control and management of this stage has been identified as a key determinant of success during the process (Yu, et al., 2005). Methanogens are responsible for all methane production during anaerobic digestion; they are therefore, very important to the overall success of the process. Furthermore, their unique attributes which include slow growth and vulnerability to inhibitions necessitates better understanding of their activities (Banning et al., 2005; Malin & Illmer, 2008; Yu et al., 2005; Zhang & Fang, 2006). A lot is now known about AD processes leading to methane production, but little is known about the methanogens responsible for this methane production. A good knowledge of these organisms, their activities and responses to different environmental changes would not only help in the planning and design of methane digester projects but would also be useful in case of system failure and maintenance (Keyser et al., 2006). Digester failures may occur as a result of excessive organic loading, presence of toxin or sudden changes in environmental or operational conditions which leads to increased stress on the microbial community (Raposo et al., 2012). One way to ensure process stability is to closely monitor the activities of microbial communities involved in the process. As a microbial mediated process, a healthy and robust methanogenic community is critical to the sustainability of the AD process. In other words, the quality and quantity of methane produced, is a direct function of the nature and activity of the methanogenic community (Steinberg & Regan, 2009).

As such, the activities of methanogens need to be closely examined in order to understand how they cope with the conditions associated with anaerobic digestion of marine materials in our attempt to optimise methane production (Banning et al., 2005; Cardinali-Rezende et al., 2009; Narihiro et al., 2009; Rastogi, et al., 2008; Steinberg & Regan, 2009). This knowledge would help in the understanding of the unique links between methanogenic population dynamics, process function and productivity during anaerobic digestion of unique feedstock such as seaweeds.

1.12 Molecular techniques used in microbial ecology studies of anaerobic digesters

Various studies have provided microbial composition and structure of different AD digesters using different molecular techniques. Techniques include denaturing gradient gel electrophoresis (DGGE) and fluorescent in situ hybridization (FISH) based on the 16S rRNA gene (Bergmann & Naturwissenschaften, 2012; Briones et al., 2009; Hu et al., 2012; Hwang etal., 2010; Ma et al., 2013; Nayak et al., 2009; Song et al., 2010; Zhu et al., 2011). These techniques provide important information on microbial composition and diversity including interactions between microbial community structure and system performance. They also allow processing of many samples simultaneously, thereby providing a snapshot of microbial interaction in environmental samples (Díez, et al., 2001).

Other techniques include quantitative polymerase chain reaction (q-PCR) techniques which allow a quick detection and quantification of the 16S rRNA or functional genes of microorganisms in environmental samples (Ma et al., 2013; Yu, et al., 2006). Quantitative knowledge of various microbial populations enable the understanding of the linkages between microbial population dynamics and process functions in AD reactors (Hu et al., 2012; Song et al., 2010; Zhu et al., 2011).

Nevertheless, since the changes in microbial community structure within AD systems and other experimental parameters such as volatile fatty acids (VFAs) or methane production does not occur concurrently in real time, it is somewhat difficult to match community structure profile with process functions (Malin & Illmer, 2008). This therefore necessitates the combination of more than one molecular technique to obtain more rounded information as a basis for monitoring process parameters, (Tabatabaei et al., 2010) as carried out in the current study.

1.12.1 Ribosomal 16S rRNA gene based studies

The 16S ribosomal RNA is a component of the 30S small subunit of prokaryotic ribosomes. It is made up of 1542 nucleotides and has 16S DNA as the coding gene which is used in the construction of phylogenies (Banning et al., 2005; Breuker & Schippers, 2013; Smith & Osborn, 2009; Steinberg & Regan, 2008). 16S rRNA gene is used for phylogenetic studies because it is highly conserved between different species of bacteria and archaea. It is present in both bacteria and archaea often as a multi-gene family and the gene function has not changed over evolutionary times. It also contains hypervariable regions that can provide species-specific signature sequences useful for bacteria and archaea identification. As a result, 16S rRNA gene sequencing has become prevalent in microbiology as a rapid and cheap alternative to phenotypic method of identification (Bercot, et al., 2011; Øvreås, et al., 1997; Skillman, et al., 2006; Yarza et al., 2014).

1.12.2 Functional gene based studies

The use of polymerase chain reaction (PCR) to amplify copies of extracted nucleic acids from environmental samples has been the key to the development of culture-independent approaches to microbial ecology. These techniques, which have been in use since the late 90s have revolutionised the studies of microbial diversity and community, structure in

terms of time and space. The use of PCR amplification combined with functional gene markers as well as DNA fingerprinting and sequence-based analyses has enabled the characterization of most of the environmental microorganisms, which are difficult or impossible to grow in culture. This has led to the discovery of new microbial linkages and lineages. In effect, targeting functional genes, which encodes specific enzymes involved in major metabolic pathways, could provide vital insights into microbial functions within an environment (Ma et al., 2013; Smith & Osborn, 2009; Traversi et al., 2012).

1.12.3 Denaturing Gradient Gel Electrophoresis (DGGE)

Conventional techniques aimed at studying microorganisms based on morphological features can hardly distinguish organisms to class level (Díez, et al, 2001; Muyzer G, 1999; Valášková & Baldrian, 2009). For an in-depth study of microbial ecology, a technique that allows processing of many samples simultaneously is vital. Fingerprinting techniques such as DGGE offers the possibility of processing a large number of samples simultaneously in order to rapidly observe community diversity over different time points and treatments (Díez, et al., 2001). During DGGE experiments, amplified DNA fragments of a specific group of organisms can be differentially separated in a gradient gel based on the nature and arrangements of the sequences. In theory, each DGGE band represents a single operational taxonomic unit (OTU), a species or a group of very closely related organisms. Therefore, DGGE is applied to analyse organism's communities in complex environments such as anaerobic reactors to obtain species diversity and relatedness (Cho et al., 2013; Demirel & Scherer, 2008; Hwang, et al., 2010; Keyser et al., 2006; Kim, et al., 2013; Munk, et al., 2010). The use of DGGE has an added advantage as selected bands can be sequenced to provide information on phylogenetic affiliation of the microbial community, without the need for cloning (Araya et al., 2003; Boon et al., 2002; Ercolini, 2004; Fry et al., 2006;

Garbeva, et al., 2001; Muyzer G, 1999; Nicolaisen & Ramsing, 2002; Throbäck, et al., 2004). However, sequences used in DGGE are usually short, and often produce less refined phylogenetic information (Díez, et al., 2001).

1.12.4 Quantitative Polymerase Chain Reaction (q-PCR)

Although studies of microbial composition and community structure of engineered AD systems are important, quantitative information of microbial population also help in the estimation of the biokinetics parameters important to process design, operation and controls (Yu, et al., 2005). A new technique; quantitative PCR has been developed to detect and quantify microbial concentration in environmental samples (Jørgensen, et al., 2013; Smith & Osborn, 2009; Swan, et al., 2010; Takai & Horikoshi, 2000).

Quantitative PCR or real time PCR is a process that monitors the formation of DNA products in real time. During this process, the target DNA sequence is amplified over a number of cycles involving denaturation-annealing-extension. But, unlike conventional PCR where only the final DNA concentration could be determined, the concentration of amplicons during q-PCR can be monitored throughout the amplification process using fluorescent reagents which bind to the amplicons as they are produced after successive cycles. The intensity of fluorescence emitted is a function of the amplicons concentration (Bergmann & Naturwissenschaften, 2012; Jørgensen et al., 2013; Takai & Horikoshi, 2000; Zhang & Fang, 2006).

In q-PCR, the threshold cycle C_t is the cycle at which the fluoresence intensity crosses over the point where amplification enters a logarithmic growth phase (Zhang & Fang, 2006). Or accroding to (Martínez et al., 2011), it is the point at which the fluorescence reaches the detection level of the instrument. The C_t value is directly proportional to the log value of

the initial concentration of the target DNA (Martínez et al., 2011; Zhang & Fang, 2006) (Fig. 1.6).

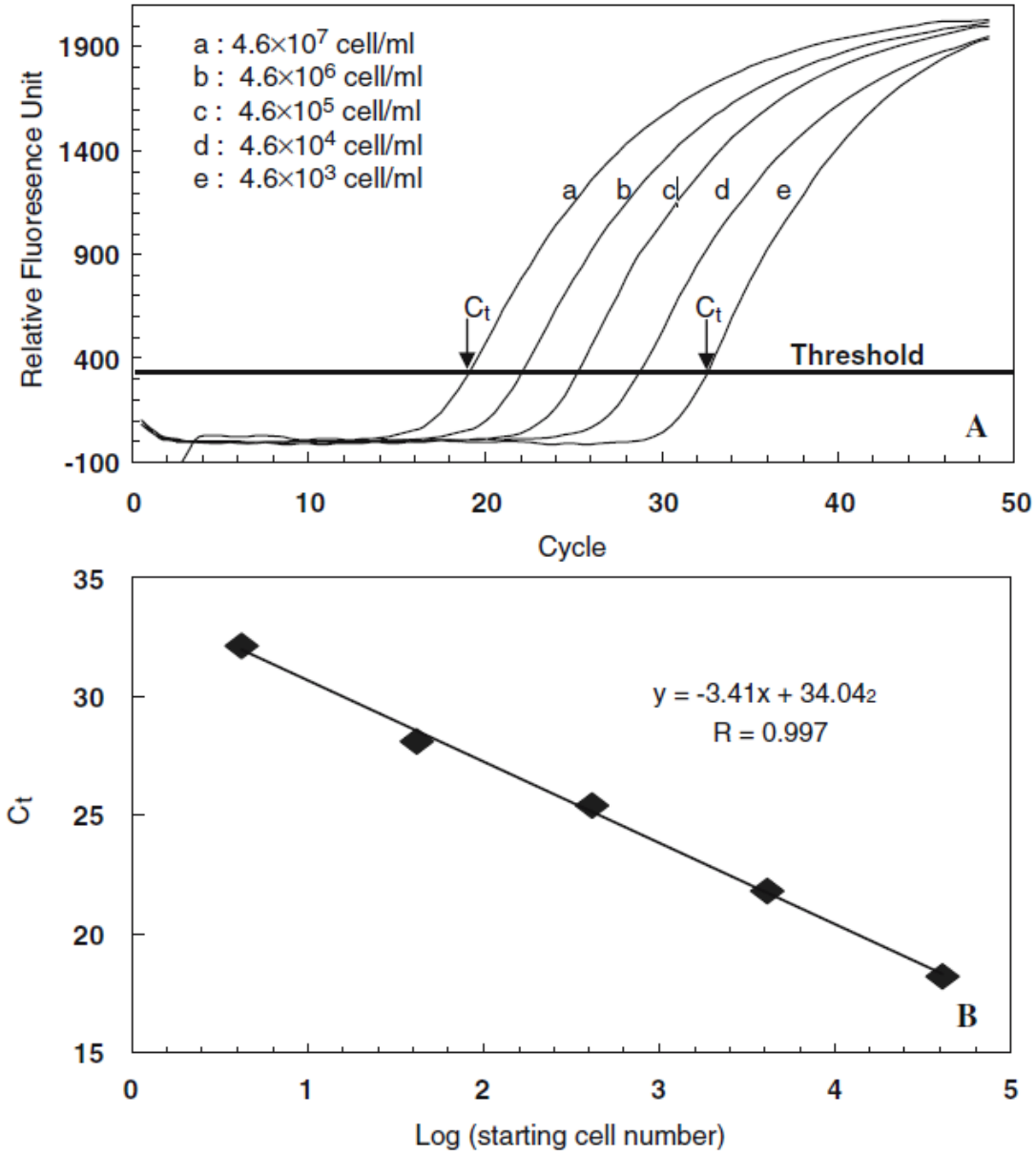

Fig. 1.6. Quantification of *Microcystis aeruginosa* PCC 7820 using q-PCR. (A) Relative fluorescence intensity of five standard solutions of PCC 7820 throughout amplification cycles where C_t represents the threshold cycle number. (B) The standard curve for q-PCR measurement of PCC 7820 (Zhang & Fang, 2006)

There are two main detection methods used in q-PCR; the non-specific method, which detects all double-stranded DNA produced during amplification and the amplicon sequence-specific (probes) method that distinguishes target sequence from non-specific amplifications such as primer-dimers. The former is the simpler and the mostly used

method involving DNA-binding fluorophores such as ethidium bromide, SYBR green, BEBO, and LCGreen. These fluorophores are DNA minor-groove binders which emit a strong fluorescent signal when associated with a double stranded DNA and when exposed to the right wavelength of light (Lloyd, et al., 2010; Martínez et al., 2011; Park et al., 2010; Smith & Osborn, 2009). Although the use of these fluorophores requires no additional oligonucleotide or chemical additions, small changes in the template sequences minimally affect them. However, the formation of primer-dimers, which is associated with the plateau stage of the process, remains a challenge. To overcome this challenge, a melt-curve analysis of the amplified DNA can be inculcated into the reaction where a melting peak profile that represents the specific product can be used to eliminate non-target sequences and prime-dimers (Martínez et al., 2011).

The second method uses different types of fluorescent probes to detect specific sequences, which add an additional level of specificity to the amplification process. These double-dye oligonucleotide probes are made to emit a signal when hybridization to a target DNA sequence occurs (Jørgensen et al., 2013; Lloyd et al., 2010; Martínez et al., 2011; Smith & Osborn, 2009; Takai & Horikoshi, 2000).

1.12.4.1 Requirements for Accuracy of q-PCR assays

Accuracy of the amplification process during q-PCR is of great importance in microbial ecology studies. In fact, whatever affects the accuracy of the quantification process hampers the use of data interpretation (Smith & Osborn, 2009; Takai & Horikoshi). Therefore, optimised and carefully performed q-PCR reaction is required to obtain reliable quantifications.

The accuracy of q-PCR process can be affected by factors such as; primer design, quality of template and presence of inhibitors (Bergmann & Naturwissenschaften, 2012; 2000;

Zhang & Fang, 2006), handling and storage of samples, primers, probes and enzymes (Martínez et al., 2011). With environmental and digester samples, care must be taken during DNA extraction to recognise the possible presence of inhibitors and sources of contamination to improve as much as possible, cleanliness of the extracted DNA (Bustin, et al., 2013; Kim et al., 2013; Smith & Osborn, 2009).

1.12.4.2 Target DNA sequences

The method or approach employed during q-PCR studies depends largely on the target organism or group of organisms. A target gene specific to the organism of interest and primer sets to recognise this gene are selected based on the specificity and coverage. The most commonly used target DNA sequence for microbial quantifications are 16S rRNA (Edwards, et al., 1989; Garbeva, et al., 2001; Nakatsu & Torsvik, 1996; Schabereiter-Gurtner, et al., 2003; Watanabe, et al., 2004) and functional genes (Hallam, et al., 2003; Morris et al., 2014; Zeleke et al., 2013). The 16S rRNA gene sequence contains conserved and variable regions according to their genetic stability, which should provide means of detecting and enumerating complex microbial populations. Organisms within a domain usually share DNA sequences especially in the most conserved regions; as such those organisms can be targeted using primer/probes specific to those sequences making identification and classification possible (Bergmann & Naturwissenschaften, 2012; Lloyd et al., 2010; Smith & Osborn, 2009; Takai & Horikoshi, 1999; Zhang & Fang, 2006). According to Yarza et al., (2014), the use of 16S rRNA gene sequence analysis enables the establishment of taxonomic thresholds that are important for the classification of, not only cultured microorganisms but also for the identification of many uncultured environmental samples.

Another approach to microbial quantification is the use of functional genes shared by organisms with similar physiological functions as the target during molecular studies. There are currently more than 14,000 DNA sequences known for over 100 functional genes in microbial ecological studies. These includes those involved in nitrification, carbon fixation, organic contaminant degradation, methane oxidation and methane production (Biddle et al., 2006; Breuker et al., 2011; Jørgensen et al., 2013; Leininger et al., 2006; Narihiro et al., 2009; Smith & Osborn, 2009; Swan et al., 2010).

Every q-PCR reaction requires a standard curve for functional or 16S rRNA gene amplification. This standard is produced from a serial dilution of a known amount of extracted genome DNA of a pure culture, or plasmid containing the target DNA or PCR amplified fragment of the sequence of interest (Goffredi, et al., 2008; Jørgensen et al., 2013; Smith & Osborn, 2009; Takai & Horikoshi, 2000; Zhang & Fang, 2006). The precision of the q-PCR process relies largely on the premise that the environmental sample under investigation shares the same PCR amplification efficiency with the selected standard solution. Therefore, it is important to assess the efficiency of the standard solutions and samples under investigation.

To assess the efficiency of the q-PCR processes, the following equation is used:

$$N_n = N_o \times (1 + \eta/100\%)^n \qquad \text{(Eq.1.8)}$$

where N_n is the number of amplified target at the end of nth cycle of amplification,

N_o; the initial number of target, and η is the PCR efficiency.

Therefore at 100% efficiency (when slope is -3.32), two DNA segments are produced for every PCR cycle.

The value of threshold cycle, C_t can then be expressed as:

$$C_t = (\log N_t - \log N_o)/\log(1 + \eta/100\%) \qquad \text{(Eq.1.9)}$$

where N_t is the number of amplified target after the threshold cycle C_t.

In practice, a reliable standard curve should have a R^2 value of more than 0.95 and a slope between −3.0 and −3.9 corresponding to PCR efficiencies of 80–115% (Smith & Osborn, 2009; Traversi, et al., 2012; Zhang & Fang, 2006) .

In q-PCR, there is a direct link between the amount of DNA present in the starting material and the number of cycles required before detection. The more the DNA copies, the faster the detection. Depending on the primers, some of the sequences might be easier to detect and copy than others. This may introduce biases into the amplification result. It is therefore important to assess the performances of all reagents, because experimental practices and instruments vary from lab to lab (Baker, 2011).

1.12.5 Cloning and sequencing

Cloning and sequencing of 16S rRNA or functional genes fragments obtained from anaerobic reactors enables in-depth characterization of active microorganisms, and provide insights into microbial diversity in nature (Díez, et al., 2001). Unlike DGGE, it enables the use of large fragment size DNA sequences which provides more refined phylogenetic information about the organism under investigation (Sanz & Köchling, 2007). Cloning and sequencing of both 16S rRNA and functional (*mcr*A) gene fragments enable potential elimination of problems associated with non-specific amplification. Therefore helping to differentiate between general archaea and methanogen communities potentially involved in the actual methane production (Steinberg & Regan, 2008). This approach has been widely used to determine the dominant microorganism in various environmental samples. For instance, Ma et al., (2013), conducted a comparative study between the clone libraries of 16S rRNA and *mcr*A genes fragments and found some unique differences between the two clone libraries. The authors concluded that the use of one or the other gene clone libraries could not have provided complete community structure. Identification of the specific

dominant group of organism using cloning and sequencing approach could provide insights into process functions. For instance, in a study by Ditchfield et al., (2012), which examined the presence of methanogenic archaea in sedimenting particulate materials and faecal pellets of natural and lab-reared copepods using the 16S rRNA gene sequences. The authors reported the dominance of methanogens belonging to the genera *Methanogenum* and *Methanobacterium* in the sedimenting particles and inferred that hydrogenotrophic methanogenesis was the preferred route towards methane production in those samples. In another study to determine archaea diversity in methane plant supplied with cattle manure and maize silage operated at mesophilic temperature, using both 16S rRNA and functional *(mcrA*) gene clone libraries, Nettmann, et al., (2008), reported the dominance of archaea belonging to the order *Methanomicrobiales* (>80%), an indication that hydrogenotrophic methanogenesis was the main route to methane formation. The authors also found similarities in the archaea composition within the two clone libraries, but reported some distinctions when clones were identified to genus level. In the current study, clone libraries of both 16S rRNA and functional *(mcrA*) gene fragments are used to understand methanogenic activities during the stage of active methanogenesis.

1.13 Project justification

Interest in marine biomass exploitation for renewable energy production has been renewed in the last decade and there is growing evidence to support its economic viability, and social and environmental benefits (Costa, et al., 2012; Hinks, et al., 2013; Miura et al., 2014; Nielsen & Heiske, 2011; Nkemka & Murto, 2010; Vanegas & Bartlett, 2013; Vergara-Fernández, et al., 2008). A number of published scenarios highlight that large-scale exploitation of marine biomass could have significant impacts on bioenergy production, but only in some coastal communities such as the western Isles of Scotland.

This is because the profitability of the process depends among other factors, on the cost of inputted feedstock for which the west coast of Scotland has a comparative advantage in terms of wild harvest or cultivation (Hermannsson & Swales, 2013; Hughes et al., 2012). Nevertheless, the overall productivity and profitability of the process is dependent on avoiding system failure while maximizing process efficiency. In order to maintain and sustain efficient process performance, a control strategy that takes into account the microbiological nature of the process is imperative. This is the main goal of the current study.

The microbial community in anaerobic digester of various substrates such as swine waste (Cook, et al., 2010), waste activated sludge (Yu et al., 2014), house-hold wastes (Cardinali-Rezende et al., 2009), fodder beet silage (Klocke, et al., 2007), wastewater (McHugh, et al., 2003; Boon, et al., 2002), maize silage (Bauer, et al., 2008) have been studied using various molecular techniques. A few other reports exist on the anaerobic digestion of seaweeds (Matsui & Koike, 2010; Migliore et al., 2012; Miura et al., 2014; Nielsen & Heiske, 2011; Nkemka & Murto, 2012; Nkemka & Murto, 2010) using different inoculum sources ranging from anaerobic sludge and mesophilic granules to anoxic sediment. However, none of the available reports contains a detailed microbial ecology study of archaea and methanogens involved in the anaerobic digestion of seaweeds.

This is therefore the first time a detailed microbial ecology study of seaweeds anaerobic digesters (under different inoculations) has been carried out.

In this study, a number of molecular approaches were employed to understand the microbial community dynamics within seaweeds anaerobic digesters and better understand the complex interactions between microbial community structure and process functions within the reactors.

1.14 Project Aim

The aim of this study is to better understand how methanogens remain viable and active, and relate our finding to process functions during anaerobic digestion of marine materials. The research focuses on improving the fundamental understanding of how methanogens cope with the conditions associated with anaerobic digestion of marine biomass while optimising methane production. It will also identify the main methanogenic groups involved in anaerobic digestion process and the effect of changing environmental conditions on the production of methane. The optimum conditions for methanogens growth and development for successful methane production during anaerobic digestion process is also investigated.

1.14.1 Objectives

To achieve our aims the following objectives and questions would be addressed:

- Determine if there are significant differences in process functions between the use of inoculum from marine environments and digested sludge during anaerobic digestion of seaweeds.
- Examine microbial population dynamics in relation to process functions during anaerobic digestion of seaweeds and cellulose.
- Observe microbial community composition and diversity as a function of changing processes and environmental conditions
- Identify the dominant archaea and methanogenic community involved in the anaerobic digestion of marine biomass under different inoculation.
- Observe microbial interactions and cooperation in relation to the maintenance and sustenance of process stability and productivity.

- Determine which of the locally available seaweeds species, *Laminaria digitata, Saccharina latissima, Fucus serattus,* produce the most methane per gram of material

1.14.2 Research questions

Chapter 3 (Test experiment)

- Are there differences in methane produced from washed and unwashed *Laminaria digitata*
- Are there differences in microbial communities between washed and unwashed *Laminaria digitata* reactors?
- If different, what are the causes?

Chapter 4 (Batch test)

- Are there differences in methane production between seaweeds inoculated with sediment or sludge?
- Are there differences in methane production between sediment and sludge inoculated seaweeds?
- How does methane production by the seaweeds compare with that of cellulose under both inoculations?
- Are there differences in methane production between sediment and sludge inoculated cellulose?
- Which is/are the most promising seaweeds in terms of methane production?

Chapter 5 (Q-PCR)

- Is there evidence to suggest microbial growth/increase as a result of substrates availability (between blank and others)?
- Are there differences in microorganism numbers between all reactors within the same inoculation treatment?
- Are there differences in microbe numbers between specific reactors across different inoculation?
- How do microbial numbers change over time between the two inoculations?
- How do microbial numbers relate to other process functions and performances?

Chapter 6 (DGGE)

- Are there significant differences in microbial composition and community structure between the inoculums (prior to AD process) and digestates during the process?
- Are there differences in microbial community composition and diversity between sediment and sludge inoculated reactors?

- Is there evidence to suggest microbial variation as a result of substrate composition?
- What are the main drivers/determinants of microbial variation during anaerobic digestion of seaweeds?
- Are there similarities between sediment and sludge inoculated microbial populations in terms of composition and community structure?
- Are there differences between seaweeds and cellulose reactors in terms of microbial community over time?

Chapter 7 (Cloning and Sequencing)

- What are the dominant archaea and methanogens communities in sediment and sludge seaweed reactors?
- Are there differences in dominant archaea (based on 16S rRNA) and methanogens (based on *mcr*A gene) communities under sediment and sludge inoculation?
- How much do archaea and methanogens communities differ between sediment and sludge inoculated seaweed reactors?
- Are there any correlations between the dominant archaea/methanogens and total methane production?

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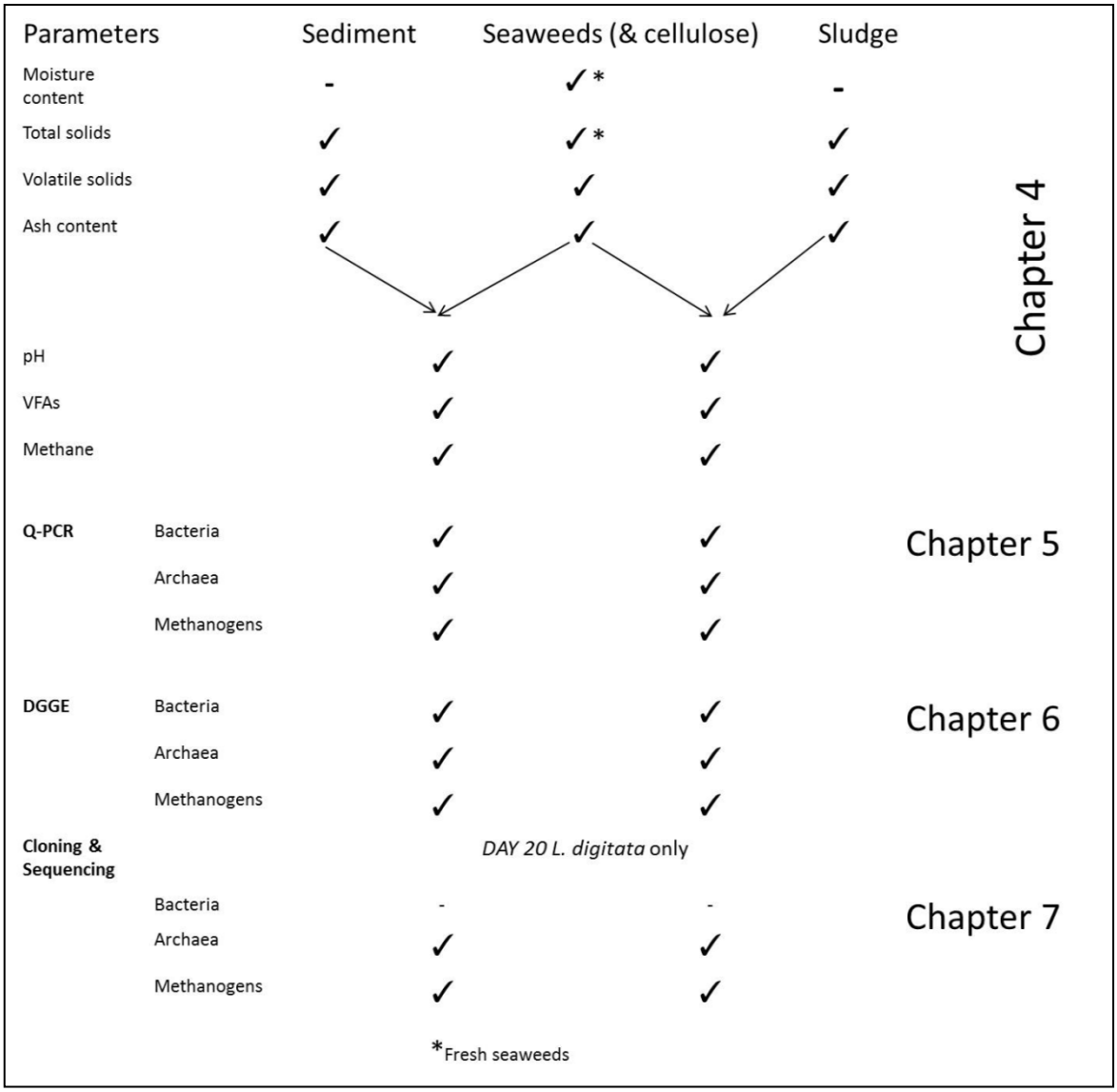
Chapter 2

2 Materials and Method

"*What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on*"

-Jacques Yves Cousteau



Methods and techniques used in this study.

2.1 Materials

2.1.1 Substrates (Seaweeds)

Seaweeds (*Laminaria digitata*, *Fucus serattus* and *Sacharrina Latissima*) (Fig. 2.1) were harvested from the beach behind Scottish Association for Marine Science, (SAMS) near Oban (56°27'09.5"N 5°26'43.2"W) in August, 2012. The seaweeds were taken to the lab and were frozen overnight before freeze-drying for 2 days. Freeze-dried seaweeds were manually grinded (to avoid the use of metallic blender which may affect the metal content of the seaweeds) into powder and sieved <1mm. Dried seaweeds were stored at room temperature until used. Experimental seaweeds were selected based on 3 considerations 1: availability on the west coasts of Scotland (Hermannsson & Swales, 2013) 2: carbohydrate content (Adams, Toop, et al., 2011; Hughes, et al., 2012) 3: feasibility and potential of large scale cultivation (Schiener, et al., 2014). Cellulose powder (Fluka, Sigma-Aldrich, Denmark) was used as the standard substrate in the controls according to (Hansen et al., 2004).

Fig. 2.1. Experimental seaweeds selected based on their high dry solid content, abundance on the coasts of Scotland and potential for cultivation (A: *Laminaria digitata*, B: *Sacharrina Latissima C: Fucus serattus*). Seaweeds were harvested fresh at low tide in August 2012 at the start of the project and used throughout the project. The time of harvest is important as it affects the compositions of the seaweeds (Adams, et al., 2011a; Schiener et al., 2014).

2.1.2 Seed inoculums

The first seed inoculum; anaerobic digested sludge was sourced from the municipal wastewater treatment plant operated at 37^{o}C in Hatton, near Dundee, UK. The second inoculum source anoxic sediment, was sourced from below fish farms in Shuna (56^{o} 35'.609 N, 05^{o} 22'.844 W), near Oban. Both inoculums were incubated at 37^{o}C for 24 hours before use. Characteristics of experimental seaweeds and inoculums are listed in Table 2.1.

Table 2.1: Characterization of experimental materials prior to anaerobic digestion

Component	*L. digitata*	*F. serattus*	*S. latissima*	Anoxic Sediment (g/L)	Digested sludge (g/L)
Moisture (wet %)	73	71	72.5	-	-
TS (wet %)	27	29	27.5	97.7	22.9
VS (dry %)	85.41	81.14	87.64	18.56	13.19
Ash (dry %)	14.59	18.86	12.36	79.14	30.8

2.1.3 Buffer solution

Non-growth synthetic buffer medium was prepared for the anaerobic digestion process to stabilise the pH of the reactor using the following compounds; 2.7g/l KH_2PO_4 (strong buffer agent), 3.5g/l K_2HPO_4 (strong buffer agent), 5mg/l $MgSO_4.7H_20$, 0.5 mg/l $CaCl_2$, 0.5 mg/l $FeCl_3$, 0.5 mg/l KCl_3, 0.1 mg/l $CoCl_2$ and 0.1 mg/l $NiCl_2$ in seawater. The medium provided the essential nutrients required by the microorganisms (Obata, et al., 2015). The seaweeds contain sufficient phosphorus and source of nitrogen (protein) to support the growth of microorganisms during anaerobic digestion (Horn, 2000).

2.1.4 Experimental Procedure

To maintain adequate mass transfer balance during anaerobic digestion batch assay in the current study, 5 g of substrates (seaweeds and cellulose) in 150 ml of non- growth medium was placed into reactor bottles and seeded with 150 ml of anoxic sediment or digested sludge. The mixtures were set up to a working volume of 300 ml in a 575 ml-capacity reactor bottles leaving a 275 ml headspace for gas measurement (Fig. 2.2). Each reactor contained ~50 g/L, which is suitable for batch test to avoid excessive acid accumulation.

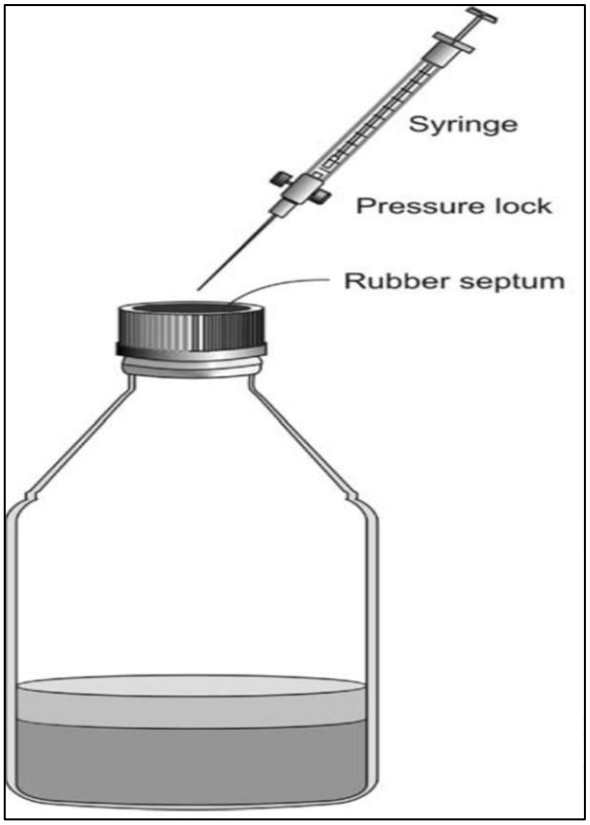


Fig. 2.2. Anaerobic digestion batch reactor used in the first part of the current study. Top of reactor bottles are tightly closed with rubber septum to allow for methane measurement and other sampling without opening the bottles (Hansen et al., 2004)

After set-up, the pH of the batch was adjusted to 7.5±0.3. Reactor bottles were sealed with septum and tightly close with aluminium caps and the headspace flushed with nitrogen (Fig. 2.3). The blanks (negative controls) containing inoculum and buffer solution were included to account for any methane produced due to residual substrates (Hansen et al., 2004). All batch tests were performed in duplicates and incubated at 37 ^{o}C for 50 days. Moisture and ash content of the seaweeds were determined as well as total and volatile solids, digested sludge and sediment were determined according to standard procedures (Siles, et al., 2010). Digestate (4 ml) was collected at regular intervals for VFA and microbial analyses while methane measurements were performed at standard temperature and pressure.

Fig. 2.3. Examples of reactor bottles (575ml) used for the batch assay, 300ml working volume was used leaving a 275ml headspace for gas analysis. Samples for analyses are taken from the top using syringes after gas measurement and then sealed with silicon material. Sampling was carried out every day for the first 10 days to monitor pH levels closely and was corrected when necessary. Gas volume measurements took into account changes in headspace volume over time.

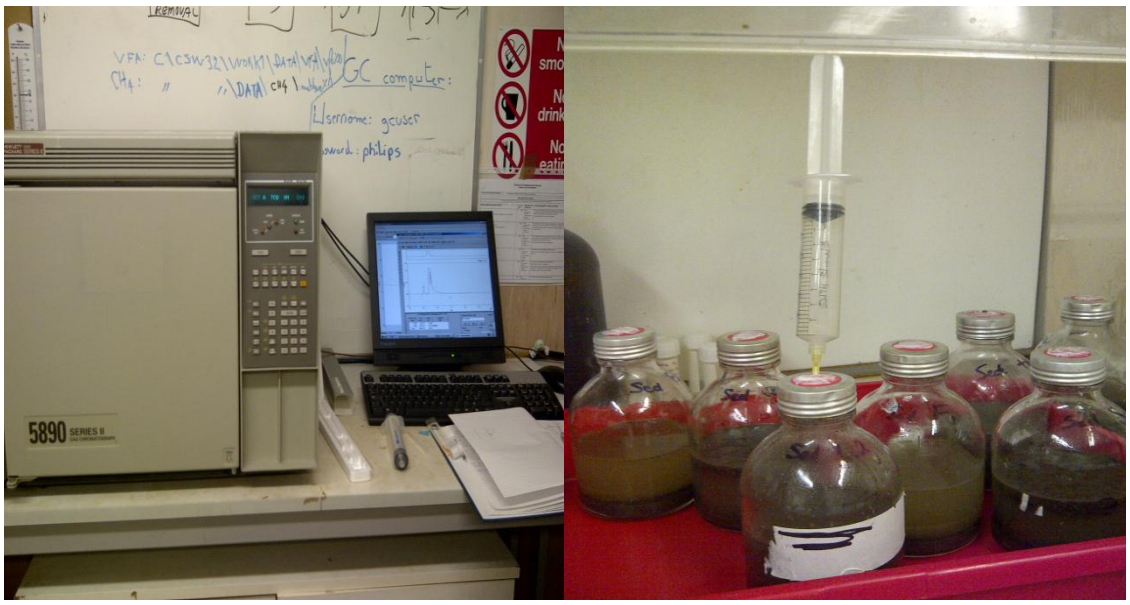


Fig. 2.4. GC-MS was used for methane concentration analysis while syringe gas-pressure displacement method was used for the methane volume quantification

2.2 Analytical Methods

2.2.1 Gas analyses

Headspace methane concentration was analysed with Gas Chromatography (HEWLETT PACKARD 5890 SERIES 11, USA) equipped with a single flame ionization detector (FID), using gas tight 100µl syringe. The injector and detector temperatures were 120^{o}C and 150^{o}C respectively. Helium was used as the gas carrier with a pressure of 200kPa. Calibration was performed using linear calibration curve based on four different methane concentrations (0.5–10% ±5% in N_2). Gas volume analysis using gas build-up methane was performed as described by Hansen et al., (2004) (Fig 2.4). Methane production determination was adjusted to standard temperate and pressure (Hansen, et al., 1999; Costa, et al., 2012).

2.2.2 pH determination

The pH of the samples was raised to 7.5±0.3 at the beginning of the experiment, but as the process progressed, the pH of the cultures fluctuated and was therefore closely monitored especially for the first 10 days. The pH was evaluated using pH meter SensIon 3 (HACH). The pH meter was calibrated before every use.

2.2.3 Total solids and Volatile solids determination

Total solids and moisture content of the seaweeds and seed inoculums was determined according to the standardised methods by oven-drying at 105 ^{o}C. Volatile solids and ash were subsequently determined by incinerating dried at 550 ^{o}C according to standard procedures, in triplicates (Schiener et al. 2014). Results are expressed as the % dry weight (Table 2.1).

2.2.4 Determination of Volatile Fatty Acids (VFAs)concentration

VFAs concentration was determined by esterification method as described by Montgomery *et al*., (1962), while calorimetric determination was done using a HACH DR/5000 (HACH method 8196). The VFAs concentration was measured in mg/l. Digestate samples (4ml) collected at intervals were centrifuged at 13000rpm for 3 min. The supernatant (0.5 ml) and 0.5 ml of distilled water (blank) were used for the VFAs analysis as follows; Ethylene glycol (1.5ml), and 0.2ml 19.2N Sulphuric acid standard solution were added to 0.5ml of the supernatant, mixed, boiled in water bath at 100^{o}C for 3 minutes and cooled. Hydroxylamine hydrochloride solution (10%; 0.5 ml) was added and shaken by hand to mix, after which 2ml of 4.5N Sodium Hydroxide solution was added and mixed. Then, 10 ml of Ferric Chloride Sulphuric acid solution was added and shaken by hand to mix. Finally, 10ml of distilled water was added to all the samples, mixed and left for 3 min. VFA concentration was measured with spectrophotometer at 495nm in duplicates.

2.3 Molecular methods for characterization of microorganisms

The failure of culture-based techniques to describe over 99% of microorganisms has led to the development of culture independent techniques, which have revolutionised microbial ecology studies (Angel, et al., 2012; Bergmann and Naturwissenschaften 2012; Fredriksson, et al., 2012; Goffredi et al. 2008; Jørgensen et al. 2013; Lloyd, et al., 2010; Martínez et al. 2011; Morris et al. 2014; Park et al. 2010; Smith and Osborn 2009). The common approach to molecular studies of microbial ecology begins with the extraction of genomic DNA from environmental samples, and then amplification of specific genes by PCR. Other downstream approaches included differentiation of amplified amplicons by molecular fingerprinting or by cloning and identification of the organisms present by DNA sequencing and phylogenetic analysis (Head, et al., 1997)

2.3.1 Deoxyribonucleic acid (DNA) extraction

DNA extraction was carried out as described by Yilmaz, et al., (2009) with some modifications. XS (10ml) buffer was made up using the following reagents: Xanthogenate powder (0.1g), DEPC (Diethylpyrocarbonate) water (6.6ml), tris- HCl pH 7.4 (1ml), 0.5M EDTA pH 8.0 (400µl), 7.5M Ammonium acetate (1ml) and 10% SDS (1ml). Samples (500µl) were placed in eppendorf tubes into which 1ml of the XS buffer was added. The mixture was mixed and incubated at 70^{o}C for 1 hour and vortexed throughout incubation. After incubation the samples were placed on ice for 30mins to precipitate the debris and later centrifuged at 13,000 x g for 15 minutes at 4^{o}C . The supernatant was removed and 1 volume of Phenol: chloroform: isoamyl alcohol (25:24:1; pH 8) added to the supernatant. The upper aqueous layer was collected and extracted again with phenol: chloroform: isoamyl alcohol. This step was repeated to provide additional clean up. The mixture was vortexed briefly before centrifuging for 10 minutes at 13,000 x g, 4ºC. The supernatant was removed, 1 volume of 14% PEG 8000 (Polyethylene glycol) 20nM MgCl2 was added, and the sample put back on ice for another 10 minutes. Samples were then centrifuged at 13,000 x g, 4^{o}C for 15mins after which PEG was removed and the samples washed with 1 ml 70% ice-cold ethanol. The ethanol was removed and samples left to air dry. Molecular grade water (100µl) was added to suspend the pellet. Nucleic acids were quantified spectrophotometrically at 260nm. Aliquots of the extracted DNA were diluted because dilution limits the effects of inhibitory substances associated with this type of samples under investigations (Traversi, et al., 2012)

2.3.2 Polymerase Chain Reaction (PCR)

All PCR runs were carried out using the PTC-200 DNA thermal cycler (MJ Research, Las Vegas, NV), reaction set-ups were performed on ice, in laminar flow to minimise contaminations.

2.3.2.1 PCR of 16S rRNA gene fragment of bacteria

PCR was carried out to obtain a small DNA fragment (<200bp) suitable for DGGE using the bacterial specific primer pair primer 2/3 (5'- ATTACCGCGGCTGCTGG-3' and 5'- CCTACGGGAGGCAGCAG -3') (Muyzer, et al., 1993). Another set of primers (pD/pF' 5'- CAGCAGCCGCGGTAATAC-3' and 5'- ACGAGCTGACGACAGCCATG -3') were used to amplify a larger fragment size (~560bp) as described by Edwards et al., (1989).

2.3.2.2 PCR of 16S rRNA gene fragments of archaea

PCR amplification of general archaea required nested PCR in order to obtain sufficient product for DGGE analysis. It has been demonstrated that the use of nested PCR can improve sensitivity and specificity of PCR, particularly when there is a high ratio of non-target to target organisms (Boon, et al., 2002; Garbeva, et al., 2003; Nakatsu & Torsvik, 1996; Øvreås, et al., 1997) such as in these samples. The first round of PCR amplification was performed to obtain a larger size fragment using archaea specific primer pair PRA46f (YTAAGCCATGCRAGT) (Øvreås, et al., 1997) and Arch1017r (GGCCATGCACCWCCTCTC) (Barns, et al., 1994) to produce a 971-bp fragment. The second round was performed using the broad specificity primer pair 344fgc (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGGHGCAG CAGGCGCGA)/ Parch519r (TTACCGCGGCKGCTG) (Banning et al., 2005) giving a product internal to the first round of ~190bp. For archaea cloning and sequencing reaction,

two rounds (using the product of the first round) of PCR was carried out to obtain a suitable amplicon length of 971-bp using the same primer pair (PRA46/Arch 1017) for both rounds as described in Table 2.2

2.3.2.3 Polymerase chain reaction (PCR) of mcrA gene fragments from methanogens

For the purpose of cloning and sequencing of methanogen community in the reactors, the *mcr*A gene fragment was amplified in a PCR as described by Steinberg and Regan (2008) to produce ~500bp amplicon using the primer pair mcrA mlas 5'- GGTGGTGTMGGDTTCACMCARTA-3' and mcrA-rev 5'–CGTTCATBGCGTAGTTVGGRTAGT- 3'. For DGGE, the same procedure was used except that the forward primer mcrA mlas has a 40-bp GC-clamp at the 5' end of the primer (5'–CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG GGTGGTGTMGGDTTCACMCARTA- 3') component to prevent completed melting of the DNA fragment and enhance separation on the gradient gel.

All PCR runs were carried out in a total 25µl volume containing 0.5µl of forward primer, 0.5 µl of reverse primer (10ρmoles/ µl), 0.1 µl of MyTaq polymerase (5u/ µl). Other components of the mix are 5µl of PCR Buffer (comprising 5mM dNTPs, 15mM $MgCl_2$, stabilizers and enhancers), 18.4 µl of molecular grade water (17.4 µl for sediment archaeal and methanogen amplification) and 0.5 µl of DNA extract (Stock, 1:10, 1:100 dilutions). Additional 1 µl of 15mM $MgCl_2$ was added to the reaction mix to (improve *Taq* efficiency) enhance sediment samples' archaeal and methanogen amplification (Schmidt, et al., 2014). Negative controls containing 0.5µl of sterile molecular grade water were included in all cases. Different dilutions were tested in chapter 3 while 1:10, 1:100 dilutions were subsequently used for archaea (and methanogen) and bacteria respectively. All primers

were obtained from Integrated DNA Technologies, (Belgium) while other reagents were obtained from Bioline Reagents Ltd, (London, UK).

The PCR conditions for different groups of organisms and primer pairs are listed in table 2.2.

2.3.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise the PCR products and check for efficiency. Loading buffer (2µl), (0.25%w/v bromophenol blue; 40% w/v sucrose in filtered sterilised water) and 5µl of sample was run on an agarose gel (1% agarose in 1 X TAE buffer (40nM tis acetate, 2nM EDTA pH 8)). Gels were run at 120V for 30 minutes. Marker (2000-100 bp) (Easy ladder I Bioline) was run on all the gels to estimate the size of the PCR products. The gel was then stained in ethidium bromide (0.8 ng/ml) solution for about 30 minutes and rinsed with sterile water. The gel was visualised and digitized using a digital imaging system (Alpha Innotech Alphaimager) with UV transillumination.

Table 2.2: Temperature cycling parameters for PCR amplification of bacterial and archaeal 16S rRNA gene fragments and methanogens' *mcr*A gene fragment with different primer pairs

Primers	Target groups	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles	Final Extension	reference
Primer 2/3*	General bacteria	3min, 95°C	30secs, 95°C	30secs, 65°C reduce 1°C every 2nd cycle	1min, 72°C	24 cycles followed by a further 15 at annealing temp. of 53°C	10min, 72°C	(Muyzer G, 1999)
Primer pD/pF'	General bacteria	3min, 95°C	1min, 95°C	1min, 55°C	1min, 72°C	30	10min, 72°C	(Muyzer,1993)
PRA46/Arch 1017[a]	General archaea	3min, 95°C	1min, 95°C	1min, 40°C	1min, 72°C	30	10min, 72°C	(Øvreås et al., 1997)
Arch 344gc/ Uni522[b]	General archaea	3min, 95°C	30sec, 95°C	30sec, 55°C	1min, 72°C	35	10min, 72°C	Amann *et al*., 1995
Mlas/ mcrA-rev	Methanogens	3min, 95°C	30sec, 95°C	45sec, 48°C	30sec, 72°C	5 cycles followed by a further 30 at annealing temp of 55°C	10min, 72°C	(Steinberg & Regan, 2008)
Mlasgc/ mcrA-rev[c]	Methanogens (DGGE)	3min, 95°C	30sec, 95°C	45sec, 48°C	30sec, 72°C	5 cycles followed by a further 30 at annealing temp of 55°C	10min, 72°C	(Steinberg & Regan, 2008)

*GC rich primer, which amplifies short fragment size as such it, is used for bacteria DGGE. [a] this primer pair amplifies a large size fragment and its used in the first round of the nested PCR for archaea amplification. [b] used in second round of nested archaea PCR for DGGE for a smaller fragment size (178bp), [c] used in single round of PCR to amplify *mcr*A gene fragment for methanogen DGGE (~500bp).

2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

Different groups of microorganisms from the anaerobic reactors from specific time points (Day 2, 13, 20, 27) were targeted during DGGE experiments namely: bacteria (16S rRNA gene), archaea (16S rRNA gene) and methanogens (*mcr*A gene). Experimental procedure depended on the target groups of organisms. Reproducibility of replicates were checked by running individual replicates on a DGGE gel, before replicates samples were pooled to capture all possible groups of microorganisms.

2.4.1 DGGE procedure for all microbial communities

DGGE was performed using an INGENYphorU-2 system (Ingeny, Netherlands). PCR products and loading buffer (40% [wt/vol] sucrose, 60% [wt/vol] 1 x Tris-acetate-EDTA [TAE], and bromphenol blue) were mixed in a 1:1 ratio. The mixture of PCR amplicons and loading buffer were applied directly to 10% (wt/vol) polyacrylamide (37:1 acrylamide: bisacrylamide) gels with a linear gradient of 40 to 80% denaturant for methanogens (~500bp) and 30 to 60% denaturant for ~ 200 bp bacterial and archaeal PCR products. Denaturant (100%) corresponds to 7M urea and 40% [vol/vol] formamide in 1 X TAE). Electrophoresis was carried out in 1 X TAE buffer (40 mM Tris-acetate [pH 7.4], 20 mM sodium acetate, 1 mM sodium EDTA) at a constant voltage of 100 V and at 60°C for 19 hours. After electrophoresis, gels were stained for 30 minutes in 1 X SYBR Gold solution (Sigma, UK) diluted 1/10000 in 1 X TAE and washed with distilled water. The gel was digitized using a digital imaging system (Alpha Innotech Alphaimager) with UV transillumination (Beckmann et al., 2011).

2.4.2 Gel analysis

Gel analysis was carried out with the software GelCompare II version 6.6 (Applied Maths, Belgium). Comparison was performed using a similarity coefficient Dice with 0.5% optimisation band matching tolerance of 0.5%. Uncertain bands were ignored. Cluster analysis using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to analyse DGGE gels. The evolutionary history of selected archaea bands was inferred using the Neighbour-Joining method, Saitou and Nei (1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches Felsenstein (1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method, Tamura et al., (2004) and are in the units of the number of base substitutions per site. The analysis involved five nucleotide sequences. Codon positions included were 1st+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted in MEGA6, Tamura et al., (2013).

2.5 Quantitative polymerase chain reaction (q-PCR)

In the current study, SYBR Green I approach was used for q-PCR amplification of bacteria, archaea and methanogens at specific points during the batch digestion process. Standard bacteria used were pure strains of *Colwellia psychrerythraea,* a *Gammaproteobacteria* generously donated by Dr Green (SAMS, UHI). Clones of *Methanomicrobiales* from this study and *mcrA* gene clones donated by Dr Purdy (University of Warwick) were used as standard for archaea and methanogens respectively.

Mass concentration of standard DNA (clone) was converted into copy concentrations using the following equations:

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}\ \text{(copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660\ \text{(g/mol/bp)}} \quad \text{(Eq.2.1)}$$

Where Avogadro's number is 6.02×10^{23} copies/mol and the average molecular weight of one DNA base pair (bp) is 660 g/mol (J. Kim et al., 2013).

For each q-PCR assay, triplicate five-point standard curve was created using data from the serial dilution of known amounts of the standards calculated from equation (2.1) above.

2.5.1 Q-PCR amplification of general bacteria in seaweeds anaerobic reactors

Primer pair E806f (5′- GGACTACHVGGGTWTCTAAT -3′) (Teske & Sørensen, 2008) with bacteria coverage rate of 95.1% (Wang & Qian, 2009) and U515r (5′ GTGCCAGCMGCCGCGGTAA -3′) with coverage rate of 99.0 % (Wang & Qian, 2009) were chosen for general bacteria q-PCR amplification because of their good coverage (Klindworth et al., 2013; Wang & Qian, 2009). A dilution of the standard corresponding to 6.04 $x10^{11}$ gene copies /ml was employed as the positive control. The amplification protocol began with hot start polymerase activation at 95 ^{o}C for 10 min, followed by 40 cycles of 95 ^{o}C denaturation for 15 s and annealing at 60 ^{o}C for 60 s. A melt curve was included to discount non-specific amplification by heating the reactions to 95 ^{o}C (0.1 ^{o}C/s) and cooled to 55 ^{o}C while fluorescence was detected at 0.3 ^{o}C interval (Nadkarni, et al., 2002).

2.5.2 Q-PCR amplification of general archaea in seaweeds anaerobic reactors

Primer pair A344f (5′- GGGGYGCASCAGGSG -3′) (Teske & Sørensen, 2008) with coverage rate of 90.8% (Wang & Qian, 2009) and A915r (5′- GTGCTCCCCCGCCAATTCCT -3′) with coverage rate of 97.1% (Wang & Qian, 2009)

were chosen for all archaea q-PCR amplification because they have good coverage within the archaea domain (Klindworth et al., 2013; Lloyd et al., 2010). They are the only pair of the 6 primer pairs tested that amplified archaea samples in a single round of PCR. A dilution of the standard corresponding to 1.930 $x10^9$ was used as the positive control. The amplification protocol begins with hot start polymerase activation at 94 ^oC for 4 min, followed by 40 cycles of 94 ^oC denaturation for 30 s and annealing at 60 ^oC for 30 s. 72 ^oC for 30 s and 85 ^oC for 10 s according to Angel et al., (2012). A melt curve was included to discount non-specific amplification by heating the reactions to 95 ^oC (0.1 ^oC/s) and cooled to 55 ^oC while fluorescence was detected at 0.3 ^oC interval.

2.5.3 Q-PCR amplification of methanogens in seaweeds anaerobic reactors

Quantitative PCR was carried out to amplify methanogens in the batch reactors by targeting *mcr*A gene which is unique to methanogens (Beckmann et al., 2011; Traversi et al., 2012). Primer pair mlas (5′- GTGGTGTMGGDTTCACMCARTA-3′) and mcrArev (5′-CGTTCHTBGCGTAGTTVGGRT -3′) was used (Steinberg & Regan, 2009; Traversi et al., 2012). A dilution of the standard corresponding to 2.62 $x10^9$ was used as the positive control. The amplification protocol begins with hot start polymerase activation at 95 ^oC for 5 min, followed by 40 cycles 95 °C for 30 s, annealing at 55 °C for 45 s, extension at 60 °C for 2 min and 83 °C for 20 s. The procedure was completed with a final elongation step at 60 °C for 7 min. Melt curve analysis to detect the presence of primer dimers and the specificity of the primers was performed after the final extension by increasing the temperature from 55 to 90°C in 0.5 °C increments every 10 s.

All amplifications were carried out in a 20µl reaction containing 2µl of DNA template, 10µl of q-PCR master mix (PrimerDesign, UK) premixed with SYBR Green I, 2.5µl of forward primer (10ρmol/µl, Bioline, UK), 2.0 µl of reverse primer (10ρmol/µl, Bioline,

UK) and 3.5 µl PCR water, according to manufacturer's instruction. PCR grade water (2 µl) was used as the control along with extraction blanks. All samples were amplified in triplicates, including the controls and standards. Details of primers used in this study are listed in Table 2.3.

Melting point, C_q and melt curve for each reaction were calculated automatically by the instrument LightCycler® 96 Software 1.1 (Roche, Switzerland).

Table 2.3. Primers utilised for q-PCR studies of microbial components of anaerobic digesters

Oligo name	target	Oligo sequence (5′-3′)	Reference
E806f	Bacteria 16S rRNA gene	GGACTACHVGGGTWTCTAAT	(Teske & Sørensen, 2008)
U515r	Bacteria 16S rRNA gene	GTGCCAGCMGCCGCGGTAA	(Wang & Qian, 2009)
A344f	Archaea 16S rRNA gene	GGGGYGCASCAGGSG	(Teske & Sørensen, 2008)
A915r	Archaea 16S rRNA gene	GTGCTCCCCCGCCAATTCCT	(Wang & Qian, 2009)
Mlas F	mcrA gene (methanogen)	GTGGTGTMGGDTTCACMCARTA	(Steinberg & Regan, 2009)
mcrA-rev - R	mcrA gene (methanogen)	CGTTCHTBGCGTAGTTVGGRT	(Steinberg & Regan, 2009)

The following primer name suffixes are used: - F – forward primer, - R – reverse primer.

2.6 Cloning and sequencing

Clone libraries were constructed by ligating the 16S rRNA fragment (archaea) amplified as described in section 2.3.2.2 and *mcrA* gene fragment (methanogen) amplified as described in section 2.3.2.3 into pCR 2.1-TOPO® vector and transformed into One Shot TOP10 chemically competent *Escherichia coli* using the TOPO TA® cloning kit according to the manufacturer's instructions (Invitrogen, CA, USA).

Agar LB plates were prepared using 15 g of agar powder, 10 g of tryptone, 5 g of yeast extract and 10g of $NaCl_2$ made up to 1 litre using sterile water. pH was adjusted to 7.5 using 10% NaOH and autoclaved for 20 min at 121 °C. The mix was cooled to 50 °C before the addition of 1 ml (50mg/ml) ampicillin. Plates were poured 2/3 full and stored at 4 °C until used.

2.6.1 Ligation

TOPO cloning reaction mix was prepared as in Table 2.4. The reagents were mixed gently, incubated for 30 minutes at room temperature, and then placed on ice.

Table 2.4: TOPO® cloning reaction

Reagents	**Quantity (µl)**
Salt solution	1
TOPO vector	1
PCR product	2
Water	2
Total	6

2.6.2 Transformation

Vials of chemically competent cells were thawed on ice, TOPO reaction mix (2µl) (from table 2.4) added and mixed gently before being incubated on ice for 30 minutes. After incubation, the cells were subjected to a heat shock at 42^{o}C for 30 seconds and thereafter

placed on ice for 2 minutes. Pre-warmed SOC medium (250µl) was aseptically added while the tubes were securely capped and put horizontally in a shaker at 200rpm for 1 hour at 37^{o}C. Aliquots from each transformation were aseptically plated on to LB /Amp (50 mg/ml) plates and incubated overnight at 37^{o}C.

Randomly selected colonies were re-streaked onto new LB plates overnight at 37°C. Selected clones were used directly in PCR reactions using vector-specific primers M13F (5'-TGTAAAACGACGGCCAGT - 3') and M13R (5'-CAGGAAACAGCTATGACC -3') (Invitrogen) as previously described (Cardinali-Rezende et al., 2009; Rastogi, et al., 2008). PCR product (5 µl) were visualised by agarose gel electrophoresis to check the sequences are the right sizes as described above (section 2.3.2.4). Clones of the correct size were purified (QIAquick spin columns, Qiagen, Crawley, UK) and sequenced using the primer M13F by Source Bioscience (Glasgow, UK). Five (5) µl of each reaction normalised to 1ng/µl per 100bp and primer (3.2pmol/µl) were used for the sequencing reactions.

2.6.3 Phylogenetic analyses of archaea and methanogen sequences

All clone sequences were viewed and manually corrected using FinchTV Version 1.4.0 (Geospiza Inc.). Sequences were aligned using Bioedit Sequence Alignment Editor (Hall, 1999). Nucleotide sequences were determined for each clone type from the clone library and were compared to the GenBank database using FASTA (Pearson & Lipman, 1988). BLAST (blastn) searches were conducted with the 16S rRNA (archaea) and *mcrA* (methanogens) sequences to determine their relationship to reference sequences in GenBank® database. Phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Confidence in the inferred relationships was assessed using Bootstrap analysis (100 replicates) (Tamura, et al., 2007).

2.7 Statistical analysis

Experimental error was determined for replicate assays and expressed in standard deviation. The significance of differences in results were determined by one-way analysis of variance (ANOVA). Statistical significant interactions were further analysed using post hoc test (Tukey) at 95% confidence interval. Differences between species and across treatments were also determined. All statistical analyses were performed using Minitab Statistical Software version 17.0.

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3 Chapter 3

"My father taught me that the only way you can make good at anything is to practice, and then practice some more"

- Pete Rose

Methane production from washed and unwashed *Laminaria digitata* during anaerobic digestion: preliminary comparative study

This chapter details initial test carried out prior to the main experiment for this project. It was to help determine the optimum experimental design and appropriate range of mesurements required. It this chapter, one of the experimental seaweeds was digested in a batch using activated sludge as the inoculum sources. A test between washed and unwashed seaweeds was also carried out to assess the impact of washing on methane production and microbial populations

3.1 Introduction

Among the several processes by which biomass resources could be utilised for energy production; anaerobic digestion (for methane production) has been reported as the most efficient and environmentally benign, leading to its widespread applications (Hughes et al., 2012; Khalid, et al., 2011; Raposo et al., 2012). However, to obtain sustainable methane production from seaweeds through anaerobic digestion and avoid system failure, it is essential to study and better understand the activities of microbial community involved in the process (Sanz & Köchling, 2007; Ruiz-filippi & Pullammanappallil, 2014) and the way they respond to the various changes throughout the anaerobic digestion process (Smith & Osborn, 2009; Traversi, et al., 2012; Vavilin, et al., 1998; Wilkins, et al., 2015). This knowledge could help to better harness the degradative abilities of certain microbial consortia associated with the process, for better and sustainable biomethane production from seaweeds especially on a large scale (Migliore et al., 2012).

Nevertheless, before embarking on the major experiments for this project, practise and test experiments were carried out to test process requirements and refine techniques and methods in order to obtain more optimised procedures towards subsequent experiments.

Therefore, the aim of the work with the current chapter was to practise some of the techniques to be used during the project, and to demonstrate batch reactor experimental set-up for subsequent experiments. The test experiment evaluated biomethane production during the anaerobic digestion of fresh washed and unwashed *Laminaria digitata* and its impact on the activities/distribution of the microbial community involved in the process.

The hypothesis for this chapter is: *there is no difference in methane production and microbial composition of washed and unwashed L. digitata during anaerobic digestion.*

3.2 Materials and Methods

3.2.1 Materials

Materials for the experiment including bottles, needles and pipes were autoclaved at 121^{o}C prior to the experiment to prevent any form of contaminations. Sediment for the practice experiment was collected from Loch Creran: a sea loch on the west coast of Scotland (56°31'41.5"N 5°20'35.8"W) as described by Ditchfield et al., (2012). Sludge for the test and practice experiment was obtained from a mesophilic waste water treatment plant in Dundee.

3.2.2 Substrate

Fresh *Laminaria digitata* (Fig.3.1) was harvested from the beach behind Scottish Association for Marine Science, (SAMS) near Oban (56°27'09.5"N 5°26'43.2"W) at low tide in February 2012. The seaweeds were immediately stored in the freezer until used.

Fig. 3.1. Fresh *Laminaria digitata* harvested at low tide and used as the experimental seaweeds for the test experiment

Some of the seaweeds were rinsed in fresh sterile water for 30 min. However, during washing the seaweeds absorbed water and became enlarged due to water absorption (Fig. 3.2). Attempts were made to rid the washed seaweed of the absorbed water by squeezing out the water by hand. Washed and unwashed seaweeds were homogenised separately. Despite water removal from the washed seaweeds (by manual squeezing), the rinsed *L. digitata* appeared softer than the unwashed and may therefore have contained less total solids.

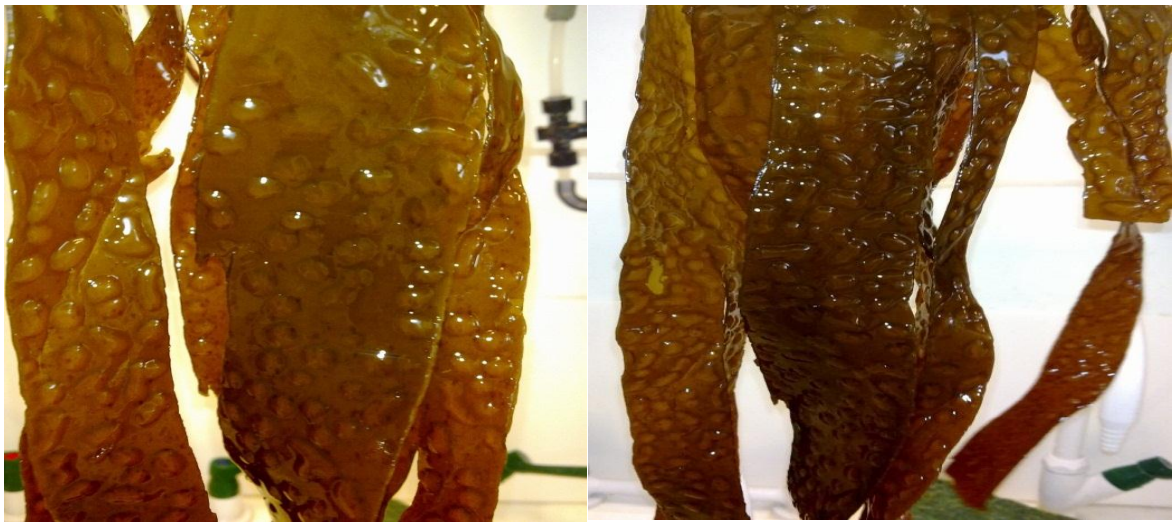

Fig 3.2. Fresh *Laminaria digitata* after water absorption

3.3 Experimental Procedure

Homogenised washed and unwashed *L. digitata* (~2g each) were weighed into 60 ml reactor bottles. Inoculum (10 ml), (anaerobic digested sludge) was added to the bottles and made up to 50 ml with sterile water in six replicates. Control samples (one for each set-up) contained inoculum and sterile water were set up to check gas production due to residual substrates in the inoculum. The reactors were operated at mesophilic conditions (37^{o}C) using a constant temperature water bath. The volume of methane was quantified by the water displacement method described by (Izumi et al., 2010; Velmurugan & Ramanujam, 2011).

3.4 Analytical techniques

3.4.1 Gas measurement

Methane concentration was analysed using 100 µl gas samples of the headspace and quantified using a gas chromatograph GC-3900 (Varian, Netherlands) fitted with a flame ionization detector (FID). Temperature settings were 80°C for the column, 120°C for the injector and 200 °C for the detector. Daily calibration of the gas chromatograph were carried out using a 1000 ppm methane standard (Scientific & Technical Gases Ltd) before methane analysis and the slope of the five-point standard curve was used to calculate methane concentration in ppm. Calibration curves obtained for all samples had a R^2 value > 0.98. The percentage (calculated from ppm values) of methane in the total gas injected was related to the total volume of methane released to obtain specific methane production.

3.5 Microbial analysis

Prior to the microbial analysis of the test anaerobic digestion experimental samples, practice microbial analysis was carried out on sediment cores and digested sludge (similar to the one to be used later). Different methods of DNA extraction were tested to assess the most suitable method, especially one suitable for optimization. Extracted DNA were quantified with Spectrophotometer, diluted (1:10, 1:100) and amplified using different primer sets through polymerase chain reaction (PCR). PCR products were visualised using agarose gel electrophoresis and then tested with denaturing gradient gel electrophoresis (DGGE) techniques to enable visualisation of diversity. Reactor samples taken at intervals (day 3, 6, 8, 10, 13, 15) from the test experiment were analysed based on optimised techniques.

3.5.1 Xanthogenate method of DNA extraction from practice and experimental samples

DNA extraction was carried out as described by Yilmaz, et al., (2009) with some modifications. XS (10ml) buffer was made up using the following reagents: Xanthogenate powder (0.1g), DEPC water (6.6ml), tris- HCl pH 7.4 (1ml), 0.5M EDTA pH 8.0 (400µl), 7.5M Ammonium acetate (1ml) and 10% SDS (1ml). Samples (500µl) were placed in Eppendorf tubes into which 1ml of the XS buffer was added. The mixture was mixed and incubated at 70^{o}C for 1 hour and vortexed throughout incubation. After incubation, the samples were placed on ice for 30mins to precipitate the debris and later centrifuged at 13,000 x g for 15 minutes at 4^{o}C. The supernatant was removed and 1 volume of Phenol: chloroform: isoamyl alcohol (25:24:1; pH 8) was added to the supernatant. The upper aqueous layer was collected and extracted again with phenol: chloroform: isoamyl alcohol. This step was repeated to provide additional clean up. The mixture was vortexed briefly before centrifuging for 10 minutes at 13,000 x g, 4ºC. The supernatant was removed and 1 volume of 14% PEG 8000 (Polyethylene glycol) 20nM $MgCl_2$ was added and put back on ice for another 10 minutes. Samples were the centrifuged at 13,000 x g at 4^{o}C for 15mins after which PEG was removed and samples washed with 1ml 70% ice-cold ethanol. The ethanol was removed while samples were left to air dry. DEPC (Diethylpyrocarbonate) water (100µl) was added to suspend the pellet. Nucleic acids were quantified spectrophotometrically at 260nm. Aliquots of the extracted DNA were diluted 1:10 and 1:100, and kept in the freezer until used.

3.5.2 DNA Extraction from samples using FAST DNA kit for soil (alternative method)

DNA was extracted based on manufacturer's instructions. Frozen digestate samples were thawed and 500µl of each sample was put into Lysing Matrix E tube. Sodium phosphate

buffer (978µl) and 122µl of MT buffer were added and vortexed for 30 seconds each. The mixture was centrifuged at 14,000 x g for 10minutes. The supernatant was transferred to clean tubes while 250µl of PPS reagent was added and thoroughly mixed. The mixture was centrifuged at 14,000 x g for 5 minutes. 1 ml of re-suspended binding matrix suspension was added to 1ml of the supernatant in clean 2 ml tubes and vortexed to mix and allow the binding of the DNA to the matrix. Approximately 600 µl of the mixture was transferred to the spin filters and centrifuged at 14,000 x g for 1 minute. The catch tubes were emptied and the remaining mixture was added to the spin filter and spun again. The flow through was again discarded. 500ul of SEWS-M solution was added to the spin filters and centrifuged at 14,000 x g for another minute. Flow through was discarded and the spin filters placed in catch tubes. The samples were left to air dry for 5 minutes after which 50µl DES (DNase/Pyrogen free water) was added and gently mixed in the filter membrane to re-suspend the silica for efficient elution of the DNA. The mixture was centrifuged at 14,000 x g for 2 minutes to transfer the eluted DNA into the catch tube ready for further applications.

3.5.3 Polymerase chain reaction (PCR) of 16*S* rRNA gene fragments from bacteria (DNA extracts from practice and test experiments).

Polymerase chain reaction (PCR) was carried out to obtain a small DNA fragment (<200bp) suitable for DGGE using the bacterial specific primer pair primer 2/3 (5'-ATTACCGCGGCTGCTGG-3' and 5'- CCTACGGGAGGCAGCAG -3') (Muyzer, et al., 1993). Another set of primers (pD/pF' 5'- CAGCAGCCGCGGTAATAC-3' and 5'-ACGAGCTGACGACAGCCATG -3') were used to amplify a larger fragment size (~560bp) as described by Edwards et al., (1989). All PCR runs were carried out in a total 25µl volume containing 0.5µl of forward primer, 0.5 µl of reverse primer (10ρmoles/ µl), 0.1 µl of MyTaq polymerase (5u/ µl) (Bioline UK). Other components of the mix are 5µl

of PCR Buffer (comprising 5mM dNTPs, 15mM MgCl, stabilizers and enhancers) (Bioline, UK), 18.4 µl of molecular grade water (Bioline, UK) and 0.5 µl of DNA extract (Stock, 1:10, 1:100 dilutions). Negative controls containing 0.5µl of sterile molecular grade water were included in all cases. The PCR program for bacterial DNA included an initial denaturation step for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 65-53°C touchdown (pDpF primer at 55°C), and 1 min at 72°C. Primer extension was carried out for 5 min at 72°C.

3.5.4 Archaea nested PCR for DNA extracts of practice and test experiments

PCR amplification of general archaea requires nested PCR in order to obtain sufficient product for DGGE analysis. It has been demonstrated that the use of nested PCR can improve sensitivity and specificity of PCR, particularly when there is a high ratio of non-target to target organisms (Boon, et al., 2002; Garbeva, et al., 2003; Nakatsu & Torsvik, 1996; Øvreås, et al., 1997) such as in these samples. The first round of PCR amplification was performed to obtain a larger size fragment using archaea specific primer pair PRA46f (YTAAGCCATGCRAGT) (Øvreås, et al., 1997) and Arch1017r (GGCCATGCACCWCCTCTC) (Barns, et al., 1994) to produce a 971-bp fragment. The second round was performed using the broad specificity primer pair 344fgc (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACGGGGHGCAG CAGGCGCGA)/ Parch519r (TTACCGCGGCKGCTG) (Banning et al., 2005) giving a product internal to the first round of ~190bp.

3.5.5 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise the PCR products and check for efficiency. Loading buffer (2µl), ((0.25%w/v) bromophenol blue; 40% w/v sucrose in filtered sterilised water) and 5µl of sample was run on an agarose gel (1% agarose in 1 X

TAE buffer (40nM tis acetate, 2nM EDTA pH 8)). Gels were run at 120V for 30 minutes. Marker 2000-100 bp (Easy ladder 1; Bioline, UK) was run on all the gels to estimate the size of the PCR products. The gel was then stained in ethidium bromide (0.8 ng/ml) solution for about 30 minutes and rinsed with sterile water. The gel was visualised and digitized using a digital imaging system (Alpha Innotech Alphaimager) with UV transillumination.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using an INGENYphorU-2 system (Ingeny, Netherlands). PCR products and loading buffer (40% [wt/vol] sucrose, 60% [wt/vol] 1 x Tris-acetate-EDTA [TAE], and bromphenol blue) were mixed in a 1:1 ratio. The mixture of PCR amplicons and loading buffer were applied directly to 10% (wt/vol) polyacrylamide (37:1 acrylamide :bisacrylamide) gels with a linear gradient of 30 to 60% denaturant for ~ 200 bp bacteria and archaeal PCR products. Denaturant (100%) corresponds to 7M urea and 40% [vol/vol] formamide in 1 X TAE). Electrophoresis was carried out in 1 X TAE buffer (40 mM Tris-acetate [pH 7.4], 20 mM sodium acetate, 1 mM sodium EDTA) at a constant voltage of 100 V and at 60°C for 19 hours. After electrophoresis, gels were stained for 30 minutes in 1 X SYBR Gold solution (Sigma, UK) diluted 1/10000 in 1 X TAE and washed with distilled water. The gel was digitized using a digital imaging system (Alpha Innotech Alphaimager) with UV transillumination (Beckmann et al., 2011).

Gel analysis was carried out with the software GelCompare II version 6.6 (Applied Maths, Belgium) as earlier described in chapter 2

3.6 Results and discussion

3.6.1 Results from test experimental samples (batch AD reactors)

The test experiment was carried out to evaluate AD process requirements in other to get familiar with some of the techniques to be used subsequently. The opportunity was used to test the differences if any, in the anaerobic digestion of washed and unwashed *Laminaria digitata* because of the high salt content of the seaweeds. The impact of washing on methane production and microbial community structure were assessed. Microbial composition and community structure were assessed using denaturing gradient gel electrophoresis (DGGE).

3.6.1.1 Methane analysis

Prior to every methane analysis, the GC was calibrated (Fig. 3.3) using 100µl gas tight syringes as described by Ditchfield et al., (2012).

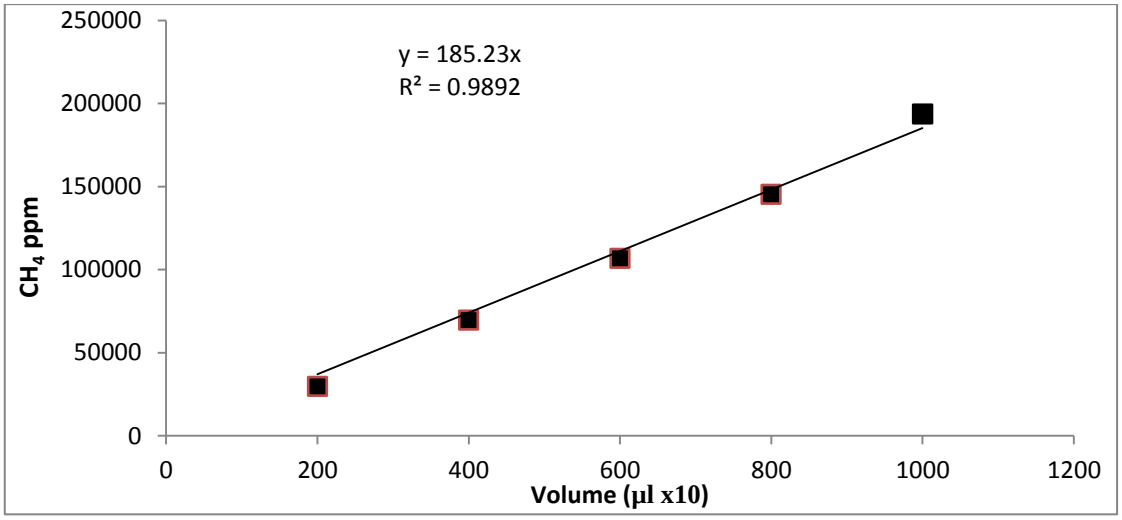

Fig 3.3. Result of GC calibration using 20-100µl of 1000 ppm pure methane. This was performed before every methane measurement.

Methane volume was determined by the volume of water displaced, whilst actual methane produced was calculated from the proportion of methane in the methane analysed. The experiment was discontinued after 15 days after some problems with the water

displacement method became apparent. Cases of water (and gas) leakages were observed between day 13 and 15 in a few batches. Also due to the small reactor size, the pressure from gas production appeared not sufficient to bring about water displacement in some of the batches, leading to long contact time between water and the methane produced. Results obtained during this time were however analysed.

Result of cumulative methane production during the 15-day anaerobic digestion showed that there was no significant difference between washed (10.72 ml) and unwashed (11 ml) *L. digitata* (Fig 3.4). Specific volumes of methane produced between each sampling point for the two treatments are shown in appendix (3.A1).

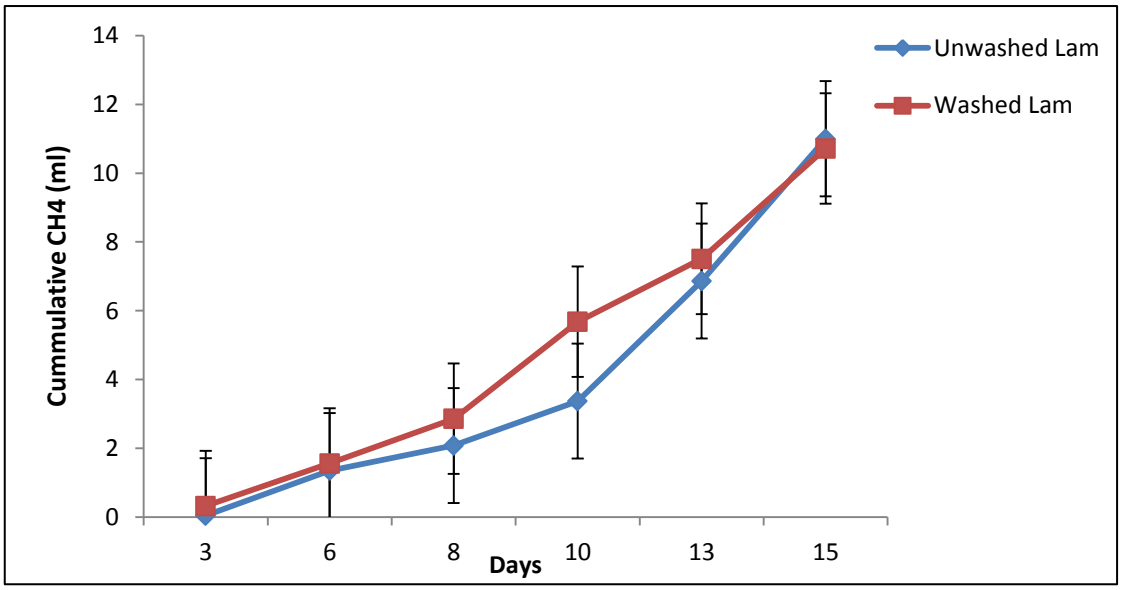

Fig. 3.4. Methane production in washed and unwashed *Laminaria digitata*

Even though the experiment was discontinued midway, the preliminary results obtained suggests that washing seaweeds (to reduce salt content) prior to anaerobic digstion, which has been carried out in a number of publised reports (Ross, et al., 2008; Marquez, et al., 2013; Park, et al., 2012; Vergara-Fernández, et al., 2008) might not be necessary. Washing seaweeds prior to anaerobic digestion results in fresh water usage and reduces the overall sustainability of the process.

3.6.2 Molecular biology analyses of practice samples (sediment and sludge)

The main aim of the practice experiments was to determine the most suitable methods to obtain high quality DNA from these samples. Two extraction methods, Xanthogenate extraction and FAST DNA soil kit, were tested in order to determine the optimum procedure for extraction of DNA from the two inoculum types. Suitability was assessed based on DNA extraction efficiency (tested by PCR), suitability for modification/optimization and cost.

Results of initial test of bacteria and archaea amplification of DNA extracted from sediment cores showed very little difference between the two extraction methods (Fig 3.5). This suggests that both methods are suitable for the types of samples being studied. Non-specific amplification (illustrated by the presence of multiple bands) occurred more noticeably in the bacteria PCR when the raw/stock extract was amplified (Fig.3.5B) than when the extracts were diluted (Fig.3.5A). This indicated that diluting DNA extracts could be beneficial to PCR, not only in reducing the amounts of debris in the extracts but also limits the effects of inhibitory materials, which may hamper the efficiency of the PCR process. The advantage of dilution is evident in the brightness of the bands of the 1:10 dilution of the DNA extract (Fig.3.5A). Traversi et al., (2012) demonstrated the benefits of DNA extracts dilution during molecular biology studies of environmental materials, which include its impact of limiting the effects of inhibitory materials.

A few nonspecific amplifications could be seen in the archaea bands of xanthogenage DNA extracts (Fig.3.5 C) which is likely as a result of carryover of first round PCR products of the nested archaea PCR. Overall the differences between the two methods of extraction in relation to PCR efficiency was not significant

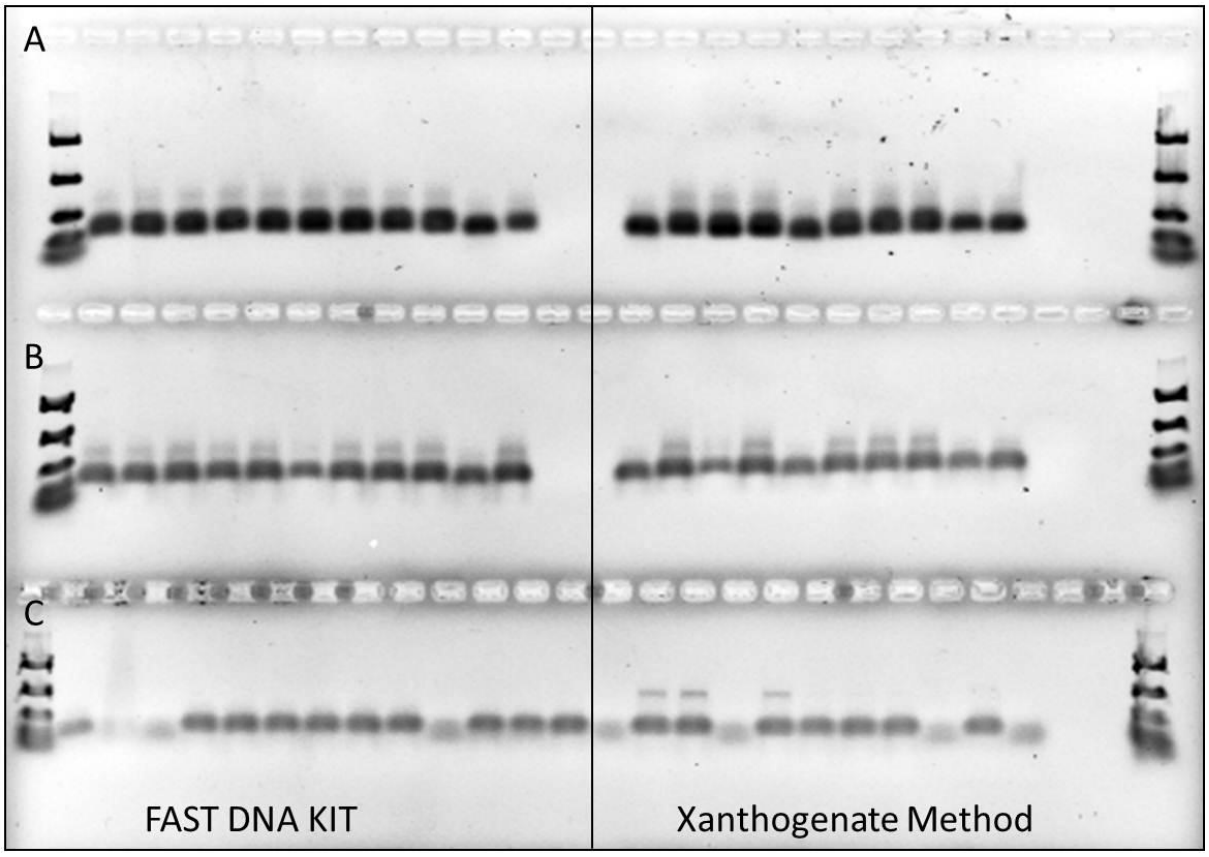

Fig 3.5. Duplicate gel image for (A) bacteria (1:10 dilution) (B) bacteria (stock DNA) and (C) archaea (stock DNA extract) using two extraction methods from sludge inoculum. The marker (easy ladder 1) was obtained from Bioline UK. A and B were subjected to DGGE analysis.

The benefit of dilutions was further highlighted by bacteria PCR amplification of DNA extracts (Xanthogenate method) from sediment cores. The result clearly showed a gradual decline in non-specific amplification and suggested that diluting DNA extracts 1:100 could be best for upstream applications (Fig. 3.6). Although there was a slight decline in the band intensity between 1:10 dilution (Fig. 3.6A) and 1:100 dilution, the 1:100 dilution of the extracted DNA is considered the most suitable for bacteria molecular biology studies. This is in consonance with published reports where the purity of DNA (PCR products) has been shown to be of more importance than the quantity of the available DNA (Bergmann & Naturwissenschaften, 2012; Bustin, et al, 2013; Kim, et al,. 2013; Kim et al., 2013; Webster, et al., 2003).

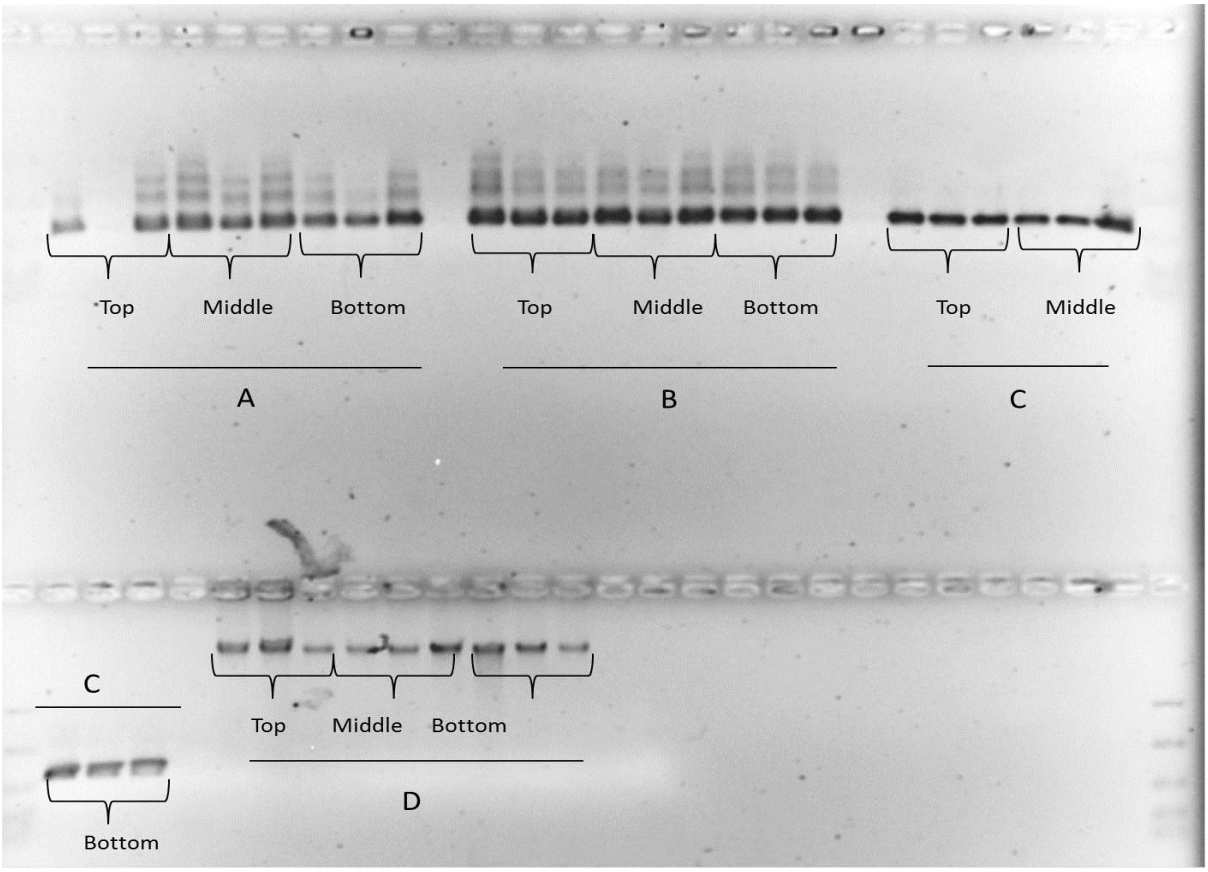

Fig 3.6. Agarose gel image of bacteria from 10-inch sediment cores (top: 1-3, middle: 3-6, bottom: 6-10 inch) showing different dilutions. A: Stock DNA extract in a PCR using primer 2/3 pair, B: 1 in 10 dilutions of DNA extract in a PCR using primer 2/3 pair, C: 1 in 100 dilutions of DNA extract in a PCR using primer 2/3 pair, D: 1 in 100 dilutions of DNA extract in a PCR using pDpF primer pair. The PCR product (C) was used for the DGGE below.

Denaturing gradient gel electrophoresis (DGGE) was carried out on some of the PCR products reported earlier (Fig 3.5 and 3.6) to check if the differences observed in the agarose gel will be evident in a gradient gel. Results obtained show that dilution (1:10 and 1:100) had little impact on microbial distribution in a gradient gel (Fig 3.7). It however further supports the choice of 1:100 DNA extract dilution as the most suitable for subsequent microbial studies.

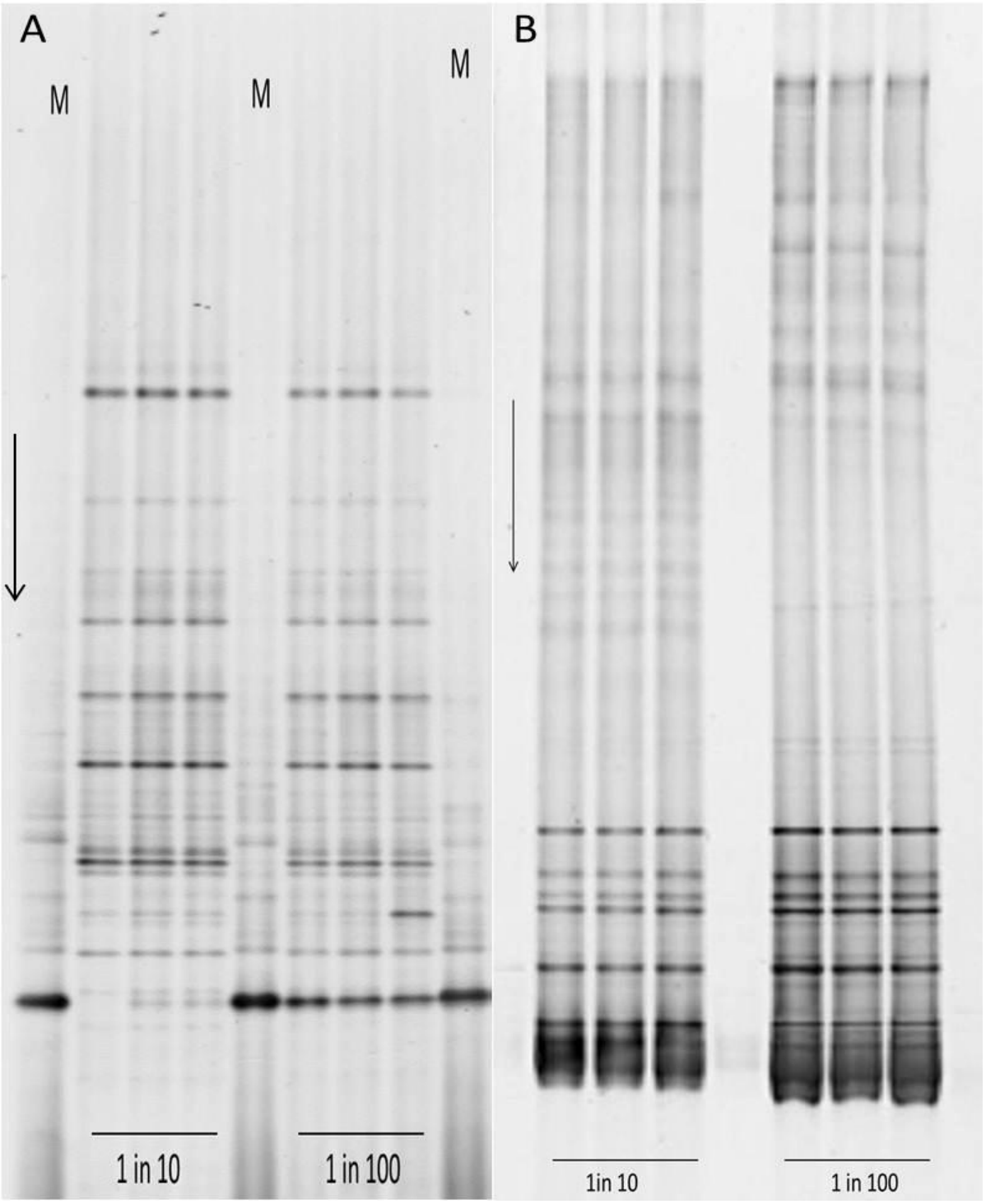

Fig. 3.7. DGGE image of bacteria in sludge (triplicates) using (A) primer 2/3 pair and (B) pDpF primer showing DNA extract dilutions. Arrows indicates the direction of gel increasing concentration gradient.

Fig. 3.8 shows bacteria distribution from 1:100 DNA extract (of sediment cores) dilution on a gradient gel and confirms its suitability as the dilution of choice for subsequent molecular studies.

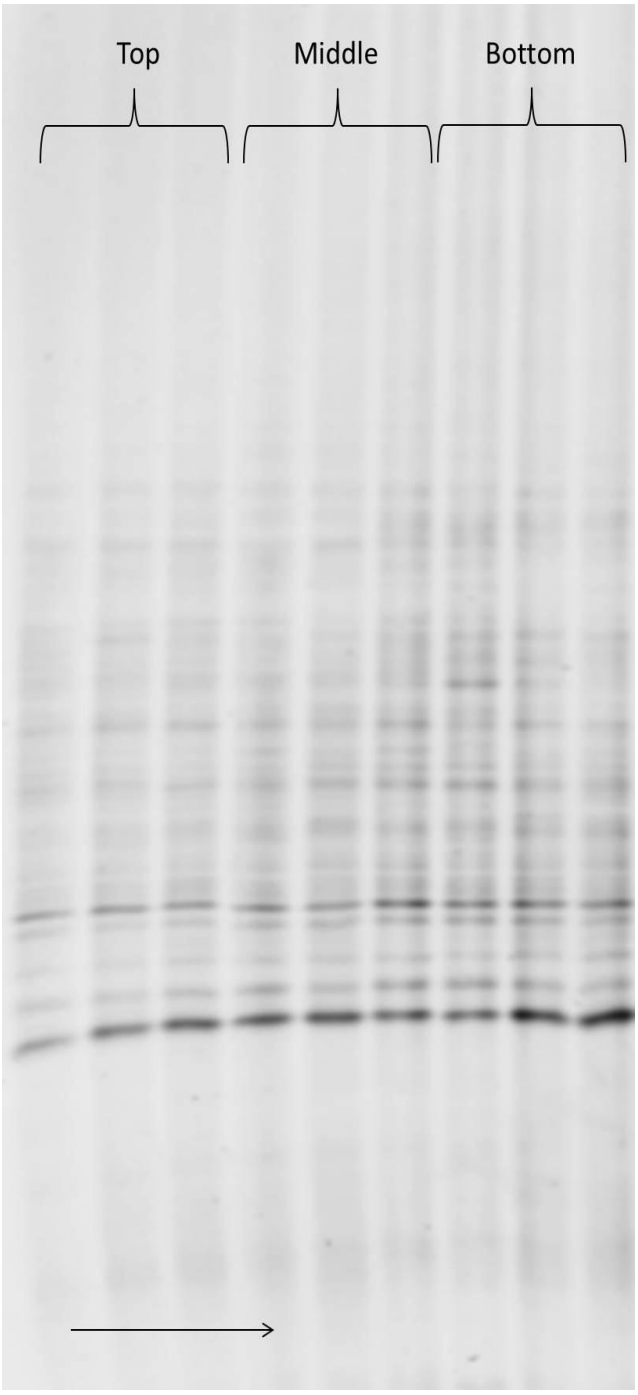

Fig. 3.8. DGGE image of bacteria in 10-inch sediment cores of 1 in 100 DNA extract dilution using primer 2/3 pair in triplicates. Arrow indicates increase in sediment depth. Top (~1-3 inch), middle (~3-6 inch) and bottom (~6-10 inch)

3.6.3 Molecular studies of bacterial and archaeal populations during the 15-day AD batch test

To assess the impact of washing on the microbial community during the 15-day anaerobic digestion, DNA was extracted from the batch reactors using a modified xanthogenate method (establised earlier). Polymerase chain reaction (PCR) was carried out as described in section 2.3.2.1 (bacteria, 1:100 DNA dilution) and 2.3.2.2 (archaea, 1:10 DNA dilution). PCR products were analysed using denaturing gradient gel electrophoresis (DGGE).

3.6.3.1 Bacteria composition and community structure in washed and unwashed L. digitata batch reactors during a 15-day anaerobic digestion

Result of bacteria DGGE analysis of the unwashed and washed showed that the bacterial communities within the two experimental set-ups appear different. For instance, at the earlier stages (day 3) of the digestion process, DGGE results showed that the unwashed seaweed reactors harboured more bacteria community with at least 11 distinct bands, compared to about 7 in the washed seaweed reactors (Fig. 3.9). Apart from richness, another noticeable difference in bacterial community between unwashed and washed seaweeds reactors is the distribution within the gel. At day 3, while bands were roughly evenly distributed between the two halves of the unwashed seaweeds reactors DGGE gel, most of the bands in the washed seaweed reactor occurred at the top half of the gel. The results indicated that the amplified DNA of most of the bacteria communities in the washed seaweeds reactors were AT rich while those in the unwashed seaweeds are more GC-rich. Similar observation was recorded in bands distribution between the unwashed and washed seaweeds reactors throughout the 15-day digestion process.

After day 3, bacterial community richness increased in all reactors (unwashed and washed) throughout the process. However, while most of the bacterial community occurred in the

bottom half of the gel in unwashed seaweeds reactors, the opposite holds in the washed seaweeds reactors DGGE gel analysis (Fig. 3.9).

Pairwise comparison of DGGE gel band composition at day 3 shows that there is only 34.86% similarity in bacterial community distribution between the two gels (unwashed and washed). In the same vein, at day 8 and 10, similarity in bacterial community distribution remained low at 33.58 and 49.06%, respectively.

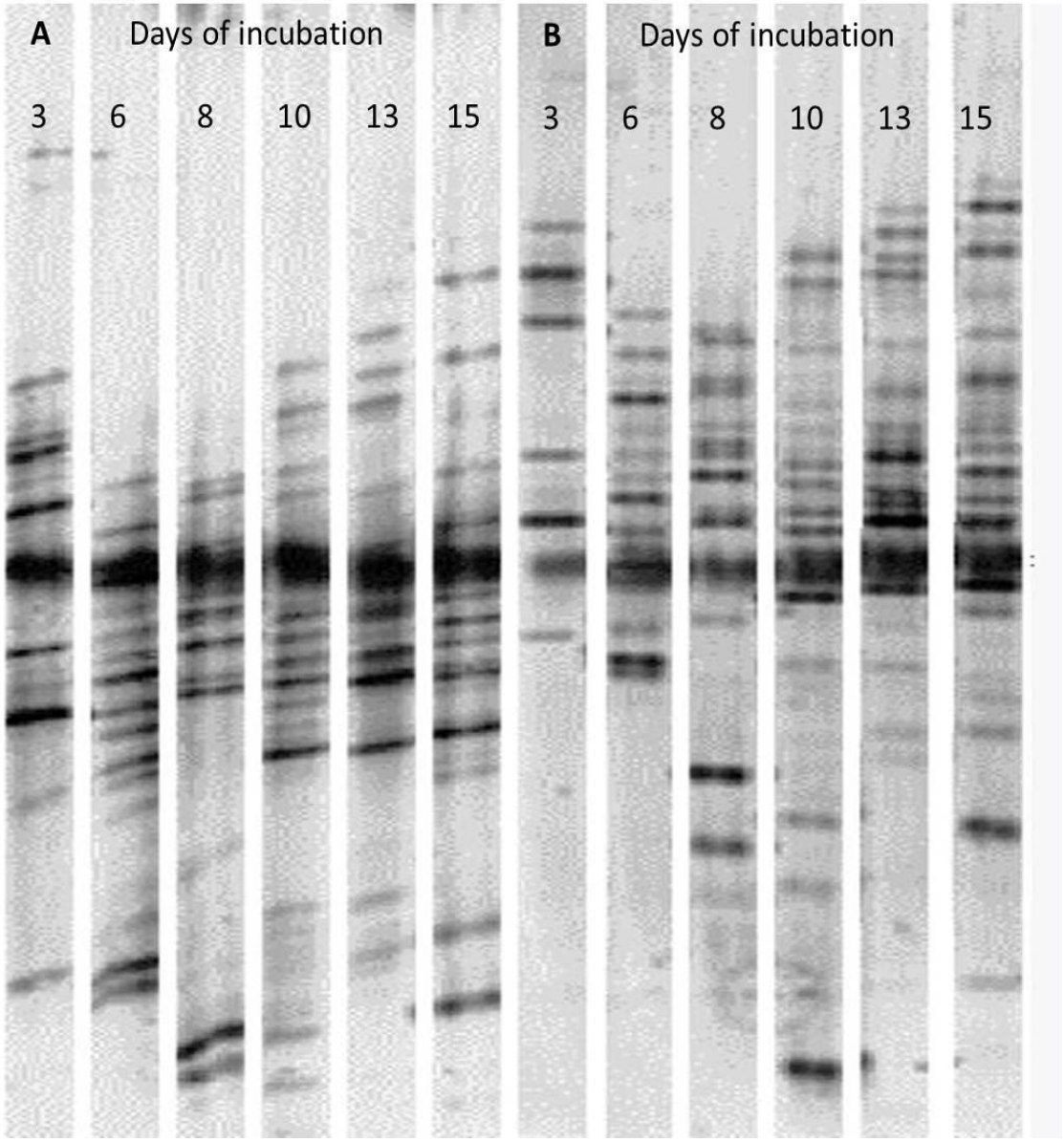

Fig 3.9. DGGE gel image of bacterial composition and community structure in (A) unwashed and (B) washed *L. digitata* batch reactors during a 15-day anaerobic digestion

However, similarity in bacterial community exceeding 50% was recorded between unwashed and washed seaweed reactors at some time points. For instance, at day 6, 13 and 15, pairwise comparison of the bacterial community were 52.75, 55.50 and 56.38% similar between unwashed and washed *L. digitata* batch reactors, respectively (Fig. 3.9). Similarity in bacterial community composition between washed and unwashed *L. digitata* batch reactors at the same time point is likely indicative of similar biological process occurring at that time.

In terms of diversity (indicated by the number of bands), unwashed *L. digitata* reactors appeared to harbour more bacterial diversity especially at the initial stages of the digestion process. A plausible explanation for this is likely that some inherent bacteria associated with the seaweeds might have been washed away during washing leading to lower initial bacterial community in the washed seaweed reactors. However, increases in bacterial community richness were observed in both unwashed and washed seaweeds reactors over time indicated by increases in band numbers and band intensity, although the pattern of band increases appeared differently between the unwashed and washed seaweeds reactors. Most of the bacteria community (bands) in the unwashed seaweed reactors occur at the bottom half of the gel and are largely unchanged during the process. These bacterial communities observed at the bottom half of the gel in the unwashed seaweed reactors were largely absent in the washed seaweed reactors. This observation suggests that many of the inherent bacteria communities in the seaweeds were GC rich and many of them might have been washed off from the washed seaweeds. Although volatile fatty acids produced by bacterial activities were not measured, production of similar amount of methane from both unwashed and washed seaweed experiments suggests that the most of the bacteria communities, which dominated the bottom half of the gel, might not have been actively involved in the process. This position is supported by their little variation over time and

their near absence in the washed seaweed reactors. However, bacterial communities found in the top half of the gel appeared to be active and possibly contributed to the process functions due to their variation and dominance over time especially in the washed seaweeds reactors. It appears that the growth of suitable bacterial community (found mainly at the top half of the gel) was stimulated in both reactors over time, to drive the process, with more diversity occurring in the washed seaweed reactors. Higher diversity recorded at the top half of the washed seaweeds gel might also be due to the stimulation of growth of bacterial communities, which thrive under low salinity levels but might not be directly involved in the methane production process.

Further research involving VFAs analysis, longer retention time would be required to determine how washing of seaweeds prior to anaerobic digestion affects bacterial community structure.

Within each of the different treatments (unwashed or washed), similarity remained high, as there were a number of consistent band throughout the process. Similarity in bacteria community within treatment was more evident in the washed treatment with at least 50% similarity across all time points whereas similarity across unwashed treatment was about at least 40% (Fig. 3.10).

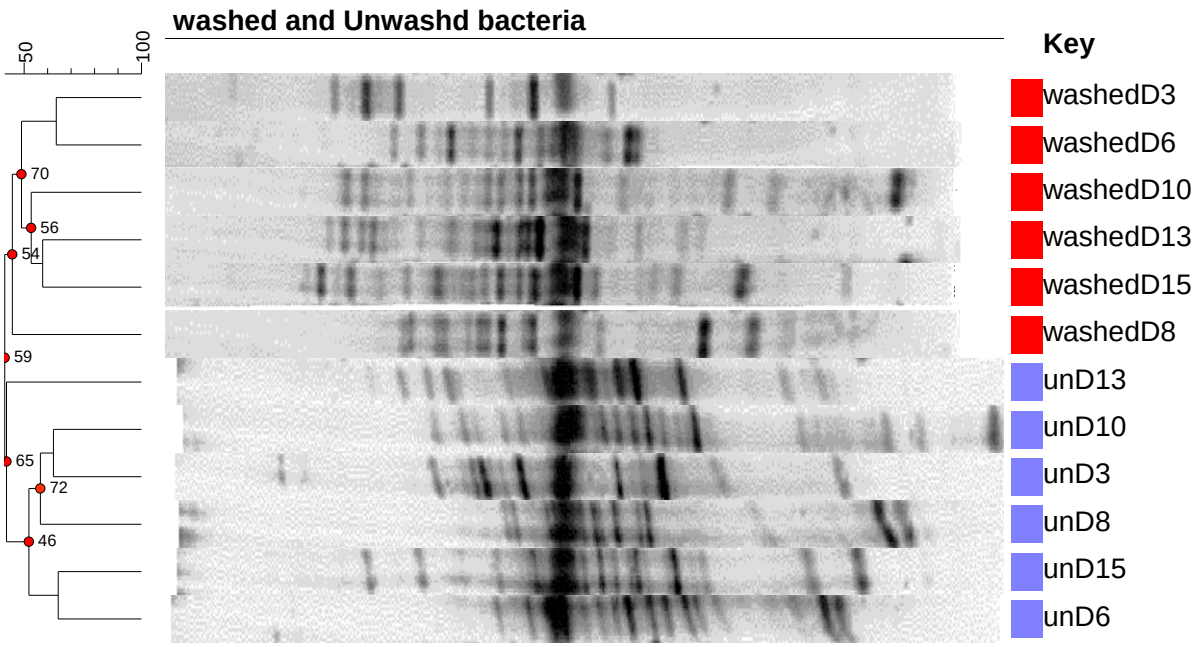

Fig 3.10. Cluster analysis of bacterial community in unwashed (top) and washed *L. digitata* batch reactors (below).

Cluster analysis of bacteria community in the reactors indicates that composition and diversity are time dependent and increase as the process progressed (Fig. 3.10). Higher bacterial diversity observed at day 13 and 15 compared to the day 3 in both treatments may result from higher microbial activity brought about by substrates availability at that stage of the process. Should the experiment have been run for longer, the results of microbial composition at the end of the process might have been different.

Results of multidimentional scalling (MDS) plot demostrates clearly, the influence of time on bacterial community distribution in washed *L. digitata* reactors. This delineation was not very evident in the unwashed *L. digitata* reactors (Fig 3.11).

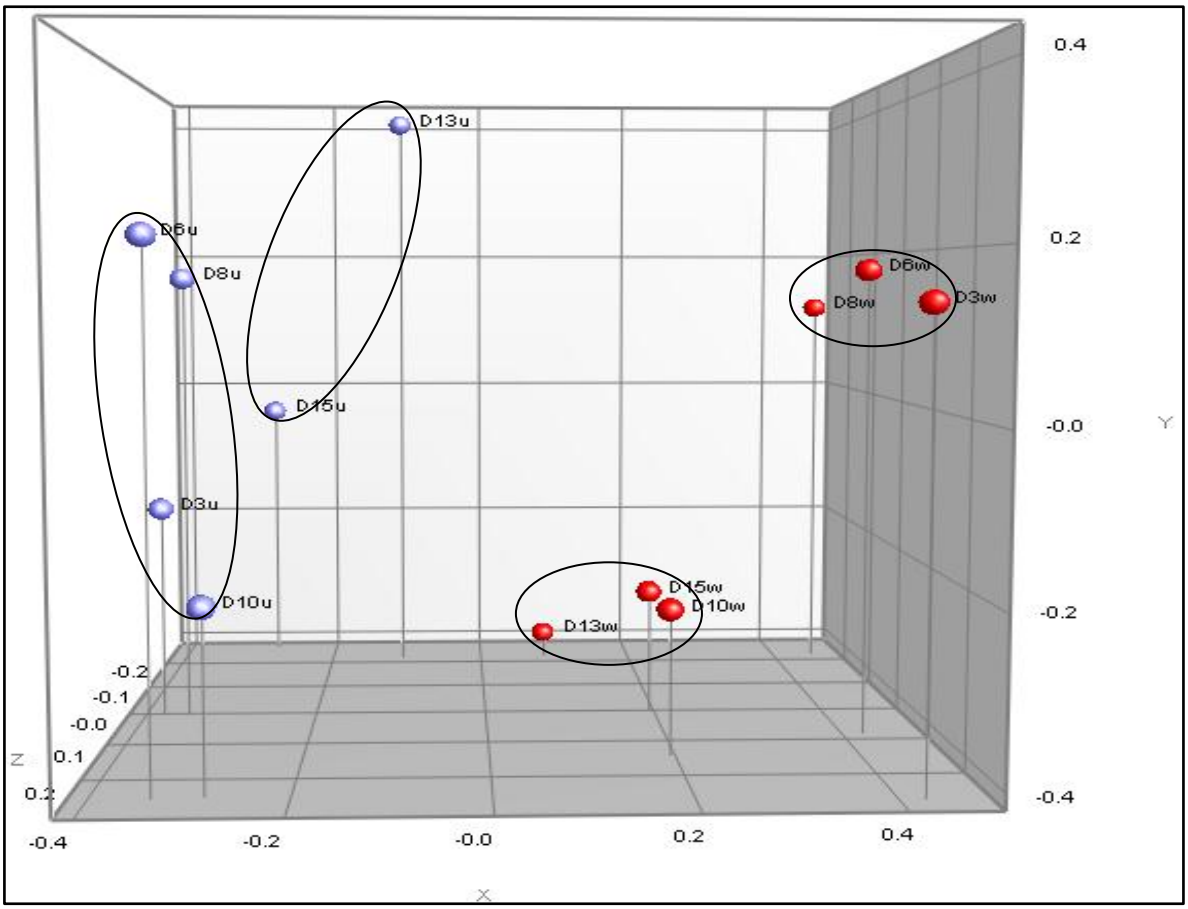

Fig. 3.11. Multi-dimentional scalling (MDS) plot of bacteria community in unwashed (blue; u) and washed (red; w) *L. digitata* batch reactors

3.6.3.2 Archaea composition and community structure in washed and unwashed L. digitata batch reactors during a 15-day anaerobic digestion

Different groups of microorganisms are responsible for specific stages of the anaerobic digesetion process. While bacteria are involved in the first three stages of the process; namely hydrolysis, acidogenesis and acetogenesis, methanogens belonging to the domain archaea are responsible for the final stage of methane production termed methanogenesis (Khalid et al., 2011).

Unlike the the bacterial community, the archaea population in both unwashed and washed seaweed reactors did not appear to be as diverse. Archaea community composition and

distribution revealed by denaturing gradient gel electrophoresis (DGGE) showed only few distinct bands in both unwashed and washed *L. digitata* batch reactors (Fig 3.12).

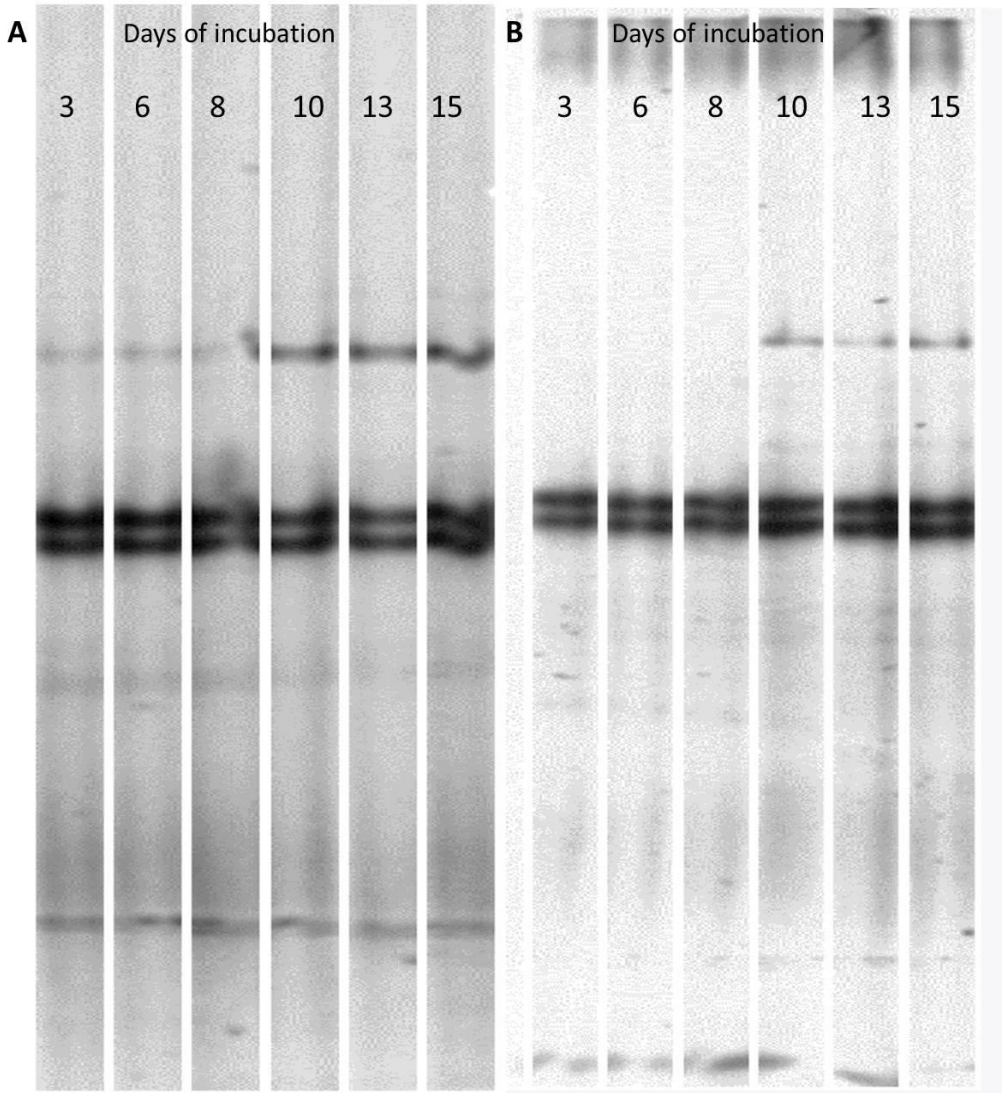

Fig. 3.12. DGGE gel image of arcaheal composition and community structure in (A) unwashed and (B) washed *L. digitata* batch reactors during a 15-day anaerobic digestion

Few bands recorded in archaea DGGE is an indication of little methanogenic archaea presence in the reactors, especially in relation to the length of the process (only 15 days).

Generally, there was little change in the archaeal community during the process which might be due to the length fo the digestion process. These results might have been different if the digesetion process have been carried out for longer. Comparison of archaea

community between unwashed and washed *L. digitata* reactors suggests that the unwashed *L. digitata* reactor harboured more diversity; having more distinct bands (Fig 3.12). The reason for this is likely that some inherent archaea in the seaweeds might have been washed off, although they may or may not be involved in methane production.

Cluster analysis of the archaea community revealed a similarity between the two treatments (unwashed and washed) (Fig. 3.13), of at least 40%. However, some differences were observed between washed and unwashed communities demonstrating that washing will affect the initial archaeal community.

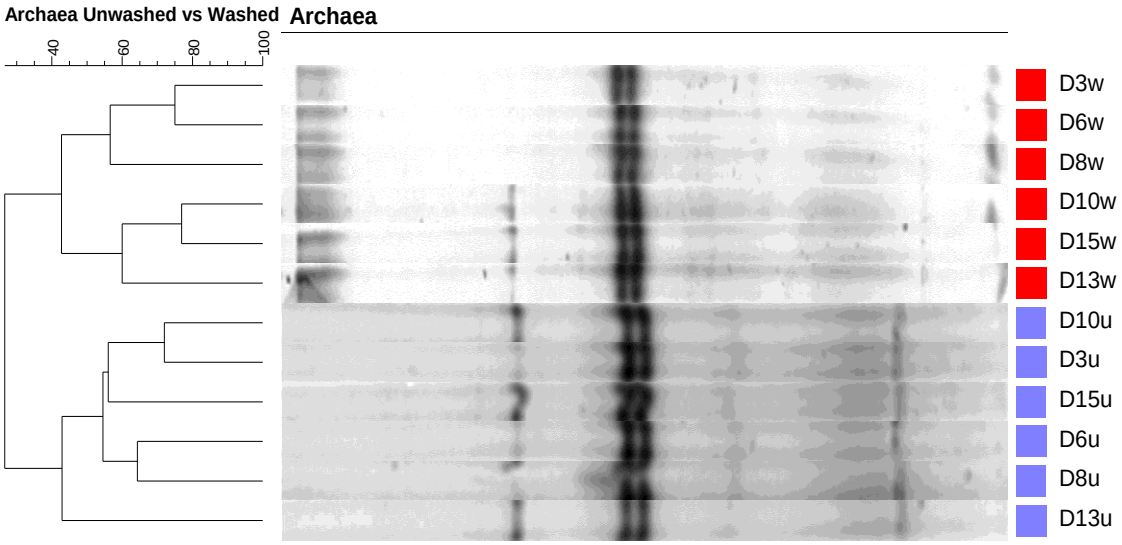

Fig. 3.13. Cluster analysis of archaea community in unwashed (u, blue) and washed (w, red) *L. digitata* batch reactors.

A pairwise comparison of archaea communities within unwashed and washed *L. digitata* batch reactors at specific time points indicates a higher similarity level than within bacteria communities. For instance, archaea community within the washed *L. digitata* batch reactors at day 3 were 67.77% similar to the unwashed substrate reactor. In the same vein, archaea community in both treatments were 71.18, 79.04 and 72.40% similar at day 6, 8, and 10 respectively (Fig 3.13). Similarity in archaea community between the treatments

increased to 81.06% at the end of the experiment (day 15). These preliminary results are suggestive of the fact that washing of seaweeds prior to anaerobic digestion have no significant impacts on the archaeal population in the reactors, although results represent only 15 days of anaerobic digesetion.

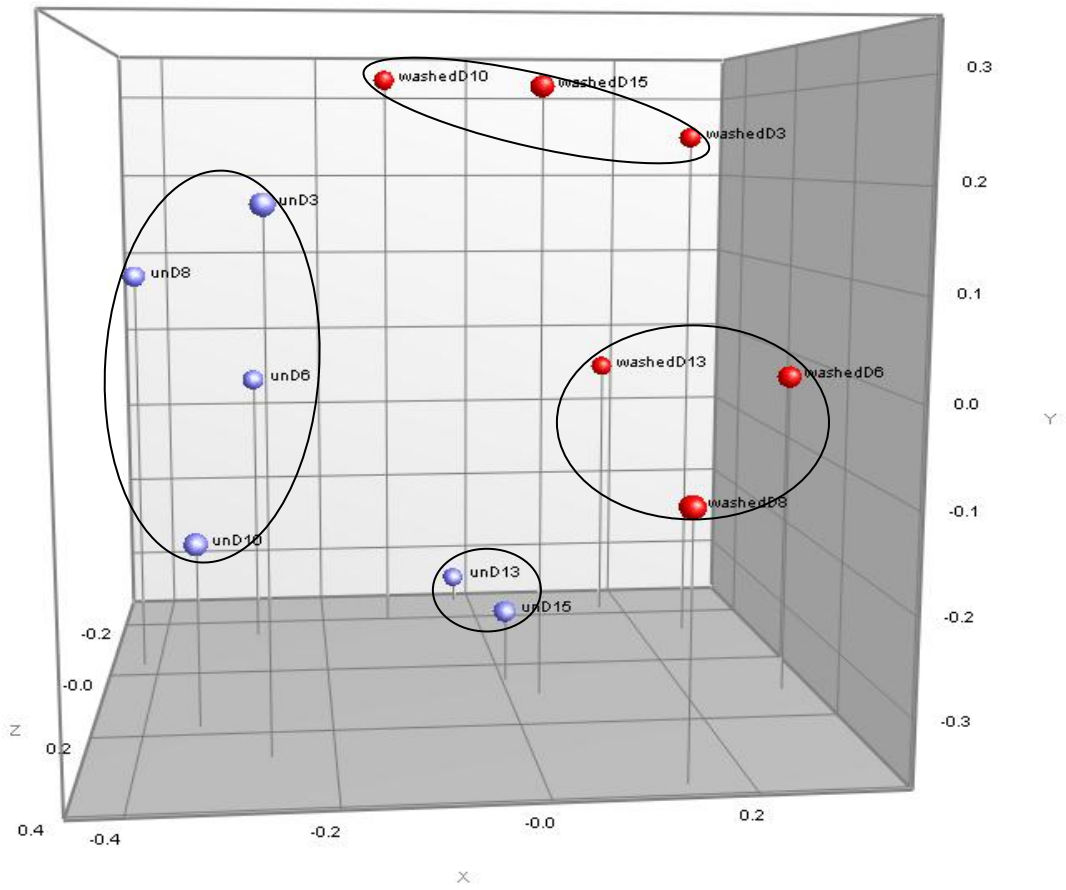

Fig. 3.14. Multi-dimentional scalling (MDS) plot of bacteria community in unwashed (blue; un) and washed (red; washed) *L. digitata* batch reactors

MDS plot used to show shifts in archaea community structure over time demonstrates the influence of time or the stage of the process as a determinant of microbial composition and community structure (Fig 3.14).

3.7 Conclusion

For the main seaweeds AD test experiment, the water displacement method of gas measurement was found to be unsuitable. Therefore, gas measurements for subsequent AD test were performed by allowing gas build up and release at intervals. Gas released are then measured as described by Hansen et al., (2004). This method has been tested on a previously research involving anaerobic digestion of seaweeds (Obata et al., 2015)

For DNA/RNA extraction for the molecular studies of microbes, Xanthogenate method of extraction was chosen based on its adaptability and ease of optimization, efficiency and low cost. Suitable DNA extract dilutions were considered; 1:100 for bacteria and 1:10 for archaea

Washing of seaweeds prior to anaerobic digestion was considered unnecessary, partly because preliminary results do not indicate any advantages and secondly in order not to undermine the sustainability of the process by minimising inputs.

Preliminary results obtained from this chapter suggest that there is no need washing seaweeds prior to anaerobic digestion, which will make the entire process more sustainable

The purpose of this experimental chapter was to learn the basic techniques associated with the measurement of gas produced during the anaerobic digestion of seaweeds and some of the techniques for the determination of microbial population involved in the process. The purpose was achieved as the basic techniques and issues associated with AD experiments were learnt, some of which were incorporated into subsequent experiments.

Although, stopped midway, preliminary results from this experiment agrees with the hypothesis that *washing of seaweeds prior to anaerobic digestion does not enhance methane production during the process.*

3.8 References

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Appendix

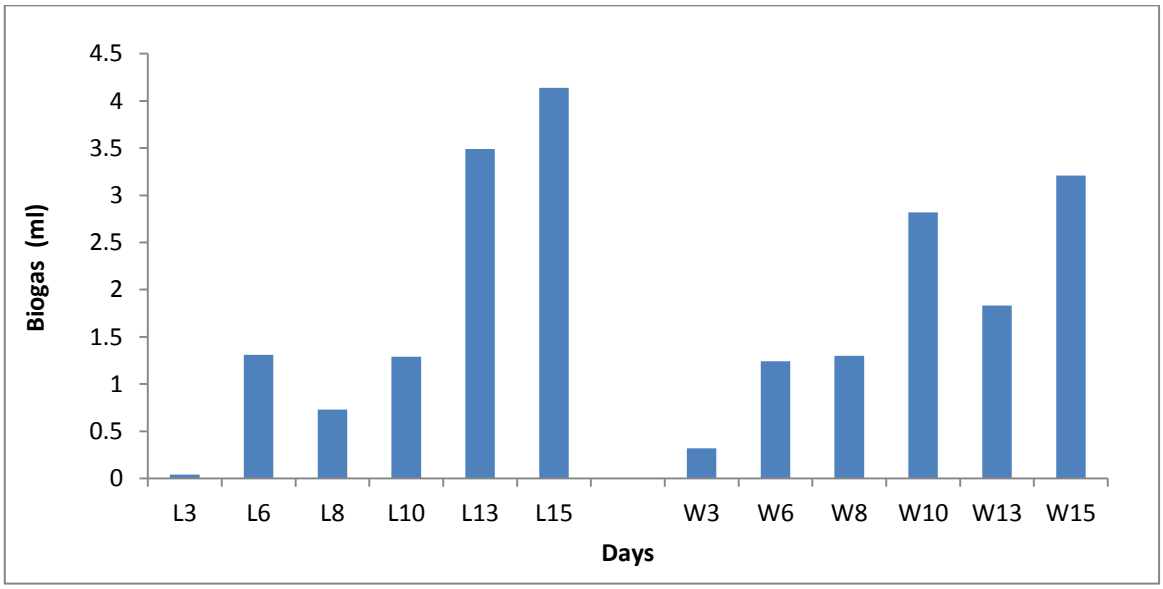


Fig. 3.A1. Volume of water displacgas produced) during anaerobic digestion of washed and unwashed *Laminaria digitata* (L= unwashed, W= washed).

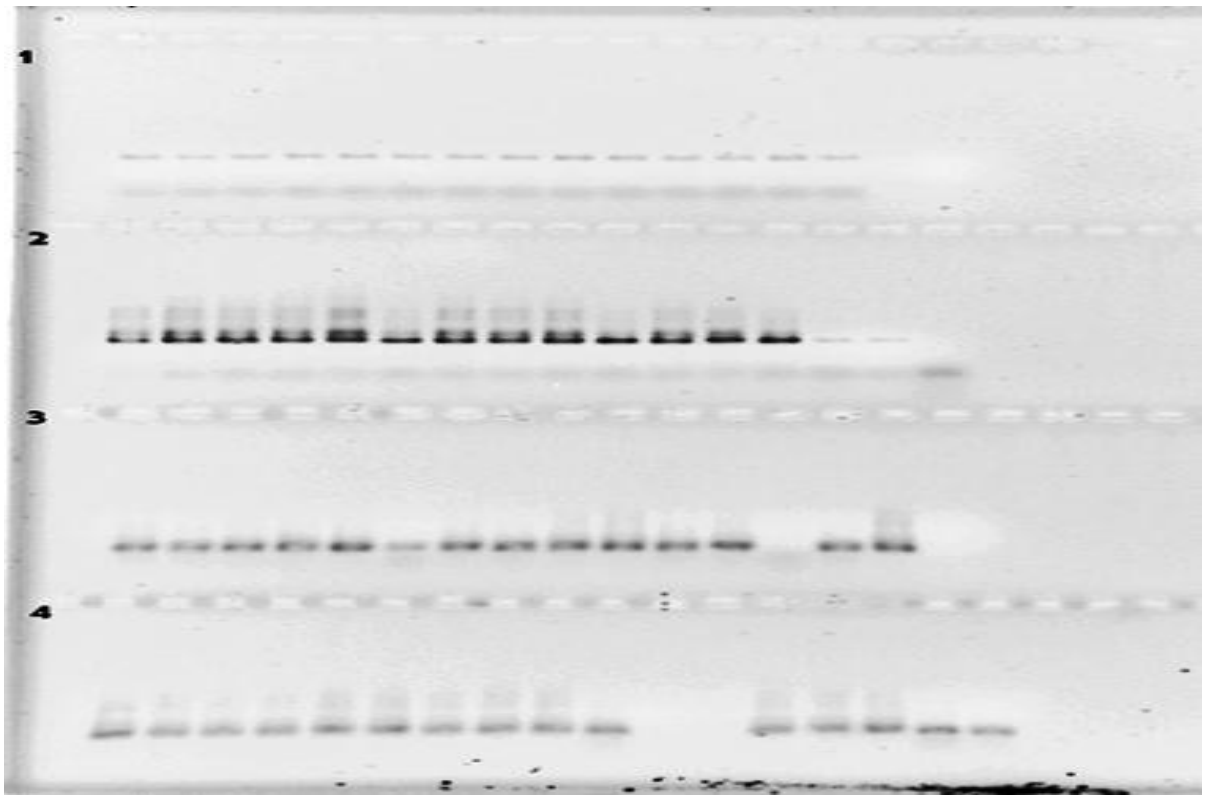


Fig. 3.A2 Agarose gel image of bacteria PCR of DNA extracted from sediment cores using FASTKIT (1) and Xanthogenate method (2); Archaea nested PCR of DNA extracted using FASTKIT (3) and Xanthogenate method (4); from the test microcosm bottles, AD set up.

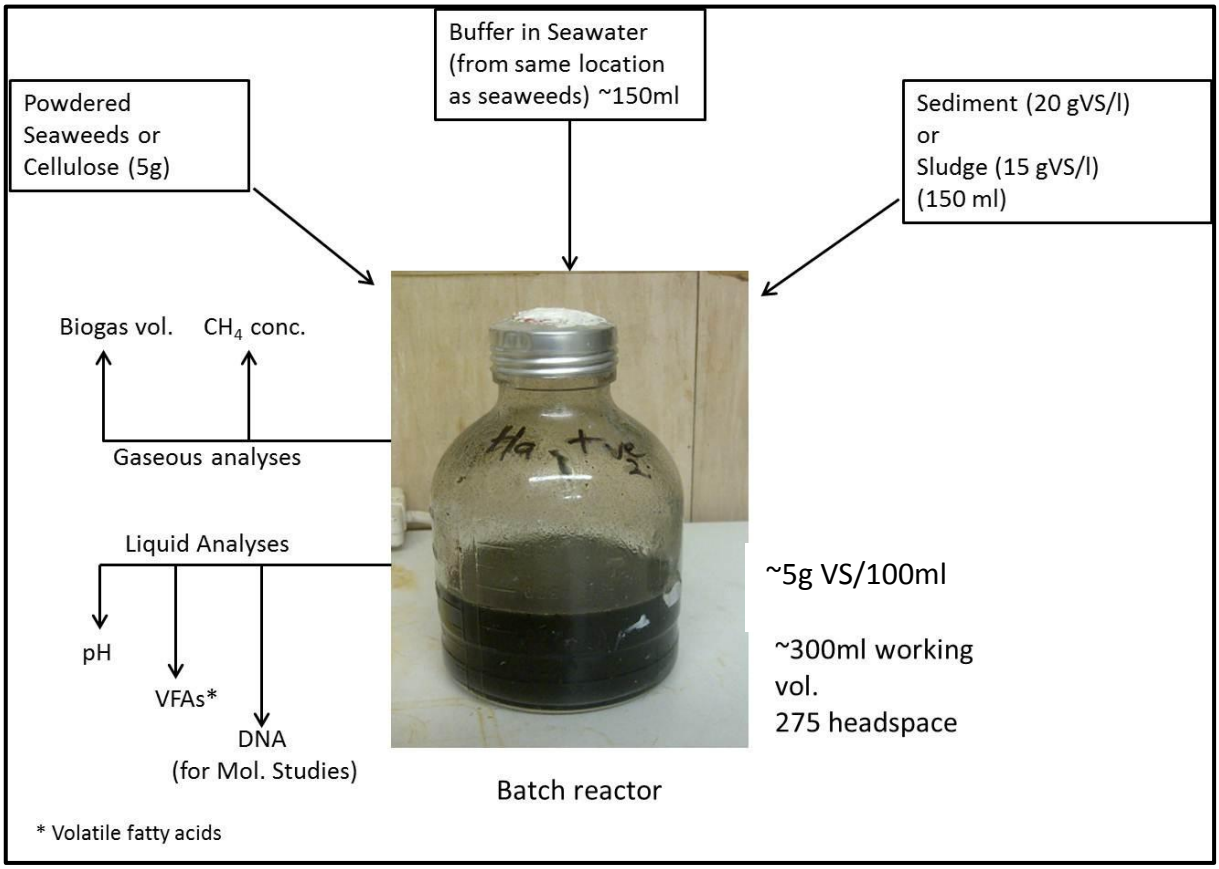
4 Chapter 4

"The important thing in science is not so much as to obtain new facts as to discover new ways of thinking about them"

- William Lawrence Bragg

Biomethane production during anaerobic digestion of seaweeds using two different inoculum sources: potential for communities on west coast of Scotland

This chapter contains report on the first part of this project where selected seaweeds were analysed and subjected to a 50-day anaerobic digestion process leading to the production of volatile fatty acids and methane. Total and volatile solids degradation was also reported.



Schematic representation of experimental procedures and analyses carried out in this chapter

4.1 Introduction

The current global quest for alternative, sustainable and renewable energy has renewed interests in a number of possible sources of renewable energy. Bioenergy appears to be a viable option because it can be sourced locally and it's one of the very few forms of renewable energy that can be stored (Manzano-Agugliaro, et al., 2013; Matsui & Koike, 2010; Singh & Olsen, 2011). Of all the alternative energy sources, biomass represents the most ready to be utilised on a large scale with minimal environmental and economic implications (Aresta, et al., 2005). However, there are concerns about growing terrestrial crops for bioenergy production which may make negligible contribution to net greenhouse gas emission and cause other problems relating to water and food shortages (Bruhn et al., 2011; Costa, et al., 2012; Hughes, et al., 2012; Nkemka & Murto, 2010). It is now obvious that utilization of mainly terrestrial biomass in the drive towards renewable bioenergy is not sustainable in the long term. Therefore, the current quest for increased utilisation of marine based biomass as a potential resource for renewable energy production is very timely. Marine biomass (seaweeds) offers an attractive option for producing renewable energy in a more sustainable manner. Apart from its ability to efficiently fix CO_2 faster than most terrestrial plants (Gao & McKinley, 1994; Wei, Quarterman, & Jin, 2013), seaweed has a number of advantages over terrestrial biomass as source of renewable energy. These include 1; lack of lignin, which makes up a bulk of terrestrial biomass; thereby making it relatively easier material for bioconversion, 2; its cultivation does not require arable land or fresh water, therefore there's no competition with food crops (Hughes et al., 2012; Nkemka & Murto, 2012; Wei et al., 2013).

This study assesses the biomethanation potential of three different seaweeds commonly found on the west coast of Scotland using two inoculum sources (anoxic sediment and

digested sludge) in a 50-day anaerobic digestion process. The idea is mainly to exploit the intrinsic degradation potential of the marine environment in other to enhance the sustainability of the methane production process in remote coastal locations. The hypothesis therefore is that: *there is no difference in the use of either of the inoculum sources*.

4.2 Methods

4.2.1 Experimental set up

Anaerobic digestion test was performed using batch reactors containing 5g of powdered freeze-dried brown seaweeds (*L. digitata, F. serratus* and *S. lattissima*) commonly found on the west coast of Scotland and 100ml of inoculums mixed in seawater. The inoculum; anoxic sediment was obtained from below fish farm in Shuna, near Oban. The use of sediment inoculum is based on the assumption that inoculum from a similar origin as the substrates would be better during anaerobic digestion of biomass. For the purpose of comparison, digested sludge (commonly used) obtained from mesophilic, wastewater treatment plant in Hatton near Dundee was used. The mixture (substrates, medium and inoculum) was made to a working volume of 300 ml in a 575 ml-capacity reactor bottles leaving a 275 ml headspace (see Fig 4a). Each reactor contained ~50 g/L, which is suitable for batch test to avoid excessive acid accumulation. The pH was adjusted to 7.5±0.3 (suitable for anaerobic digestion (Raposo, et al., 2012)) at the start of the experiment for all batches using drops of 35% NH_4OH or HCl. The reactor bottles were sealed with septum while the headspace was flushed with nitrogen to create anaerobic conditions. Cellulose powder (Fluka, Sigma-Aldrich, Denmark) was used as the standard substrate in the controls according to Hansen et al., (2004) while the blanks contain just inoculum in medium to discount the methane produced due to residual substrates in the inoculums. The batch tests were performed in duplicates. Moisture and total solid content of the fresh seaweeds were determined as well as volatile solids and ash content of the freeze-dried seaweeds, digested sludge and sediment according to standard procedures (Sluiter et al., 2008). Digestate (4 ml) was collected at intervals for VFAs and microbial analyses.

4.2.2 Analytical methods

Headspace methane concentration was analysed with Gas Chromatography (HEWLETT PACKARD 5890 SERIES 11, USA) equipped with a single flame ionization detector (FID), using gas tight 100µl syringe. The injector and detector temperatures were 120^{o}C and 150^{o}C respectively. Helium was the gas carrier with a pressure of 200kPa. Calibration was performed using linear calibration curve based on four different methane concentrations (0.5–100% ±5% in N_2). Methane production determination was adjusted to standard temperate and pressure (Costa, et al., 2012; Hansen, et al., 1999). Gas volume analysis was performed as described by Hansen et al., (2004). The pH was evaluated using pH meter SensIon 3 (HACH) which was calibrated before every use.

To determine the amount of total solids present and the amount of volatile solids available for bioconversion during the experiment, total solids content of fresh seaweeds was determined by drying at 105^{o}C for 24 hours. After which volatile solids content was determined by incinerating samples at 550^{o}C for 2 hours according to standard methods (Schiener, et al, 2014; Sluiter et al., 2008). All preliminary analyses were performed in triplicates.

Volatile fatty acids concentration was determined by esterification method as described by (Montgomery, et al., 1962) while calorimetric determination was done using a HACH DR/5000 Direct Reading spectrophotometer at 495nm (HACH method 8196). VFA concentration was measured in mg/L

Full description of experimental process and procedures are in Chapter 2.

4.3 Results and discussion

To determine the amount of solids available for biodegradation, moisture and total solid content of experimental seaweeds were first analysed followed by ash and volatile solids content. During the 50-day AD batch test, pH, VFAs and methane production were measured as a function of process performance.

4.3.1 Determination of moisture, ash, total solids and volatile solids of substrates

The results of the moisture content analysis of the fresh seaweeds indicate that highest moisture was found in *Laminaria digitata* (73%) followed by *Sacharrina Latissima* (72.5%) while *Fucus serattus* (71%) hold the least amount of moisture (Fig. 4.1). However, ANOVA and pairwise comparison of moisture content shows that there's no significant difference between the three seaweed species (P>0.268).

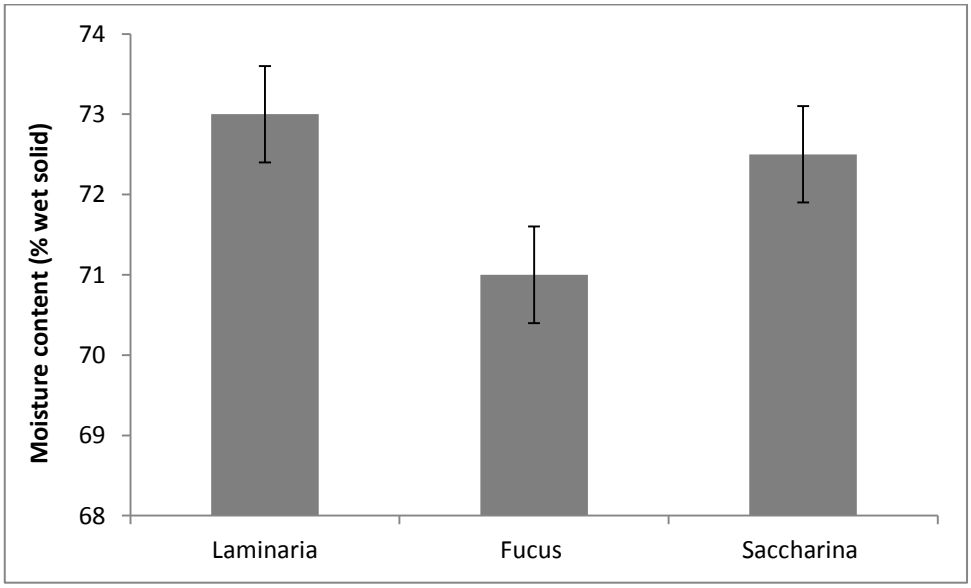


Fig. 4.1 Moisture content of the wet experimental seaweeds determined in triplicate, samples were weighed before and after 4 days of freeze drying. n=3, error bars are standard deviation from the mean

Total solid content of the wet seaweeds ranges from 27% in *Laminaria sp*, to 29% in *Fucus sp* (Fig. 4.2). Literature shows that other seaweeds possess lower amounts of total solids, for instance, Costa et al., (2012) reported 16% total solids for *Ulva sp* and 21% for *Gracilaria sp* while Matsui & Koike, (2010) obtained 11% total solids from *Ulva sp*. Nevertheless, the time of harvest may be the determining factor of the amount of solids present in seaweeds (Adams, et al., 2011a).

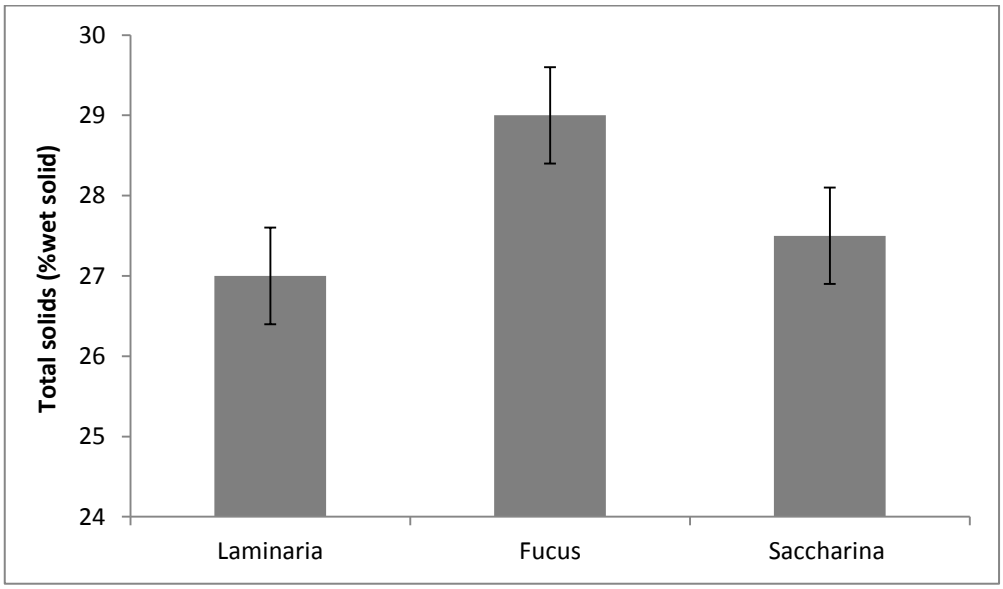


Fig. 4.2. Total solid content of the wet experimental seaweeds determined in triplicate, n=3, error bars are standard deviation from the mean. Samples were weighed before and after 24 hrs in the oven at 105^{o}C.

Results of ash content of the seaweeds showed that *F. serattus* has the highest amount of ash (18.86%) while *L. digitata* and *S. latissima* have 14.49% and 12.36% respectively (Fig. 4.3). Analysis of variance and Tukey's post hoc test highlighted significant differences in ash contents of the 3 seaweeds. High ash content of *F. serattus* compared to the other two seaweeds means there is less solid available for microbial degradation which may impact methane production.

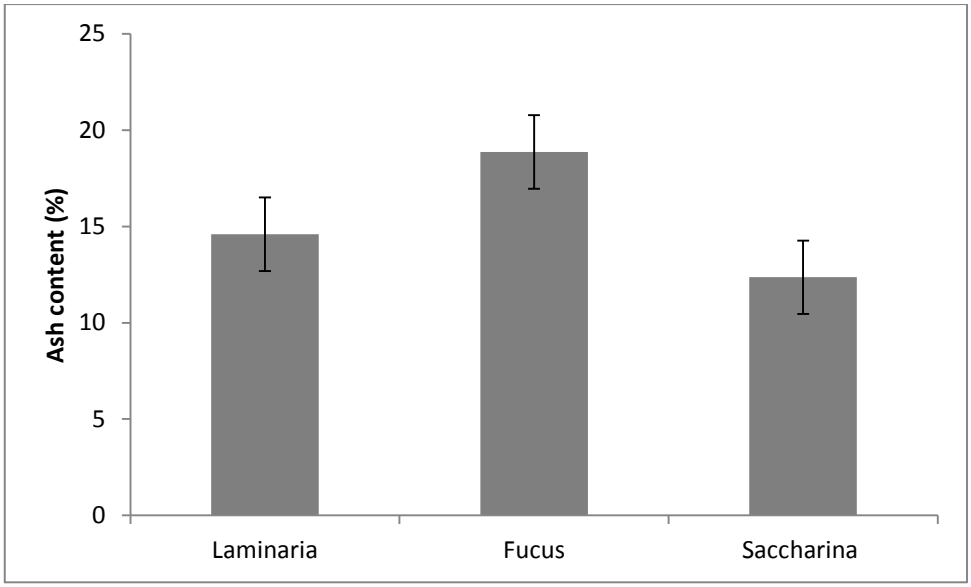


Fig. 4.3. Ash content of the freeze-dried experimental seaweeds determined in triplicate; samples were weighed before and after 24 hrs in the oven and a further 4 hours in the furnace at 550^{o}C. n=3, error bars are standard deviation from the mean

The proportion of volatile solids in any biomass meant for anaerobic digestion is one of the most important parameters that determine the amount of methane produced from the process. Volatile solids represent the amount of solid amenable for microbial degradations. Therefore, the more volatile solids present in a given substrate, the more methane is expected to be produced.

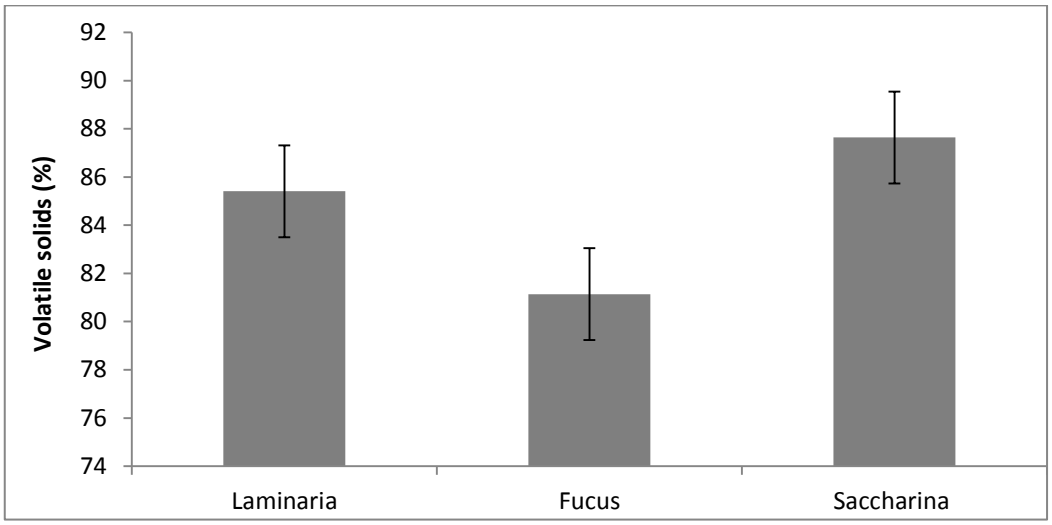


Fig. 4.4. Volatile solids content of the freeze-dried experimental seaweeds determined in triplicate. n=3, error bars are standard error. Samples were weighed before and after 24 hrs in the oven (105^{o}C) and a further 4 hours in the furnace at 550^{o}C.

Of the 3 seaweeds studied in the current experiment; *S. latissima* possess the highest amount of volatile solids (87.64%), followed by *L. digitata* (85.41%) while *F. serattus* has the least proportion of volatile solids (81.14%) (Fig. 4.4). Although *F. serattus* has the highest amount of total solids, it has the least amount volatile solids, which mean there are recalcitrant solids present in *F. serattus* than in the other 2 experimental seaweeds. Statistical analysis of the 3 seaweeds using Analysis of variance and Tukey's post hoc test highlighted significant differences in the mean volatile solid contents of the 3 seaweeds ($P<0.00001$).

There are a few reports on seaweeds composition and its variations with seasons, which are some of the factors that affect their bioconversion to energy fuels. A study by Schiener et al., (2014) measuring variation in seaweeds components recorded lower moisture for seaweeds in the summer months than in winter months. Variability in macro algal/seaweeds content with seasons is an important factor to be considered when seaweeds are to be used for bioenergy production as this affects the composition and quantity of materials available for bioconversion (Adams, et al., 2011a). Experimental seaweeds for this study harvested in August (summer) are thought to contain high amount of usable carbohydrates and very low amount of metals and other recalcitrant elements which may impact bioconversion to biomethane (Adams, et al., 2011b).

4.3.2 Volatile acids production during anaerobic digestion of substrates using various inoculum sources

Volatile fatty acids (VFAs) are important intermediates in the formation of biomethane during anaerobic digestion processes. VFAs are produced in the second stage of anaerobic digestion termed acidogenesis where diverse groups of fermentative bacteria such as Clostridia ferment soluble organic monomers into organic acids, CO_2 and H_2 (Traversi, et

al., 2012). Steady production of VFAs by the fermenters and concomitant utilization by methanogens is important during methane production so as to avoid the accumulation of VFAs and its inhibitory effects on methanogens at high concentrations (Wei, Kitamura, & Li, 2005).

4.3.2.1 VFAs production from experimental samples during AD using anoxic sediment

VFAs concentrations were generally low (below 40mg/L) in all of the reactors inoculated with anoxic sediment until after day 8 of the digestion process, although VFAs production commenced as early as day 2. Rapid increase in VFAs production was observed in all batches after day 8, which reached its peak on day 23 in all 3 seaweeds and day 35 in cellulose reactors. Peak VFAs production on day 23 of anaerobic digestion in *L. digitata, F. serratus, and S. latissima* was 2514, 2934, 4154 mg/L respectively, while peak VFAs production in cellulose reactors occurred later at day 35 measuring 1551mg/L (Fig.4.5). Peak VFAs produced are all below the concentration (>5000mg/L) at which it becomes inhibitory to methanogens (Khalid, et al., 2011). ANOVA and Tukey's post hoc tests on peak VFA production in all reactors show that differences observed between the mean VFA produced in cellulose (day 35), *L. digitata, F. serratus* (day 23) batches were not significant ($P>0.1329$), however, significant difference was observed in VFAs produced between cellulose and *S. latissima* ($P<0.028$) (Fig. 4.6). Additionally, statistical analysis of VFAs concentration on day 23 indicates that while there was no significant difference between all three seaweed reactors ($P>0.05$) the difference between the three seaweeds reactors and cellulose is statistically significant ($P<0.05$).

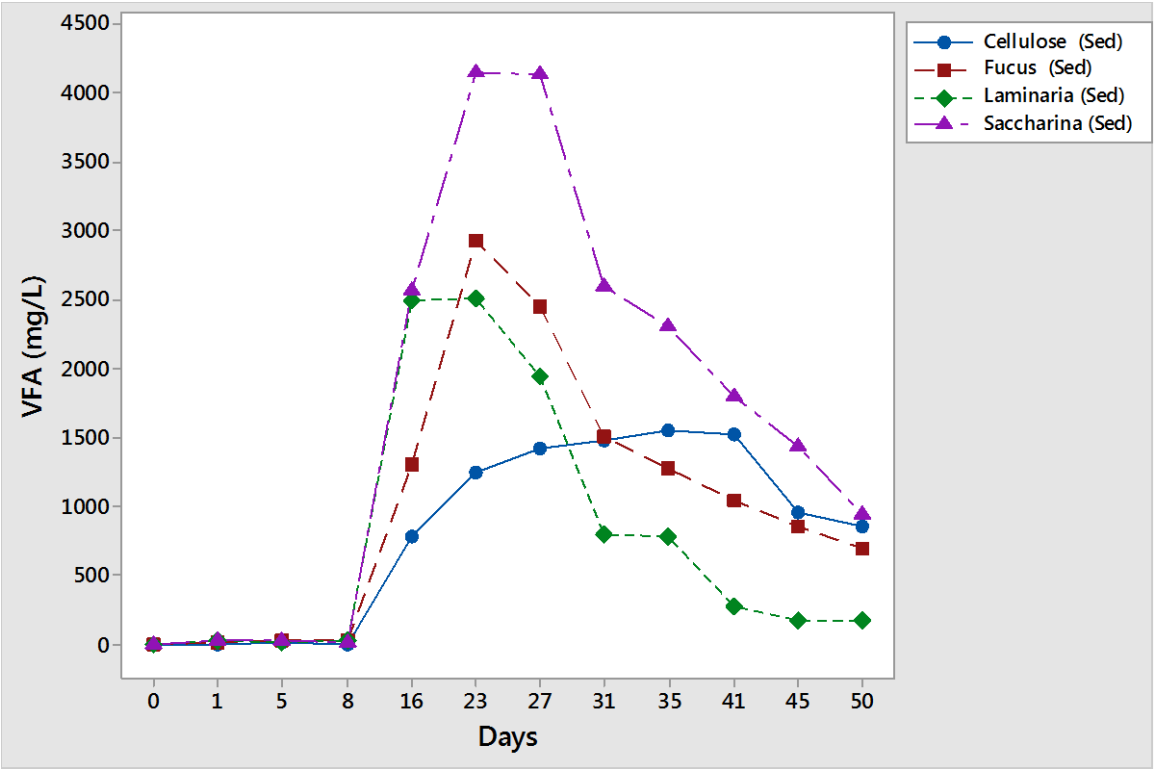


Fig. 4.5. Volatile fatty acid production during anaerobic digestion of seaweeds using anoxic sediment as the source of inoculum. Esterification methods by Montgomery et al., (1962) was used to measure fatty acids produced in each batch. Mean VFAs produced by the blank batches was subtracted from the mean of the other batches.

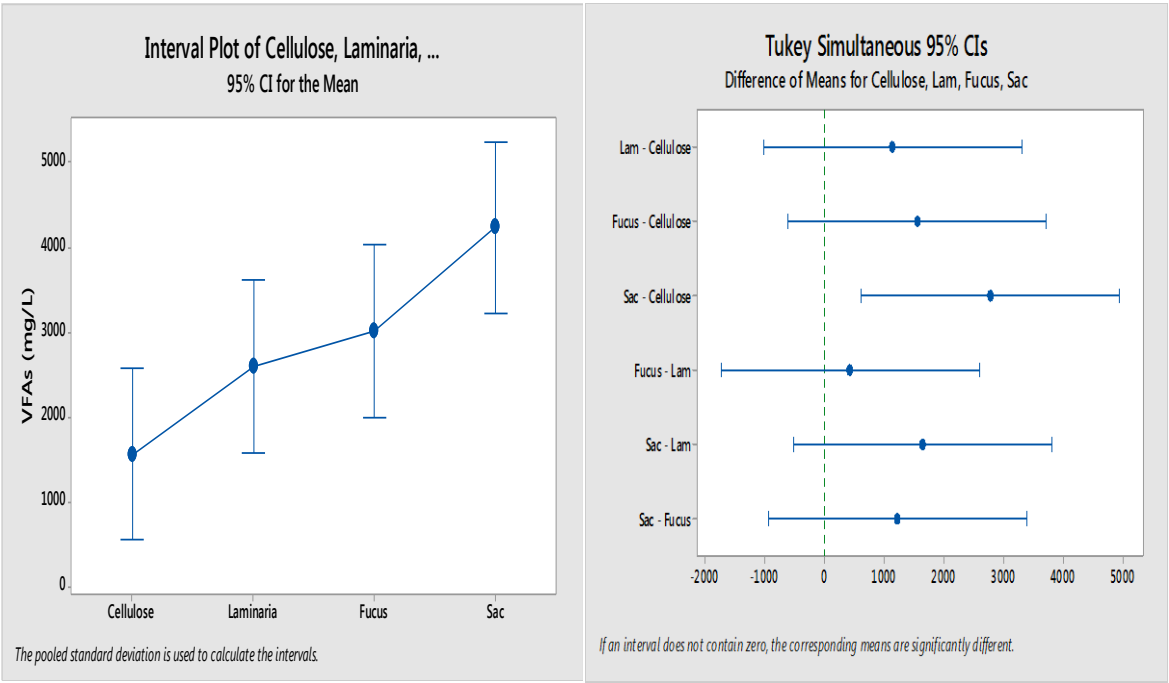


Fig. 4.6. Interval plot and Tukey simultaneous post hoc test at 95% confidence interval to statistically analyse differences in mean of VFAs produced in all reactors inoculated with anoxic sediment.

4.3.2.2 VFAs production from anaerobic digestion of substrates using digested sludge

Until after day 8, VFAs concentration was generally low (below 70mg/L) in all sludge inoculated reactors, although VFAs production commenced after 24 hours of incubation. As observed in sediment-inoculated samples, peak VFAs concentration was recorded at day 23 in *L. digitata* and *S. latissima* and at day 27 for cellulose and *F. serratus* reactors during the 50-day anaerobic digestion process (Fig.4.7). The observed similarity in performance is an indication that both inoculums sources contained the type of fermentative bacteria required for efficient VFAs production.

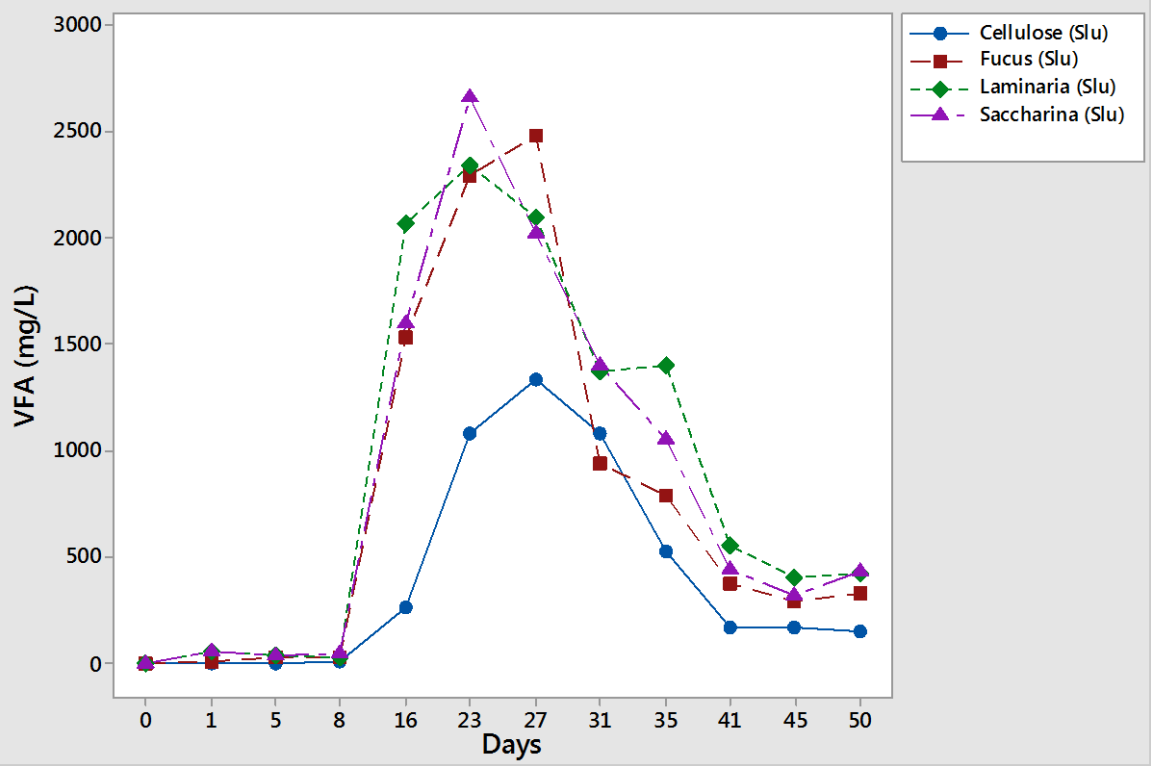


Fig. 4.7. VFA production from experimental samples during AD using digested sludge as the source of inoculum. Esterification methods by Montgomery et al., (1962) was used to measure fatty acids produced in each batch. Mean VFAs produced by the blank batches was subtracted from the mean of the other batches.

VFAs concentration appeared higher in all experimental seaweeds than in cellulose even though peak VFAs was recorded 4 days later in cellulose and *F. serratus*. Peak VFA recorded in cellulose and *F. serratus* reactors were 1330mg/L and 2483.5mg/L on day 27 while *L. digitata, and S. latissima* produced 2336, 2661 mg/L respectively on day 23. Statistical analysis of variance at 95% confidence interval and Tukey's post hoc test show that the difference in mean peak VFAs production between all the three seaweeds and cellulose is not significant (P>0.135) (Fig. 4.8). Analysis of variance in mean VFA production on day 23 in all reactors also show that the differences observed is not statistically significant (P>0.0978)

Gradual decline in VFAs concentration over time is thought to be due to the activities of methanogens resulting in copious methane production after day 7.

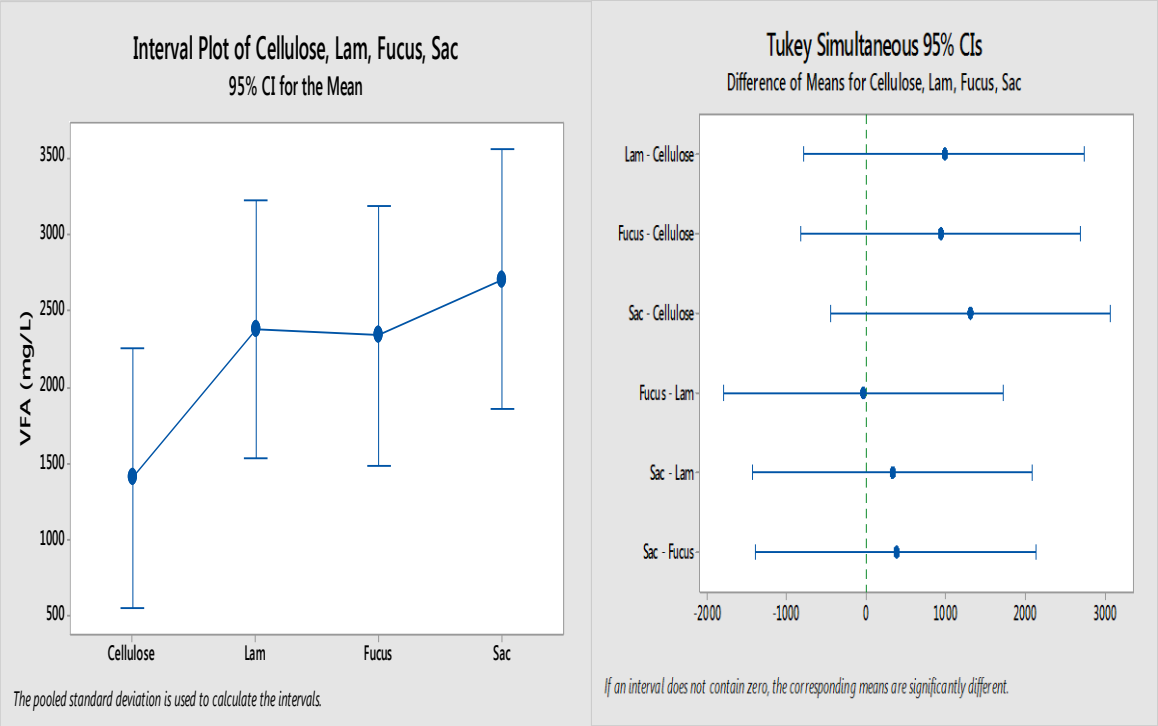


Fig. 4.8. Interval plot and Tukey's simultaneous post hoc test at 95% confidence interval to statistically analyse differences in mean of VFAs produced in all reactors inoculated with digested sludge.

4.3.2.3 Comparative VFAs formation in all substrates under different inoculation

To determine differences in VFAs production based on the source of inoculum, statistical analyses were performed on peak concentration as well as the length of time required to achieve peak VFA concentration in all reactors. Analysis of peak VFAs concentration in cellulose reactors on day 35 (sediment) and day 27 (sludge) indicates higher VFA concentration in sediment-inoculated batches (1551mg/L) than in sludge inoculated ones (1330mg/L). Nevertheless, the peak VFA in sediment-inoculated cellulose occurred 8days later. This observation suggests that the conditions within the cellulose reactor may be sub-optimal for some of the fermentative bacteria of sediment origin, which are not readily exposed to cellulose in situ. ANOVA and pairwise post hoc test however revealed that the observed difference is not statistically significant (P=0.417) (Fig. 4.9), just as there is no significant difference in VFAs concentration in cellulose reactors on day 27 (P>0.815) using either sediment or sludge as the source of inoculum. However, ANOVA shows that difference in VFAs concentration on day 35 was significantly higher in sediment than sludge inoculated cellulose reactors (P<0.027). This observation is likely as a result of accumulation of VFAs due to lower rate of removal by methanogens during periods of active methanogens rather than higher rate of production (Fig. 4.5).

Analysis of peak VFAs concentrations in all three seaweeds reactors (*L. digitata*, *F. serratus* and *S. latissima*) under the two different inoculations at day 23 show the differences observed in peak VFA concentration across inoculation are not statistically significant (P>0.05) (Fig. 4.9).

It can therefore be inferred from the above statistical analyses that the two sources of inoculums employed in the current study performed in a similar manner regarding volatile fatty acids formation across all experimental substrates.

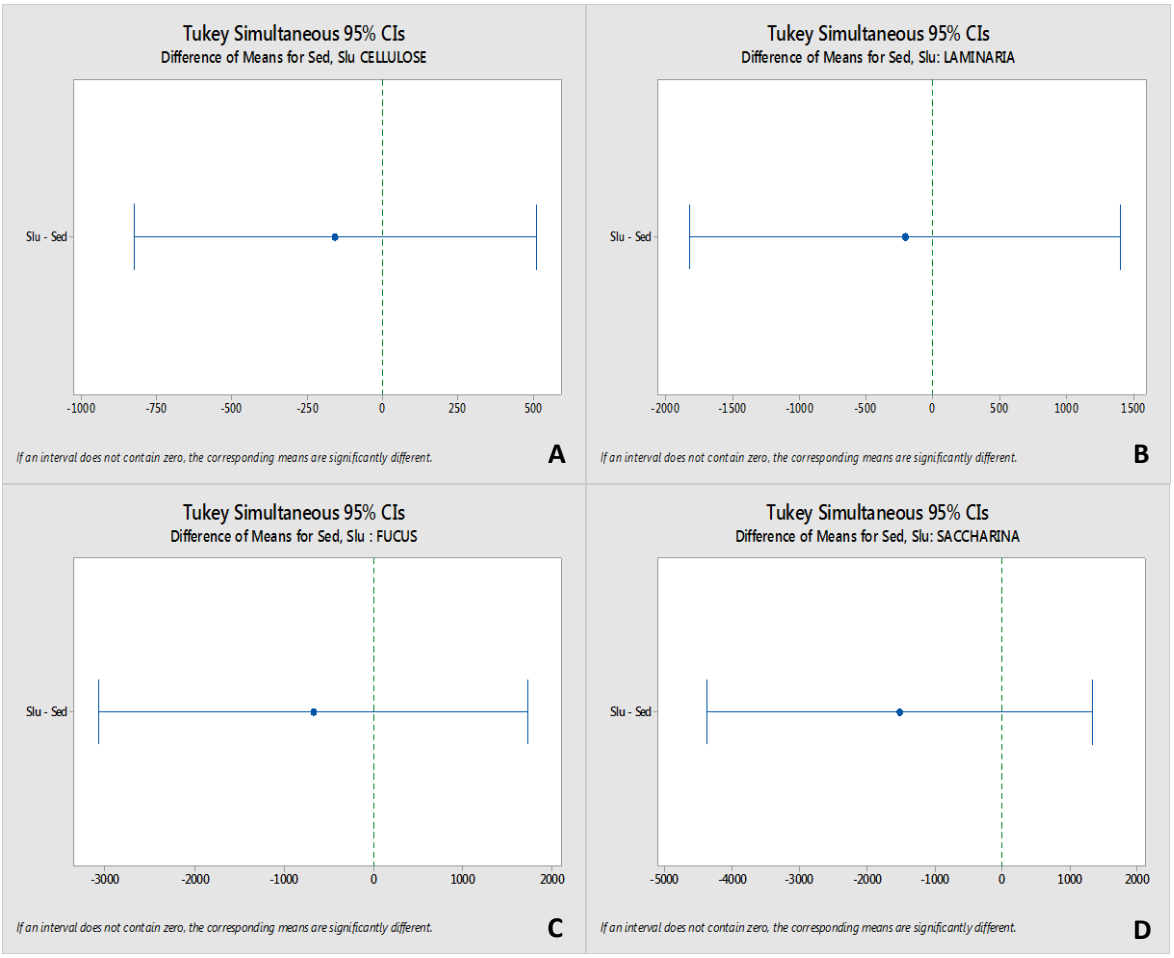


Fig. 4.9. One-way ANOVA and Tukey's pairwise post hoc analyis of differences in mean VFA production between different inoculum sources of all experimental substrates. The test conducted at 95% confidence interval shows if there are differences in mean values recorded and determines if these differences are statistically significant (Significance level $\alpha = 0.05$). **A**: Cellulose, **B**: *L. digitata*, **C**: *F. serratus*, **D**: *S. latissima*

The results obtained agree with the submission by Hanssen et al., (1987); Migliore et al., (2012) that inoculums derived from anaerobic marine environments can performs as well as those derived from digested sewage sludge and sometimes better.

VFAs formation by fermentative bacteria and its accumulation, its impacts on pH and microbial population dynamics would be assessed in subsequent chapters.

4.3.3 pH variation during anaerobic digestion of seaweed substrates

During anaerobic digestion, pH remains one of the most critical factors that determines process functions and stability (Chanakya, et al., 2006). Unlike other parameters or factors that affect methane production during anaerobic digestion of biomass, optimum pH requirement of various microorganisms involved in the process differ considerably (Khalid, et al., 2011; Raposo, et al., 2012). It is therefore very important to monitor pH variation to ensure it is within range at different stages of the digestion process. To ensure a uniform start, pH of all reactors was adjusted to ~7.5 at the start of the digestion process.

4.3.3.1 pH variation during anaerobic digestion of substrates using anoxic sediment as the source of inoculum

A sharp drop in pH was observed in all three seaweeds batches after 24 hours of anaerobic digestion unlike in cellulose and blank batches where pH stayed above 7.2. This may be due to the presence of readily hydrolysable sugars present in the seaweeds which could be easily hydrolysed and fermented resulting in faster acid formation and resultant drop in pH (Wei et al., 2013). However, drop in pH did not go below 6 in any of the reactors during the hydrolysis and acidogenesis stages of anaerobic digestion. An indication of the efficacy of the buffering capacity of the buffers included in the experimental set up. The lowest pH recorded in seaweeds reactors were 6, 6.15, and 6.2 for *F. serratus*, *L. digitata* and *S. lattisima* respectively (Fig. 4.10). These were within the range of optimum pH for this stage of the digestion process (Chanakya et al., 2006; Khalid et al., 2011). After the initial drop, steady rise in pH was observed in all reactors, despite increasing VFAs production. This may be as a result of the buffering provided by ammonia accumulation during substrates degradation (Weiland, 2010) or production of HCO_3^- ions as a result of SO_4^{2-}

reduction to H_2S as well as the buffers included in the medium (Fang, et al., 2011; Nkemka & Murto, 2010).

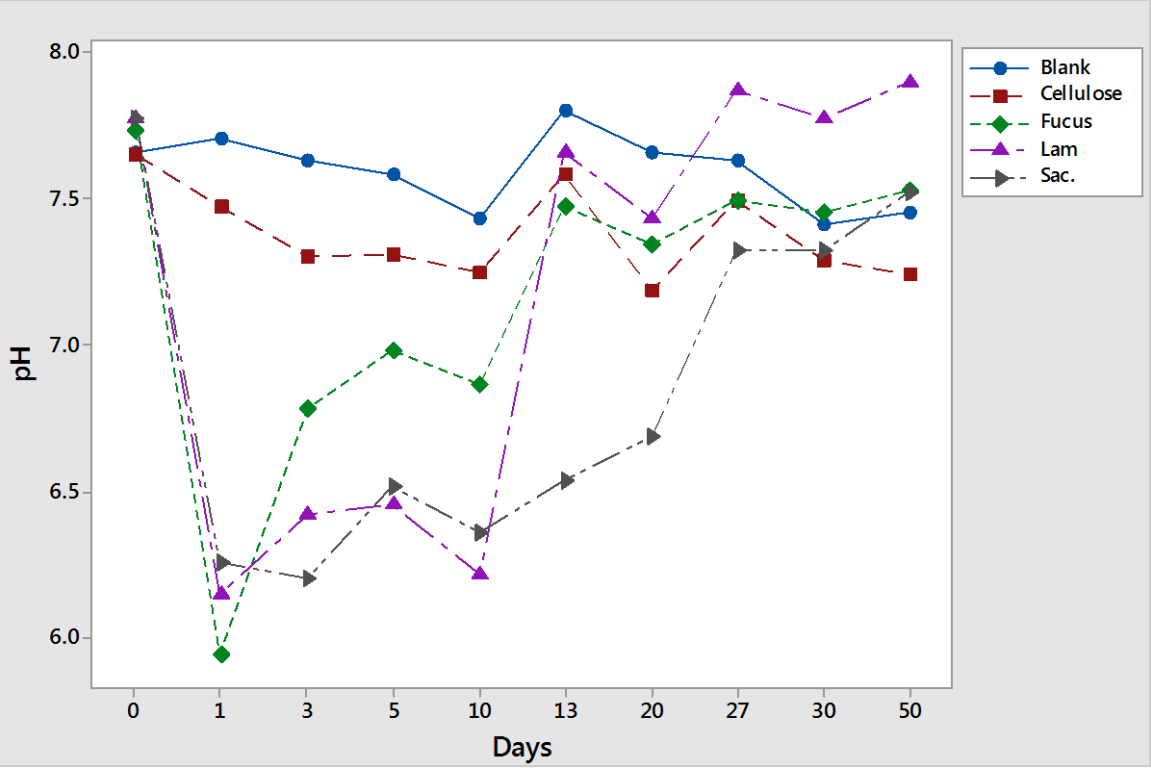


Fig. 4.10. pH variation in batch reactors during anaerobic digestion of substrates using anoxic sediment as the source of inoculum.

4.3.3.2 pH variation during anaerobic digestion of seaweed substrates using digested sludge as the source of inoculum

Like sediment inoculated reactors, drastic drops in pH were observed in all three seaweeds reactors inoculated with digested sludge after 24 hours of anaerobic digestion. The drops in pH were more severe in batches containing *S. lattisima* and *L. digitata* where pH dropped to 5.3 and 5.4 respectively (Fig. 4.11). The pH of these 2 batches was readjusted to ~7 using drops of to using 10M NaOH to halt further drops in pH which was achieved according to Hanssen et al., (1987). This readjustment of pH was performed in batches where pH dropped below 6. The pH recorded for the third seaweeds *F. serratus*, at day 2 was 6.7 while cellulose and blank reactors recorded pH 7.53 and 7.55 respectively,

(Fig.4.11). The reason for these drops in seaweeds reactors might be connected to the easily hydrolysable sugars present in seaweeds resulting in faster acid production (Hughes et al., 2012; Wei et al., 2013).

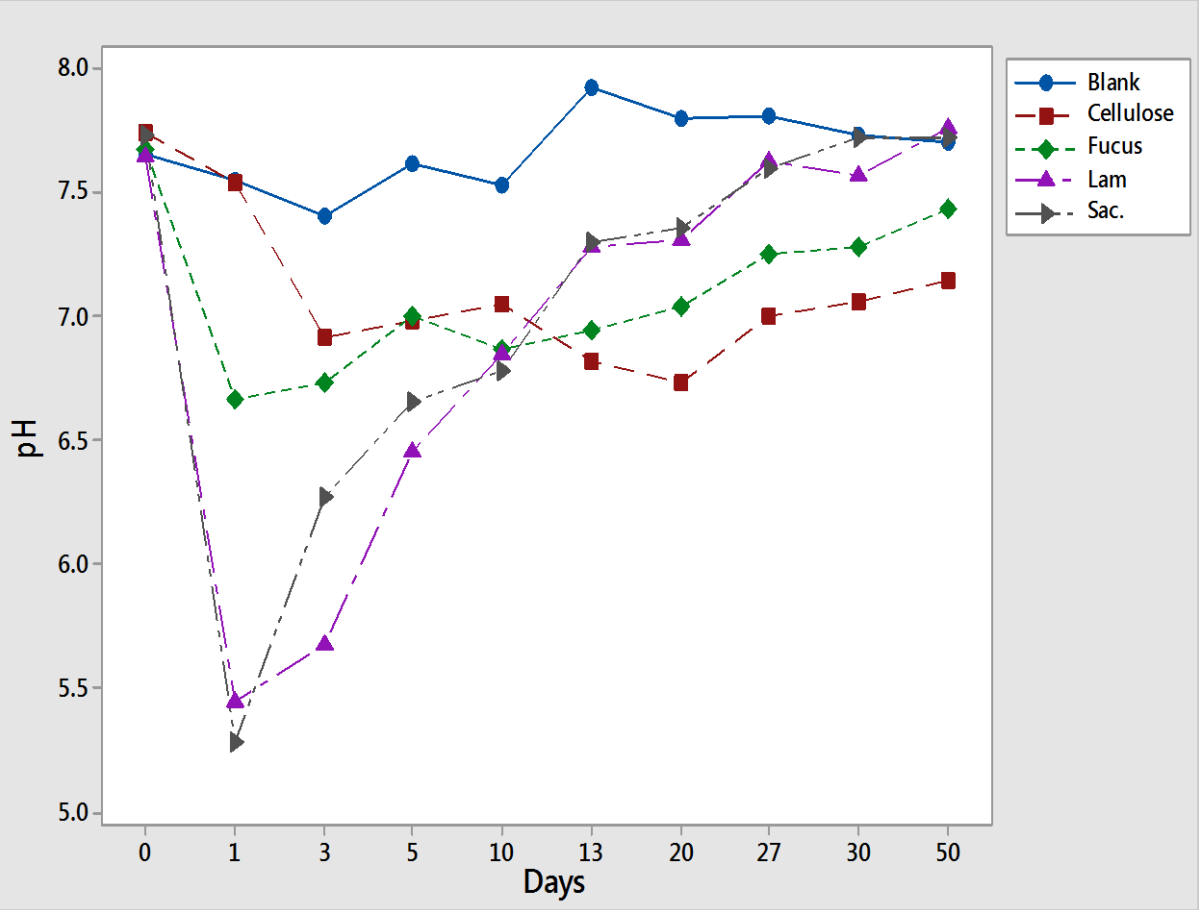


Fig. 4.11. pH variation in batch reactors during anaerobic digestion of seaweed substrates using digested sludge as the source of inoculum. pH below 6 was readjusted to 7 to avoid system failure.

The steady rise observed in pH after the initial drop coincides with the start of methane formation as a result of fatty acids utilisation. Although VFAs continued to be produced throughout the process, fatty acids accumulation did not occur because VFAs produced are likely being concomitantly utilised for methane production leading to the observed relative stability in pH after day 5 in all reactors (Fig. 4.11).

The stability of anaerobic digestion process depend on a number of factors, with pH and temperature being the most reported (Khalid et al., 2011; Raposo et al., 2012). Unlike temperature, which is mainly externally controlled, the pH of the digestion process is mostly dependent on the internal workings of the process. One of the internal workings of the systems which aid pH stability is the cooperation among various groups of microorganisms resulting in active interactions between acidogenesis and methanogenesis leading to prompt utilisation of fatty acids produced for methane production (Chanakya et al., 2006; Wei et al., 2013).

4.3.4 Methane production during anaerobic digestion of substrates using different inoculum sources

One of the objectives of the current study is to demonstrate the feasibility and sustainability of biomethane production from locally sourced seaweeds in batch reactors inoculated with anoxic sediment sourced from the same environment. Moreover, for comparison, the commonly used source of inoculum (digested sludge) was employed. Therefore, one of the hypotheses of this chapter is that *there is no difference between the use of the two sources of inoculum in relation to methane production*. In this section, methane production potential of the three seaweeds was evaluated, and comparison was made between the different inoculations.

4.3.4.1 Methane production during anaerobic digestion of marine biomass using anoxic sediment as the source of inoculum

Methane produced was below detection in the all reactors inoculated with anoxic sediment in the first 24 hours of anaerobic digestion, as gas produced composed almost entirely of CO_2. However methane production was recorded in all reactors after 48 hours (day 2) including blank reactors. Methane production in blank batches was as a result of residual biomass present in the sediment used as the source of inoculum. This was discounted from methane produced in reactors containing experimental substrates to account for actual methane production from substrates biodegradation, following the procedure described by Costa et al., (2012). All experimental substrates produced considerable amount of methane after day 2 except cellulose reactors where methane production remained low throughout the 50-day digestion (Fig. 4.12). One plausible explanation for this is the lack of conversion of most of the VFAs produced into methane by the methanogens in the sediment-inoculated cellulose reactors, possibly due to inhibition as a result of VFAs accumulation as shown in Fig 4.5

Specific methane volume recorded at intervals showed early methanogenesis occurring in all three seaweeds. This suggests that marine sediments utilised (as source of inoculum) in this study contain sufficient and active methanogens for methane production from seaweeds at seawater salinity conditions (Migliore et al., 2012; Miura et al., 2014).

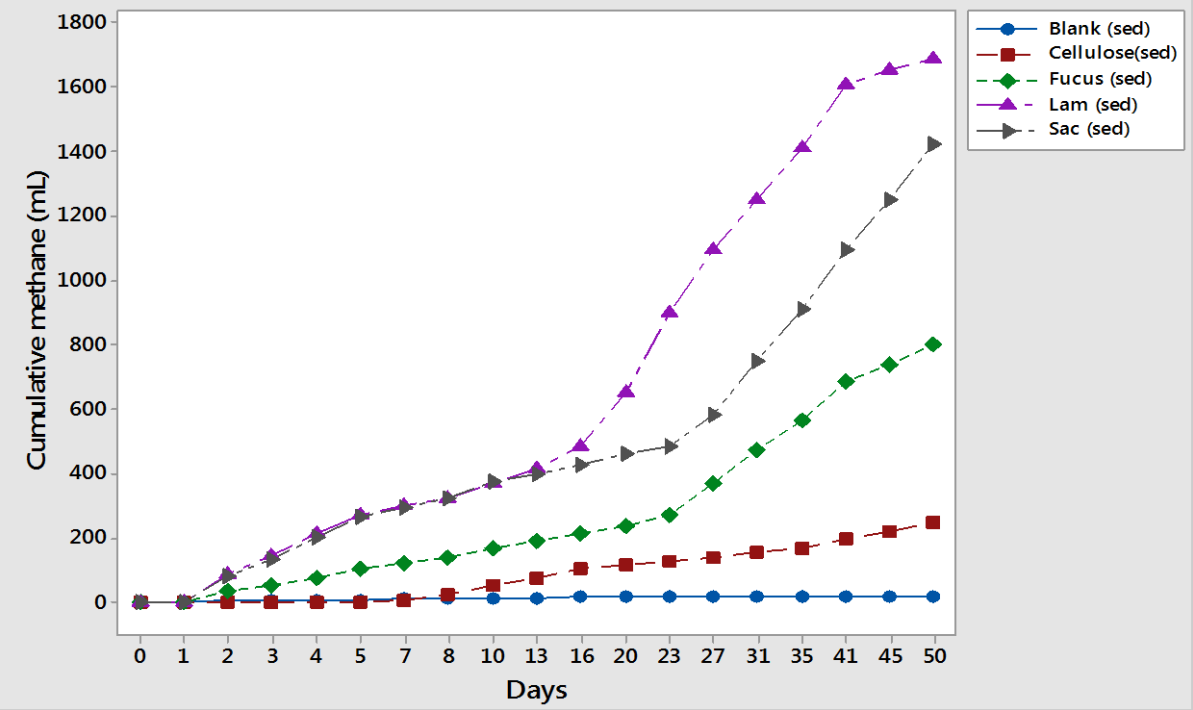


Fig. 4.12. Cumulative methane production during 50-day anaerobic digestion of substrates using anoxic sediment as the source of inoculum.

Measurement of cumulative methane production showed a steady increase in methane production in the first 10 days of anaerobic digestion especially in all three experimental seaweeds. *L. digitata* and *S. latissima* led off and produced similar amount of methane until after day 13 when drastic increase in methane production occurred in *L. digitata* reactors. Exponential increases in methane production began in *S. latissima* and *F. serratus* after day 23, but the rate of methane production was much higher in *S. latissima* leading to more methane production in the former (Fig.4.12). Cumulatively, all experimental seaweeds performed better and produced significantly more methane than cellulose

(P<0.00002). Specifically, *L. digitata* produced about 7 times more methane than cellulose when inoculated with anoxic sediment. Similarly, *S. latissima* and *F. serratus* also produced 6 and 3 times more methane than cellulose, respectively.

Comparison between the three experimental seaweeds shows that cumulatively, *L. digitata* produced significantly higher methane than *S. latissima* (P<0.002) which in turn produced more methane than *F. serratus* (P<0.0001) (Fig. 4.13). Methane produced in *L. digitata* reactors was more than double that of *F. serratus* under the same anoxic sediment inoculation. Cumulative methane production recorded for the three experimental seaweeds and cellulose inoculated with anoxic sediment occurred in this order: *L. digitata* > *S. latissima* > *F. serratus* > cellulose (Fig.4.12).

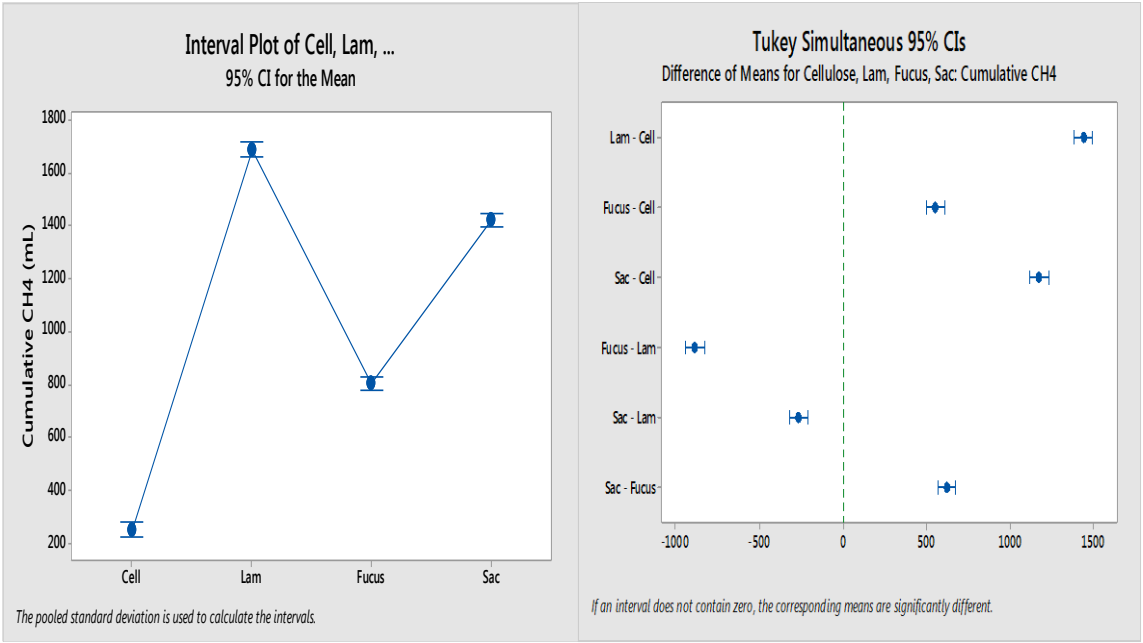


Fig. 4.13. Interval plot and Tukey's simultaneous post hoc test at 95% confidence interval to statistically analyse differences in mean of cumulative methane produced in all reactors inoculated with anoxic sediment.

4.3.4.2 Biomethane production during anaerobic digestion of substrates using digested sludge as the source of inoculum

Digested sludge from wastewater treatment plants remains the most commonly utilized source of inoculum during anaerobic digestion of biomass for methane production, mainly due to availability and uniformity in features (Chanakya et al., 2006; Khalid et al., 2011; Raposo et al., 2012). In the current study, digested sludge was used as the source of inoculum in anaerobic digestion on three different seaweeds and cellulose at seawater salinity conditions.

Methane production started earlier in sludge inoculated reactors at day 1 (after 24 hours) in all three seaweeds *L. digitata*, *S. latissima* and *F. serratus* compared to sediment inoculated reactors (day 2). In cellulose reactors, methane production commenced at day 2 and gradually increased until day 8 where exponential increase began and continued for most part of the digestion process (Fig. 4.14).

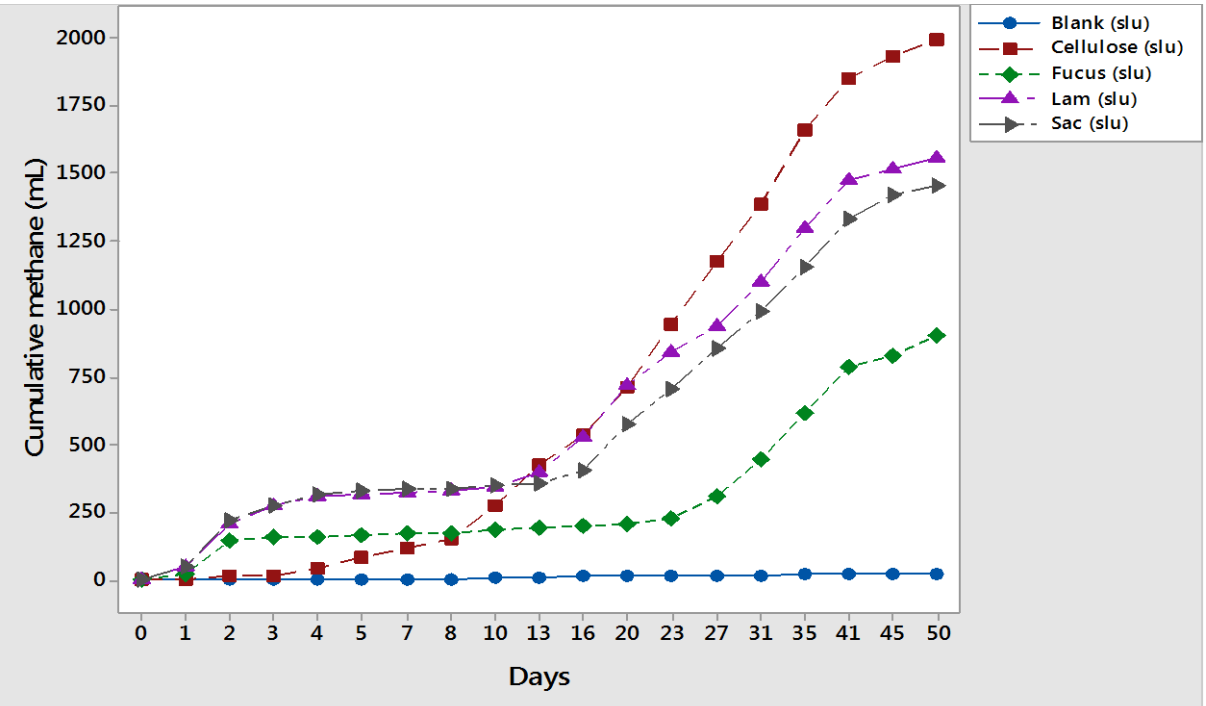


Figure 4.14. Cumulative methane production during 50-day anaerobic digestion of substrates using digested sludge as the source of inoculum.

Initial surge in methane production observed in the three seaweeds reactors plateaued after day 4 in *L. digitata* and *S. latissima* reactors until after day 10 where exponential increases in were recorded. Methane production pattern in these two seaweeds (*L. digitata* and *S. latissima*) were similar throughout the 50-digestion except that the former produced more methane cumulatively. The observation is however different in *F. serratus* reactors where methane production plateaued after day 2, until after day 20 when exponential increase began (Fig. 4.14). The long lag phase within *F. serratus* reactors affected cumulative methane production and it is likely due to inhibition of methanogens by inhibitory components of the seaweed such as polyphenols. Although present in most brown seaweeds, polyphenols concentration in Fucus sp. (up to 14%) is significantly higher than in Laminaria and Saccharina spp. (<2%) (Kelly & Dworjanyn, 2008; 2011). Higher cumulative methane was produced in *L. digitata* and *S. latissima* than in *F. serratus* sludge inoculated batch reactors, similar to the observation in sediment inoculated seaweeds reactors (Fig. 4.12).

Unlike sediment inoculated cellulose with cumulative methane production of only 50ml/gVS, sludge-inoculated cellulose produced more methane than the three experimental seaweeds, an indication that microbes in digested sludge were better adapted to utilise cellulose than those in anoxic sediments. This confirms the submission by Raposo et al., (2012) that inoculums favour the type of environments relevant to the test.

Cumulatively, cellulose produced significantly higher volume of methane (1996.5ml) than *L. digitata* (1557.05ml) which is the best performing seaweed ($P<0.0186$). ANOVA analysis of the difference recorded in methane production between *L. digitata* (1557.05ml) and *S. latissima* (1455.71ml) shows that the observed difference is not statistically significant ($P>0.6281$). However, statistical analyses highlighted that the differences observed in cumulative methane production between *L. digitata* and *F. serratus*

(P<0.00446) and between *S. latissima* and *F. serratus* (P<0.00823) are statistically significant (Fig. 4.15).

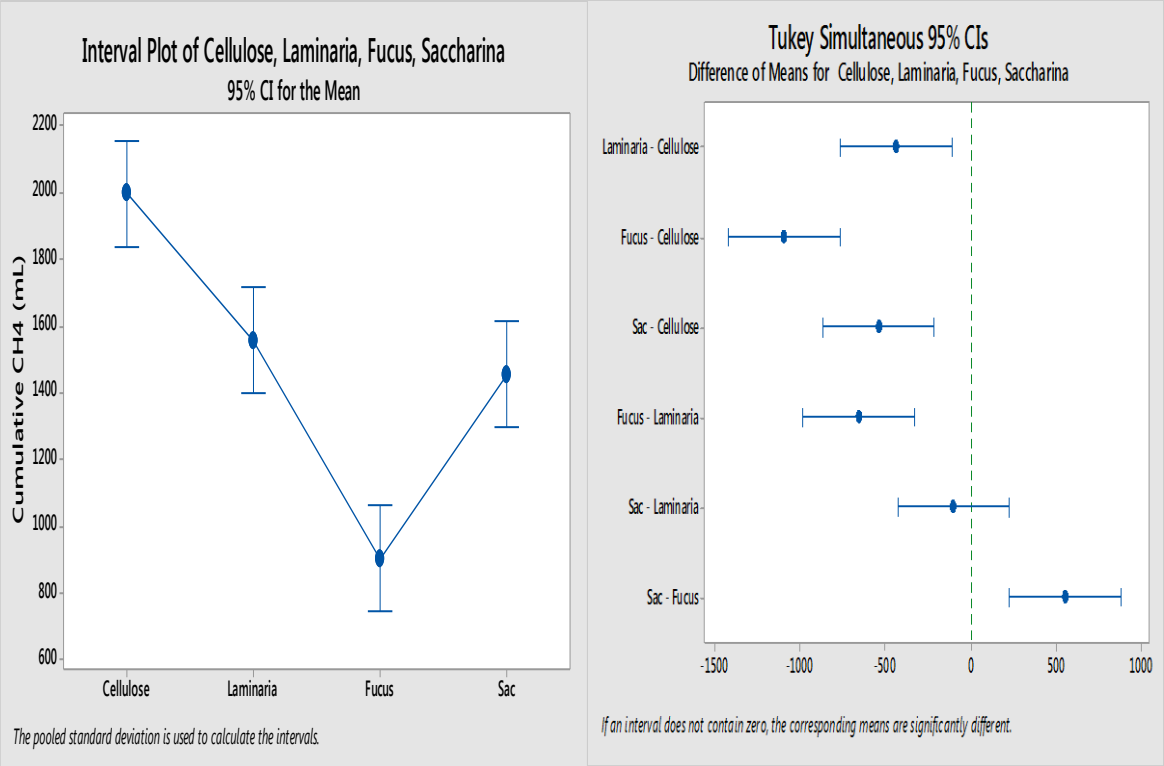


Fig. 4.15. Interval plot and Tukey's simultaneous post hoc test at 95% confidence interval to statistically analyse differences in mean of cumulative methane production in all reactors inoculated with digested sludge.

Cumulative methane production recorded for cellulose and the three experimental seaweeds inoculated with digested sludge occurred in this order; cellulose > *L. digitata* ≥ *S. latissima* > *F. serratus* (Fig. 4.14).

4.3.4.3 Specific methane production in all reactors under 2 distinct inoculations during 50-day anaerobic digestion

To obtain specific methane production in all reactors, cumulative methane produced by each of the substrates was divided by the total volatile solids present in the starting materials. Volatile solids of substrates utilised during anaerobic digestion account for all solids available for microbial degradation and by extension methane production.

Results of specific methane production in reactors inoculated with anoxic sediment show that *L. digitata* produced the highest amount of methane (395.1 ml/ gVS), while *S. latissima*, *F. serratus* and cellulose produced 324.55, 197.66 and 50.1 ml/ gVS, respectively (Fig. 4.16). The order of specific methane production is similar to that obtained when cumulative methane production was measured i.e. *L. digitata* ≥ *S. latissima* > *F. serratus* > cellulose.

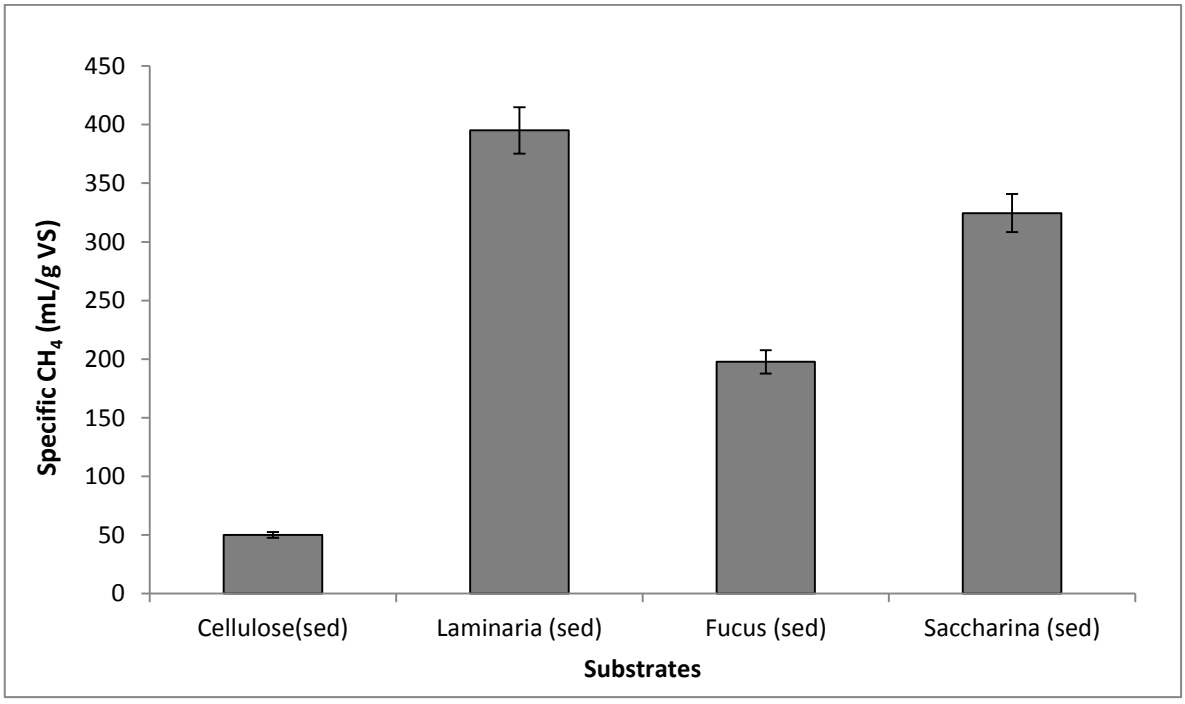


Fig. 4.16. Specific methane production during 50-day anaerobic digestion of substrates using anoxic sediment as the source of inoculum calculated in relation to the total amount of volatile solids in the substrates. Error bars represent standard deviation from the mean.

One-way ANOVA and Tukey's pairwise comparison of specific methane production in reactors inoculated with anoxic sediment showed that there is no significant difference in specific methane production in *L. digitata* and *S. latissima* ($P>0.072$). Specific methane produced in *L. digitata* and *S. latissima* are significantly higher than the values obtained for *F. serratus* ($P<0.00202$) and cellulose ($P<0.00072$), a trend similar to that obtained in cumulative methane production in sediment reactors.

Unlike the observation in sediment-inoculated reactors, the highest specific methane was produced in cellulose reactors inoculated with digested sludge. However, among the experimental seaweeds, *L. digitata* produced highest specific methane (365 ml/gVS) followed by *S. latissima* (322 ml/gVS) and then *F. serratus* (223 ml/gVS) (Fig. 4.17).

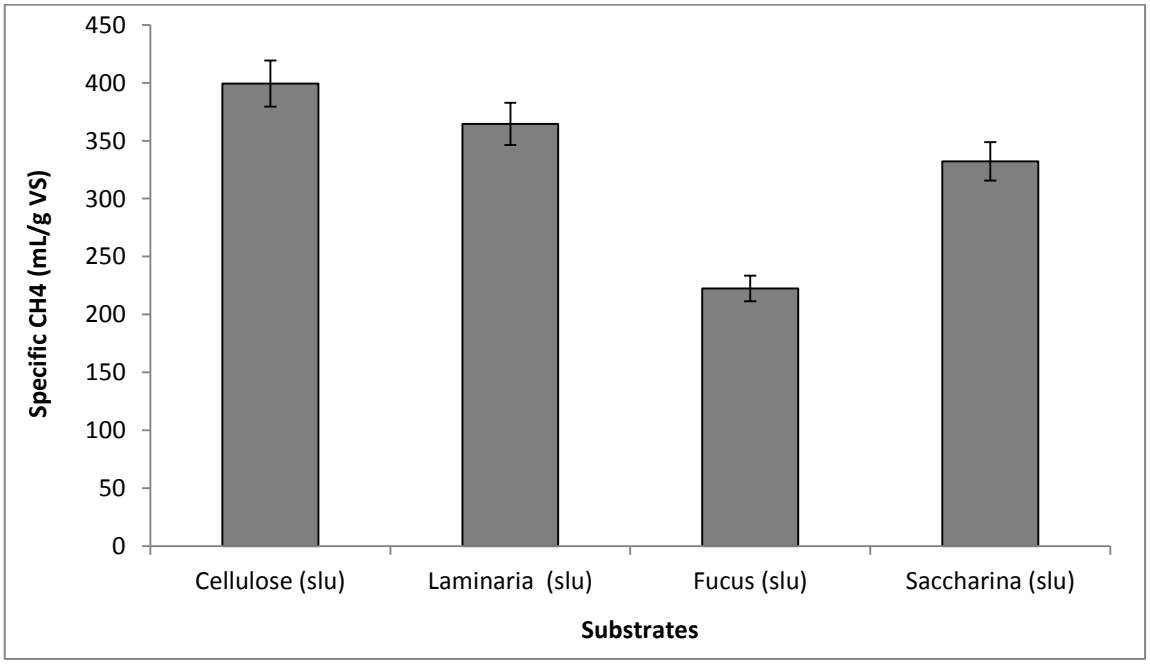


Fig. 4.17. Specific methane production during 50-day anaerobic digestion of substrates using digested sludge as the source of inoculum calculated in relation to the total amount of volatile solids in the substrates. Error bars represent standard deviation from the mean.

One-way ANOVA and pairwise comparison of specific methane production between sludge inoculated cellulose and *L. digitata* showed that the observed difference is not statistically significant ($P>0.123$). There is also no significant difference in specific

methane production between sludge inoculated *L. digitata* and *S. latissima* ($P>0.319$) but specific methane production in *L. digitata* and *S. latissima* is significantly higher than *F. serratus* under sludge inoculation ($P<0.015$).

Published reports on methane (methane) production of various substrates with different operational parameters abound in literature. Nonetheless, reviews by Raposo et al., (2012) and (Khalid et al., 2011) provided extensive biomethane production potentials (in ml CH4/g VS added) of several substrates such as cellulose (379-419), food wastes (396), fruit and vegetable waste (420) glucose (335-351), *Jathropha curcus* (80-968), kitchen waste (370-450), maize residue (229-363), organic fraction of municipal solid wastes (180-530) and papers (84-340). Others include; lignin rich organic waste (200), municipal solid waste (360) starch (340), rice straw (350), water hyacinth (60-350), wheat straw (227-396) and energy crops such as switch grass (191-309), sugar cane (230-300) and sorghum (228-538).

Results of methane production from seaweeds in the current study compares favourably with those reported above and corroborates the need to revisit and exploit seaweeds for biomethane production on a large scale (Hughes et al., 2012).

However, limited reports (Table 4.1) exist on anaerobic digestion studies involving seaweeds as the sole substrate. More so is the anaerobic digestion of seaweeds using anoxic sediment as the source of inoculum to potentially exploit the degradative potential of the marine environment (Migliore et al., 2012; Miura et al., 2014). A study to examine renewable methane production from *L. digitata* by Chynoweth, et al., (2000), using sludge as the source of inoculum in a conventional reactor operated at 35^{o}C, reported maximum methane production of 280 ml/g VS which is 30% less than that obtained in the current study. Nielsen & Heiske, (2011) used roughly chopped *S. latissima* (0.4g VS)

inoculated with sludge from reactors treating cattle manure (4% VS) operated under thermophilic conditions (55^{o}C) for 34 days and obtained maximum methane production of 340 ml/g VS of seaweed added. This is similar to methane produced in the current study under both sediment (325 ml/g VS) and sludge (332 ml/g VS) inoculation. In that study, they concluded that *S. latissima* is one of the most suitable marine biomass feedstock for anaerobic digestion. That conclusion ties in with observations in this study

A similar bioenergy potential test of macerated *Ulva lactuca* species of seaweed using inoculum effluent from cattle manure in a reactor operated at 52^{o}C produced 271 ml/g VS after 42 days of incubation (Bruhn et al., 2011). Similarly, using cow manure as the source of inoculum during a two-stage anaerobic digestion of *Macrocystis pyrifera* and *Durvillea antarctica* in a reactor system, Vergara-Fernández et al., (2008) reported peak methane production of 181 and 179 ml/g VS respectively. The similarity recorded in maximum methane production in that study was said to be as a result of similarity in both algal compositions.

Conversely, the use of anoxic sediment as the sole source of inoculum in a 35days batch digester operated at 37^{o}C, Miura et al., (2014) reported methane production of 420 mg/ g-COD from *Saccharina japonica*. Another study by Migliore et al., (2012) using anoxic sediment as the source of inoculum during anaerobic digestion of a mixture of red (*Gracilariopsis longissima*) and green (*Chaetomorpha linum*) seaweeds yielded 380 ml/g VS of methane. The authors concluded that the use of sediment considerable enhanced methane production.

Contrary to the above reports on the suitability of anoxic sediment for efficient methane production from seaweeds, Costa et al., (2012) in an experiment to assess the biomethanation of *Ulva sp* and *Gracilaria sp* during a 50-day AD process reported that the

use of anoxic sediment had no positive impact on methane production. In that experiment however, seaweeds were not digested alone, but co-digested with mixed primary and secondary sludge. Since sediment was not used as the source of inoculum for solely seaweeds digestion, the impact on methane production of those seaweeds could not be fully assessed.

Although seaweeds, time of harvest and operational conditions differ, methane produced in the current study compare favourably with those obtained in literature irrespective of the source of inoculums.

4.3.4.4 Comparative methane production by all four sets of reactors under different inoculation

The two sources of inoculum employed in the current study displayed some forms of differences and similarities depending on the substrates digested. For instance, methane production was significantly higher in cellulose reactors inoculated with digested sludge (399 ml CH_4/g VS) than those inoculated with anoxic sediment (50.1 ml CH_4/g VS) ($P<0.003$). Apparently, methane production in sludge inoculated cellulose reactors was 8 times more than what was produced in sediment inoculated cellulose reactors. Another interesting scenario is the similarity observed in methane production pattern in all three experimental seaweeds irrespective of the source of inoculum. Although sediment inoculated, *L. digitata* produced more methane /gVS than sludge inoculated ones while a little higher methane was produced in *S. latissima* and *F. serratus* reactors inoculated with sludge than in sediment inoculated ones. However, analysis of variance (ANOVA) and Tukey's pairwise post hoc test showed that the differences observed in methane produced between sediment and sludge inoculated *L. digitata* ($P>0.375$), *F. serratus* ($P>0.237$) and *S. latissima* ($P>0.772$) reactors are not statistically significant (Fig.4.18).

These results suggest that seaweeds are much more amenable to biodegradation by diverse groups of microorganisms (based on their unique composition) than substrates such as cellulose.

The above scenario partly corroborates the conclusion from the study by Miura et al., (2014) who evaluated the use of marine sediment as a source of inoculum for methane production from seaweeds, that marine sediments are better sources of inoculum than others from non-marine origin when methane is to be produced from marine materials such as seaweeds.

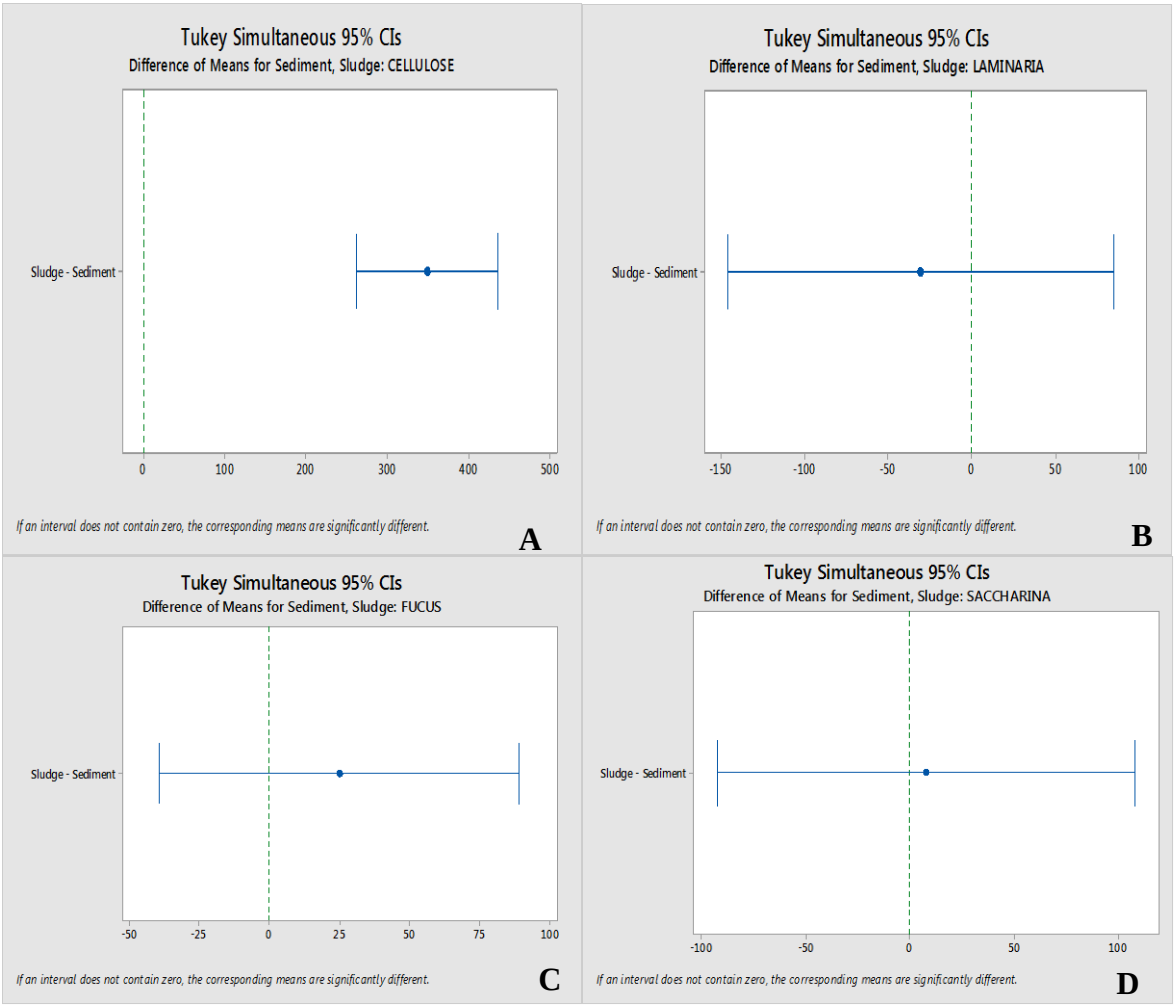


Fig. 4.18. One-way ANOVA and Tukey's pairwise post hoc analyis of differences in mean specific methane production between different inoculum sources of all experimental substrates. The test conducted at 95% confidence interval shows if there are differences in mean values recorded and determines if these differences are statistically significant (Significance level $\alpha = 0.05$). **A**: Cellulose, **B**: *L. digitata*, **C**: *F. serratus*, **D**: *S. latissima*

Generally, these observations support the hypothesis that; *there is no difference in methane production when either anoxic sediment or digested sludge is utilised as the source of inoculum during anaerobic digestion of seaweeds*.

Table 4.1: Summary of methane yields during anaerobic digestion of seaweeds using various inoculum sources in literature and in the current study

Substrate (Seaweeds)	Type of seaweed	Inoculum	Process conditions	Methane yield (ml/g VS)	Reference
C. linum + *G. vermiculophylla* (2:3 w/w)	Green and Red	Anoxic sediment	Batch, 35^{o}C, 57 days	380	(Migliore et al., 2012)
Chaetomorpha linum	Green	Sludge from cattle manure reactor	Batch, 53^{o}C, 34 days	166	(Nielsen & Heiske, 2011)
Durvillea antarctica	Brown	Cow manure	Two-phase batch, 37^{o}C, 31 days	179	(Vergara-Fernández et al., 2008)
Enteromorpha sp	Green	Digested sludge (WWTP)	Batch, 37^{o}C, 82 days	154	(Costa et al., 2012)
Fucus serratus	Brown	Bovine slurry	120ml Batch, 35^{o}C, 32 days	60	(Vanegas & Bartlett, 2013)
Gracillaria vermiculophylla	Red	Sludge from cattle manure reactor	Batch, 53^{o}C, 34 days	132	(Nielsen & Heiske, 2011)
Gracillaria vermiculophylla	Red	Digested sludge (WWTP)	Batch, 37^{o}C, 82 days	182	(Costa et al., 2012)
Laminaria digitata	Brown	Sludge from food waste treatment plant	Batch, 55^{o}C, 50days	200 [a]	(Matsui & Koike, 2010)
Laminaria digitata	Brown	Bovine slurry	120ml Batch, 35^{o}C, 32days	163	(Vanegas & Bartlett, 2013)
Laminaria digitata	Brown	Digested sludge	Batch, 35^{o}C, 36 days	240	(Adams, et al., 2011b)
Laminaria digitata	Brown	Bovine slurry	1L, Batch, 35^{o}C, 109 days	246	(Vanegas & Bartlett, 2013)

Substrate (Seaweeds)	Type of seaweed	Inoculum	Process conditions	Methane yield (ml/g VS)	Reference
Laminaria digitata	Brown	Digester slurry	Conventional reactor, 35^{o}C, 30days	280	(Chynoweth et al., 2000)
Laminaria hyperborea	Brown	Mixed	Continuous reactor, 35^{o}C, 108days	230	(Hinks, et al., 2013)
Macrocystis pyrifera	Brown	Cow manure	Two-phase batch, 37^{o}C, 31 days	181	(Vergara-Fernández et al., 2008)
Mixture of red and brown seaweeds	Red and brown	Sewage sludge	Batch, 37^{o}C, 35 days	121	(Nkemka & Murto, 2012)
Saccharina japonica	Brown	Anoxic sediment	Batch, 37^{o}C, 35 days	420[b]	(Miura et al., 2014)
Saccharina latissima	Brown	Sludge from cattle manure reactor	Batch, 53^{o}C, 34 days	340	(Nielsen & Heiske, 2011)
Saccharina latissima	Brown	Bovine slurry	120ml Batch, 35^{o}C, 32days	245	(Vanegas & Bartlett, 2013)
Saccharina latissima	Brown	Bovine slurry	1L, Batch, 35^{o}C, 109 days	565	(Vanegas & Bartlett, 2013)
Saccorhiza polyschides	Brown	Bovine slurry	120ml Batch, 35^{o}C, 32days	175	(Vanegas & Bartlett, 2013)
Saccorhiza polyschides	Brown	Bovine slurry	1L, Batch, 35^{o}C, 109 days	468	(Vanegas & Bartlett, 2013)

Substrate (Seaweeds)	Type of seaweed	Inoculum	Process conditions	Methane yield (ml/g VS)	Reference
Ulva lactuca	Green	Sludge from cattle manure reactor	Batch, 53^{o}C, 34 days	152	(Nielsen & Heiske, 2011)
Sagassum spp+ sea grass	Brown	Anoxic sediment	1.5L batch, 27.5^{o}C, 85days	94.33	(Marquez, et al., 2013)
Ulva lactuca	Green	Sludge from cattle manure reactor	Batch, 52^{o}C, 42days	271	(Bruhn et al., 2011)
Ulva lactuca	Green	Digested sludge (WWTP)	Batch, 37^{o}C, 82days	196	(Costa et al., 2012)
Ulva lactuca	Green	Bovine slurry	120ml Batch, 35^{o}C, 32days	110	(Vanegas & Bartlett, 2013)
Ulva lactuca	Green	Bovine slurry	1L, Batch, 35^{o}C, 109 days	191	(Vanegas & Bartlett, 2013)
Ulva lactuca	Green	Sludge treatment plant	Batch, 55^{o}C, 50days	150[a]	(Matsui & Koike, 2010)
Fucus serratus	Brown	Anoxic sediment	Batch, 37^{o}C, 50days	198	This study
Laminaria digitata	Brown	Anoxic sediment	Batch, 37^{o}C, 50days	395	This study
Saccharina latissima	Brown	Anoxic sediment	Batch, 37^{o}C, 50days	325	This study
Fucus serratus	Brown	Digested sludge	Batch, 37^{o}C, 50days	223	This study
Laminaria digitata	Brown	Digested sludge	Batch, 37^{o}C, 50days	365	This study
Saccharina latissima	Brown	Digested sludge	Batch, 37^{o}C, 50days	332	This study

[a] ml/g TS: [b] mg/g-COD, WWTP: Wastewater treatment plant

4.4 Conclusion

This study has demonstrated the potential of seaweeds mainly: *L. digitata* and *S. latissima* to contribute significantly to the current move to exploit marine biomass for renewable energy production. It also suggests that the inherent potential of the marine ecosystem could be maximised by using anoxic sediment sourced from the same ecosystem as the seaweeds as a rich source of inoculum for sustainable methane production. It has further shown that marine biomass compare favourably with most of the currently employed feedstock for methane production, and coupled with its added advantages in bioremediation, CO_2 sequestration and job creation, the potential could be enormous.

However, despite the current gains and advancement in research towards sustainable marine bioenergy production via anaerobic digestion, marine biomass exploitation might not become very popular nationally or globally. Potential large-scale exploitation could be limited to coastal areas with comparative advantage for its exploitation, such as those rural coastal communities and islands on the west coasts of Scotland.

This chapter has established the suitability of seaweeds (especially *L. digitata* and *S. latissima*) as suitable feedstock for sustainable biomethane production. The next chapter of this study will look into quantitative studies of various microorganisms present in the reactors at different time points to obtain information about microbial interactions that led to the recorded methane performances across all the reactors (in this chapter).

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Appendix

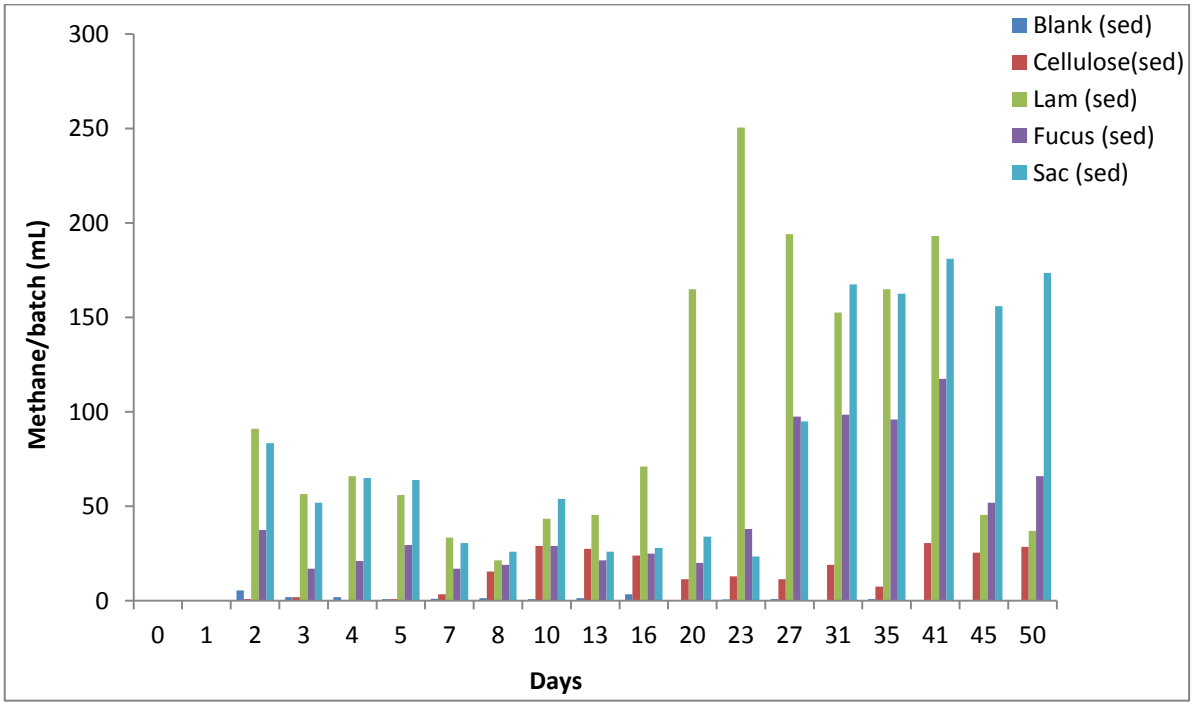


Fig 4.A1. Methane production by reactors during 50-day anaerobic digestion of seaweeds using anoxic sediment as the source of inoculum.

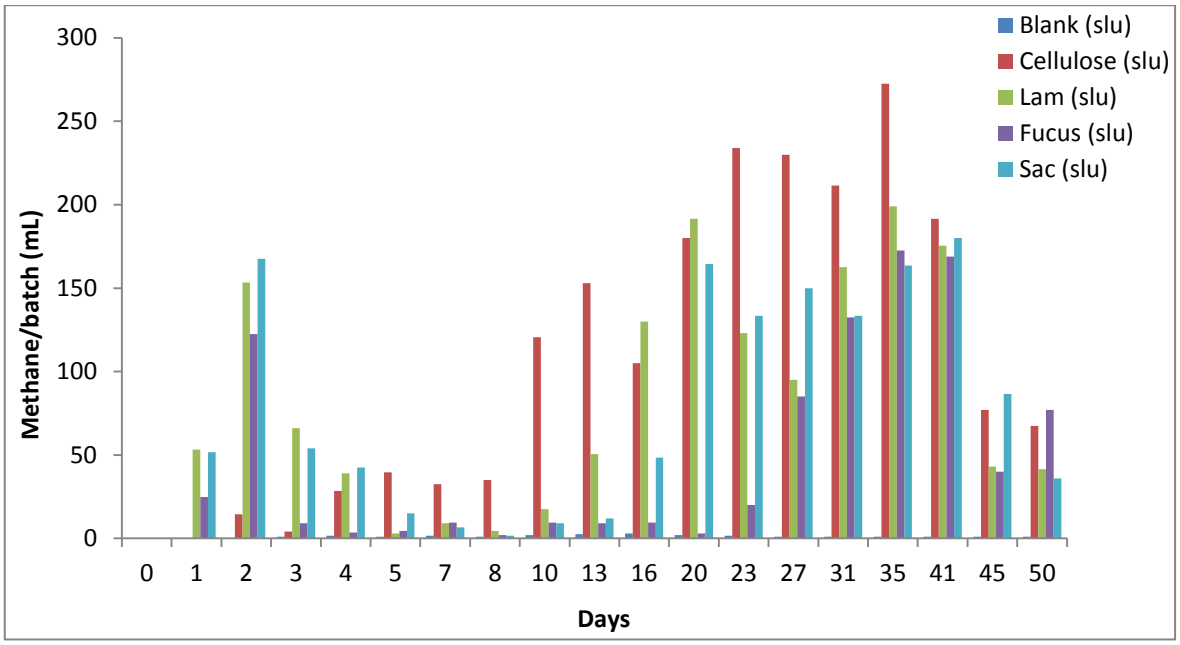


Fig 4A2. Methane production by reactors during 50-day anaerobic digestion of seaweeds using digested sludge as the source of inoculum.

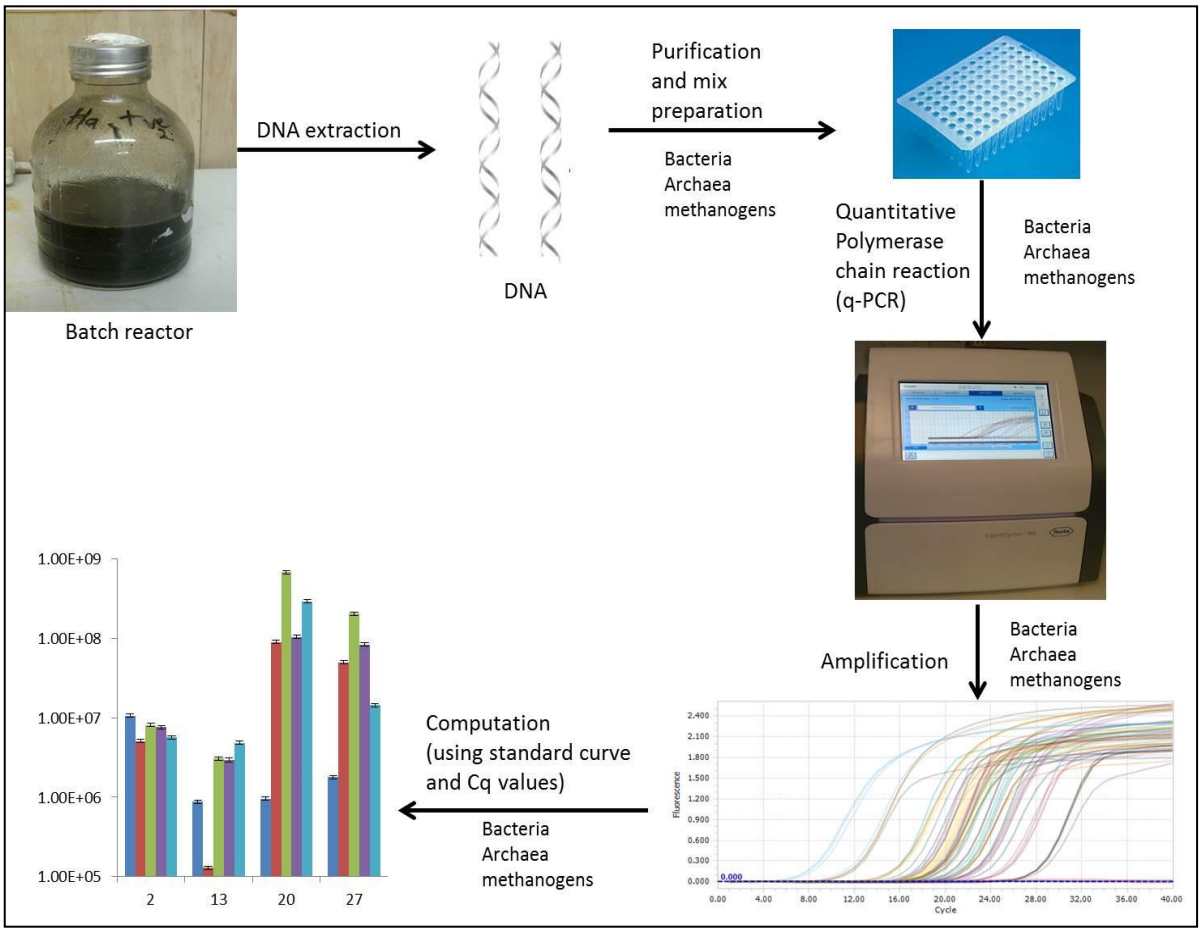
5 Chapter 5

"When you measure what you are talking about, and express it in numbers, then you know something about it".

–Lord Kelvin

Microbial population dynamics during anaerobic digestion of seaweeds using quantitative polymerase chain reaction (q-PCR) techniques

This chapter provides quantitative data of the various organisms present in the batch reactors under various inoculations. Quantitative PCR was used to measure organisms' gene copies/number at different points during the digestion process. This will give an indication of growth and responses of microbes to the changing environmental and operational conditions in the reactors.



Schematic representation of processes and techniques employed in this chapter

5.1 Introduction

5.1.1 Background

The importance of process optimisation during anaerobic digestion of biomass has been widely demonstrated in literature (Appels et al., 2011; Carlsson, et al., 2012; Duan, et al., 2012; Izumi et al., 2010; Prajapati, et al., 2013; Singh & Olsen, 2011; Zeng, et al., 2010; Zhong et al., 2012). While process optimization is important, it is also equally important to have a good knowledge of the microbial composition, behaviour and interactions during the process (Calli, et al., 2006; Cook, et al., 2010; Demirel & Yenigün, 2006; Franke-whittle, et al., 2014; Zhang et al., 2012). The complexity of biological systems involved in the anaerobic digestion of biomass necessitates the need to perform in-depth molecular studies in order to better understand the microbial interactions and functions in the systems (Yu, et al., 2006). Understanding of microbial functions and interactions within AD systems will require that organisms' identity, numbers and activities be well established. As such, a sound knowledge of the quantity (number) of organisms involved in biological processes is important to determine or estimate process kinetics which is a key factor to process design and maintenance (Beckmann et al., 2011; Biddle et al., 2006; Hu, et al., 2012; Song, et al., 2010). Quantitative polymerase chain reaction (q-PCR) allows the quantitative monitoring of double stranded DNA formation during PCR in real time, and could therefore be used to estimate organism numbers in environmental samples (Jørgensen, et al., 2013; Lloyd, MacGregor, & Teske, 2010; Martínez et al., 2011). It is one of the few techniques available in molecular biology that allows for numerical quantification and provides information on organism numbers at specific points in the digestion process (Bergmann & Naturwissenschaften, 2012; Morris et al., 2014; Narihiro et al., 2009; Smith & Osborn, 2009; Traversi, et al., 2012). Consequently, the popularity of real time quantitative PCR has increased considerably since it's commercialisation in 1996

(Zhang & Fang, 2006). Now, a large number of kits are commercially available for preparing, amplifying and analysing samples, as instruments have become more affordable over the years. Two different approaches are employed in q-PCR: the first involves a non-specific method, where all double stranded DNA produced during PCR are detected, while the second uses specific oligonucleotide probes to differentiate target sequences from other non-specific amplifications like primer dimers (Morris et al., 2014; Narihiro et al., 2009; Smith & Osborn, 2009; Takai & Horikoshi, 2000; Zhang & Fang, 2006). Irrespective of the mode of detection employed during q-PCR, the underlining principle remains the same as double stranded DNA formation is monitored through fluorescence in real time thereby enabling the quantification of DNA copies produced as a function of organism numbers (Bustin, et al., 2013; Jørgensen et al., 2013; Lloyd et al., 2010; Smith & Osborn, 2009; Takai & Horikoshi, 2000). In the current study, SYBR Green I approach (non-specific method) was utilised to quantify bacteria (target: 16S rRNA gene), archaea (target: 16S rRNA gene), and methanogen (target: *mcr*A gene), numbers in our anaerobic batch reactors at different time points (day 2, 13, 20 and 27) (See section 1.12.4 Chapter 1). This method has been previously used to quantify microbial populations in different environmental samples. For instance, Steinberg & Regan, (2009) used the approach to quantify *mcr*A gene in different digesters as well as in acidic peat. Other reported uses of this approach include quantification of microbial population in swine wastewater lagoon (Cook, et al., 2010), food spoilage (Martínez et al., 2011), wastewater treatment plants (Kim, et al., 2013) and sediments (Zeleke et al., 2013).

5.1.2 Reproducibility and requirements for accuracy of q-PCR assays

Accuracy of the amplification process during q-PCR is of great importance in microbial ecology studies. In fact, whatever affects the accuracy of the quantification process hampers the use of data interpretation (Smith & Osborn, 2009; Takai & Horikoshi). Therefore, optimised and carefully performed q-PCR reaction is required to obtain reliable quantifications. The accuracy of q-PCR process can be affected by factors such as; primer design, quality of template presence of inhibitors (Bergmann & Naturwissenschaften, 2012; 2000; Zhang & Fang, 2006), handling and storage of samples, primers, probes and enzymes (Martínez et al., 2011). With environmental and digester samples, care much be taken during DNA extraction to recognise possible presence of inhibitors and sources of contamination to improve as much as possible cleanliness of the extracted DNA. This is important because any inhibitors and contaminations in the DNA will affect the efficiency and reliability of the q-PCR results (Roose-Amsaleg, et al., 2001). The precision of the q-PCR process relies largely on the premise that the environmental sample under investigation shares the same PCR amplification efficiency with the selected standard solution. Therefore, it is important to assess the efficiency of the standard solutions and samples under investigation. To assess the efficiency of the q-PCR processes, the following is used

$N_n = N_o \times (1 + \eta/100\%)^n$

where N_n is number of amplified target at the end of nth cycle of amplification, N_o; the initial number of target, and η is the PCR efficiency. Therefore at 100% efficiency (when slope is

-3.32), two DNA segments are produced for every PCR cycle. The value of threshold cycle Ct can then be expressed as:

$C_t = (\log N_t - \log N_o)/\log(1 + \eta/100\%)$

where N_t is the number of amplified target after the threshold cycle C_t.

In practice, a reliable standard curve should have a R^2 value of more than 0.95 and a slope between −3.0 and −3.9 corresponding to PCR efficiencies of 80–115% (Smith & Osborn, 2009; Traversi, et al., 2012; Zhang & Fang, 2006) .

5.1.3 Biases in q-PCR amplification of the 16*S* rRNA and *mcr*A gene approach

There are a few potential biases, which could hamper the results and interpretation of molecular biology data. Some of the potential biases include those introduced from DNA extraction, differences in PCR amplification or random PCR errors. Despite the suitability of the 16S rRNA gene for microbial quantification using specific primers and probes (Edwards, et al., 1989; Watanabe, et al., 2004; Ercolini, 2004), the presence of multiple 16S ribosomal RNA operon in microorganisms especially bacteria undermines the accuracy of the amplification results. Reports estimated only 15% of bacteria have a single copy of the 16S rRNA gene while half of the currently analysed bacteria genomes harbour 5 or more copies (Větrovský & Baldrian, 2013). For instance, the standard bacteria used in this study (pure strain of *Colwellia sp.* Gammaproteobacteria) contains up to six copies of the targeted 16S rRNA gene (Větrovský & Baldrian, 2013). Therefore, results of q-PCR amplification of the bacteria in this study could be high by a factor of as much as six or more. The multiplicity of the 16S rRNA gene is much lower in archaea, which may harbour from one to four copies of the 16S rRNA gene. However more than 57% of sequenced archaea genome contain a single copy of the 16S rRNA gene (Lee, et al., 2009). Some groups of archaea such as members of the *Methanosarcina* and *Methanocella* have been reported to possess up to three copies of the 16S rRNA gene (Angel, et al., 2012).

Although all methanogen genome contains one copy of the *mcr*A gene, members of the *Methanococcales* and *Methanobacteriales* also contain a copy of the gene the isoenzyme

Mrt (Steinberg & Regan, 2009) which is also amplified by the primers (mlas and mcrA-rev), as a result methanogen numbers could be overestimated especially when the two orders above are dominant. Furthermore, amplification of *mcr*A gene present in anaerobic methane-oxidizing euryarcheota (ANME) (Goffredi, et al., 2008), may also result in overestimation of the *mcr*A gene copies of the methanogens in sediment-inoculated reactors due to their prevalence in marine sediment (Goffredi et al., 2008; Kubo et al., 2012).

Potential biases from DNA extraction, primer specificity and multiple numbers of the 16S rRNA gene (in bacteria and archaea) as well as nonspecific amplification of *mcr*A gene in methanogens may introduce error margins higher than recorded and undermine the direct interpretation of the results obtained in this study. However, results of this chapter are to illustrate microbial abundance (number), variations and responses to other physical and chemical parameters during anaerobic digestion processes, despite the biases and uncertainties associated with the processes involved.

5.1.4 Differences in microbial community quantification in relation to the source of inoculums

Differences exist between anoxic sediment and digested sludge based on origin and environmental parameters such as ambient temperature. While sediment was sourced from below fish farm at 6-9^{o}C, digested sludge came from a wastewater treatment plant operated at 37^{o}C. There are also a few similarities between the two sources of inoculum; both are from anaerobic conditions and are exposed to substantial amount organic nutrients. It should be interesting to observe how these differences and similarities aid their performance when used as the sources of inoculum for similar substrates.

This is the first time, to our knowledge that this approach will be used to quantify bacteria, archaea and methanogen in seaweeds anaerobic reactors, using our kind of inoculum.

The main objectives of this chapter are to:

- Obtain quantitative data of organisms in different reactors.
- Determine if substrates influence microbe's numbers across reactors.
- Determine if the source of inoculum is a determinant of microbe's numbers across reactors.
- Relate microbial numbers to other parameters such as VFA and methane production.

5.2 Methods

SYBR Green I approach was used for q-PCR amplification of bacteria, archaea and methanogens numbers at Day 2, 13, 20 and 27 of the digestion process, the point where substantial microbial activities were suspected based on physical and chemical analyses (chapter 4). Bacteria, archaea and methanogens (gene copy numbers) present in the sources of inoculum (anoxic sediment and digested sludge) were also quantified using the same approach.

For standard DNA samples, mass concentration of standard DNA (clone) was converted into copy concentrations using the following equations:

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}\ \text{(copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660\ \text{(g/mol/bp)}}$$

Where Avogadro's number is 6.02×10^{23} copies/mol and the average molecular weight of one DNA base pair (bp) is 660 g/mol (Kim, et al., 2013; Song et al., 2010).

Serial dilution (1:10) of the standard was performed to obtain a standard curve (Fig 5.1)

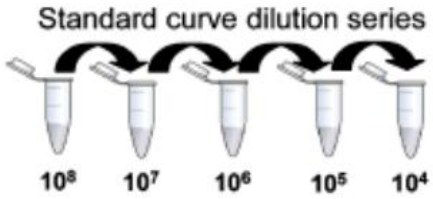


Fig. 5.1. Serial dilution of pure strains (bacteria) or clones (archaea and methanogens) for standard curve formation

After the creation of standard curves, q-PCR efficiency was calculated using the following equation in all reactions: Efficiency = $10^{(-1/\text{slope of standard curve})} - 1$ (Zhang & Fang, 2006).

5.2.1 Q-PCR amplification of bacteria, archaea and methanogens in the sources of inoculum and seaweeds anaerobic reactors

The amplification process was carried out in a 20µl reaction mix containing 2µl of DNA template (1:10 dilution to minimize PCR inhibition according to Roose-Amsaleg, et al., (2001)), 10µl of q-PCR master mix (PrimerDesign, UK) premixed with SYBR Green I, 2.5µl of forward primer and 2.0 µl reverse primer and 3.5 µl PCR water. Reaction mix was prepared in all cases according to manufacturer's instruction. PCR grade water (2 µl) was added to the control mix along with extraction blanks as controls. A dilution of the standard (known) corresponding to 6.04 $x10^{11}$ (bacteria), 1.930 $x10^{9}$ (archaea), and 2.62 $x10^{9}$ (methanogen) copies /ml (obtained using equation 1 above) was used as the positive controls. All samples were amplified in triplicates, including the controls and standards. The amplification protocol began with hot start polymerase activation at 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C denaturation for 15 s and annealing at 60^{o}C for 60 s (bacteria), 60^{o}C for 45 s (archaea) and 55^{o}C for 45 s (methanogens) (details in Table 5.1). A melt curve was included at the end of the amplification to discount non-specific amplification by heating the reactions to 95^{o}C (0.1^{o}C/s) and cooling to 55^{o}C while fluorescence was detected at 0.3^{o}C interval (Nadkarni, et al., 2002). Melting point, C_q and melt curve for each reaction were calculated automatically by the instrument LightCycler® 96 Software 1.1 (Roche, Switzerland). Primers for each group of microorganisms, their coverage and process conditions are listed in table 5.1.

Full description of experimental procedures and methods can be found in chapter 2.

Table 5.1. Primers used for q-PCR studies of microbial components of anaerobic digesters

Oligo name	Target	Oligo sequence (5′-3′)[a]	Coverage (%)	Reference	PCR conditions
E806F	Bacteria 16S rRNA gene	GGACTACHVGGGTWTCTAAT	95.1	(Teske & Sørensen, 2008)	40 cycles of 95^{o}C denaturation for 15 s and annealing at 60^{o}C for 60 s.
U515R	Bacteria 16S rRNA gene	GTGCCAGCMGCCGCGGTAA	99.0	(Wang & Qian, 2009)	
A344F	Archaea 16S rRNA gene	GGGGYGCASCAGGSG	90.8	(Teske & Sørensen, 2008)	40 cycles of 95^{o}C denaturation for 10 s and annealing at 60^{o}C for 45 s
A915R	Archaea 16S rRNA gene	GTGCTCCCCCGCCAATTCCT	97.1	(Wang & Qian, 2009)	
Mlas F	*mcrA* gene (methanogen)	GTGGTGTMGGDTTCACMCARTA	most	(Steinberg & Regan, 2009)	40 cycles 95°C for 30 s, annealing at 55°C for 45 s, extension at 60°C for 2 min and 83°C for 20 s. final elongation at 60°C for 7 min.
mcrA-rev - R	*mcrA* gene (methanogen)	CGTTCHTBGCGTAGTTVGGRT	most	(Steinberg & Regan, 2009)	

The following primer name suffixes are used: - F – forward primer, - R – reverse primer, [a] M=A+C, R=A+G, S=G+C, V=A+G+C, H=A+C+T, D=A+G+T, B=G+T+C.

5.3 Results and discussion

The reliability of the q-PCR was assessed by a number of factors such as; standard curve, efficiency, slopes, the R^2 value and the melt curve analysis (Table 5.2). The slopes obtained from the standard curve of q-PCR runs of the three reactions (bacteria, archaea and methanogens) range from -3.33 in archaea to -3.58 in bacterial quantification, with the concomitant efficiencies of 91, 95 and 100% for during the quantification of bacteria, methanogens and archaea, respectively (Table 5.2). R^2 values were >0.99 while the Y-intercept ranges from 38.36 for methanogens to 47.81 for bacterial quantification (Table 5.2).

Table 5.2. Summary of the standard curve parameters for q-PCR of bacteria, archaea and methanogens

Organisms	**Bacteria**	**Archaea**	**Methanogens**
Parameters			
Slope (mean)	-3.58	-3.3262	-3.4550
Efficiency % (mean)	90.5	100	95
R^2 (mean)	0.99	0.99	0.99
Y-intercept (mean)	47.81	48.69	38.36

Results of the q-PCR amplification parameters (Table 5.2) fall within the range of parameters for a reliable q-PCR reported in literature (Jørgensen et al., 2013; Narihiro et al., 2009; Park et al., 2010; Takai & Horikoshi, 2000; Zhang & Fang, 2006) which include efficiency ranging between 80-115%, slope between -3.0 and -3.9, the R^2 value greater than 0.95.

5.3.1 Q-PCR quantification of the different microbial populations present in the sources of inoculum (anoxic sediment and digested sludge)

Results of bacterial q-PCR amplification shows that ninety-one percent (91%) efficiency was achieved during the amplification, with a slope of -3.58 and R^2 value at 0.99 (Table 5.2). The specificity of the primer pair was confirmed by the result of the melt curve analysis, which shows a single melting peak for all reactions ranging between 85-89°C (Fig 5.2).

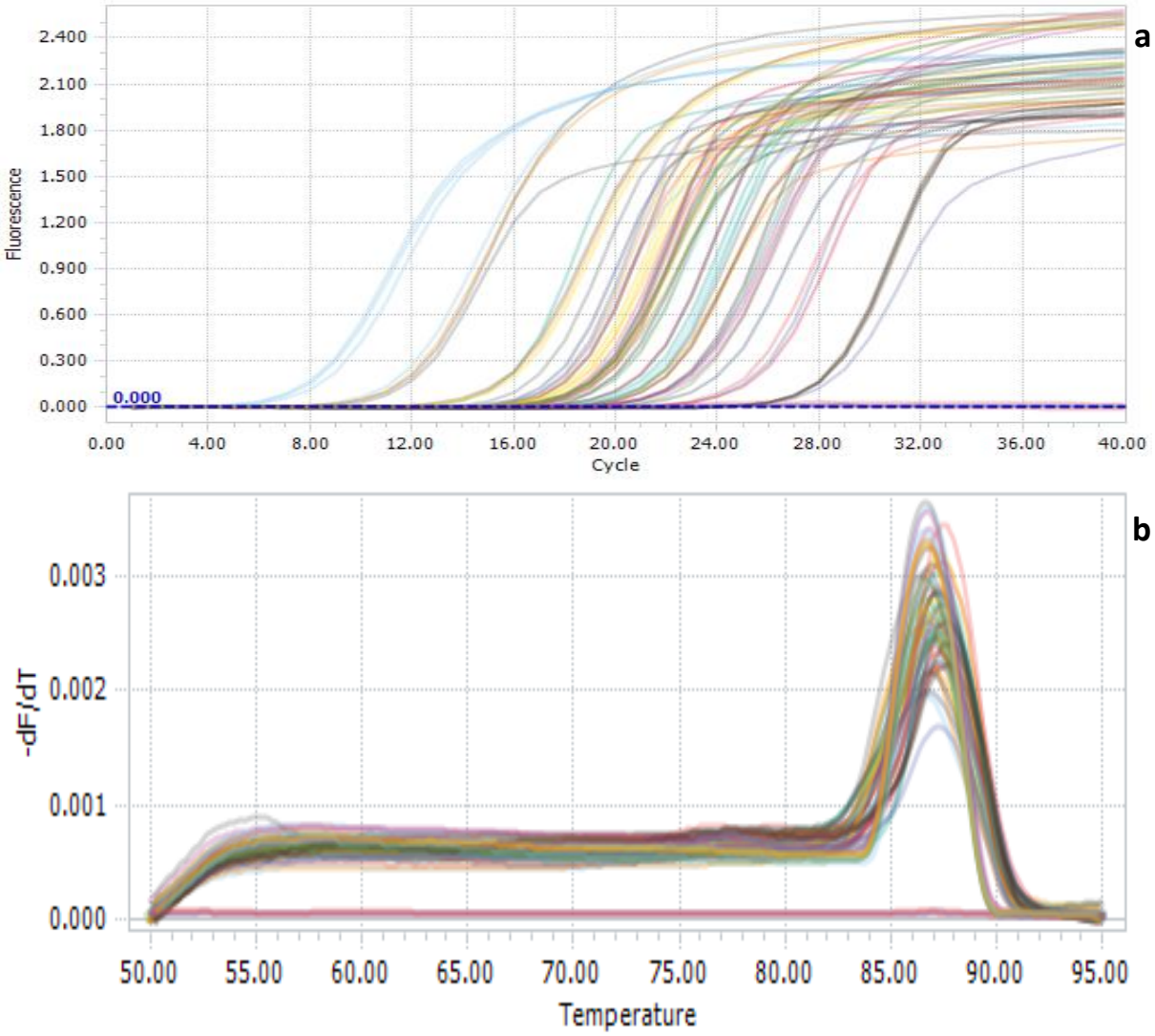


Fig. 5.2 Amplification curves (a) and melting curve analysis (b) of the melting peaks for all bacteria q-PCR reactions. Melt curve analysis was performed by heating the reactions to 95°C (0.1°C/s) and cooled to 55°C while fluorescence was detected at 0.3°C interval.

Results of melt curve analysis indicates no or minimal non-specific amplification because melting temperature (T_m) of q-PCR products is >82 ^oC while T_m of primer-dimer is ~75 ^oC. This is consistent with published reports (Bergmann & Naturwissenschaften, 2012)

5.3.1.1 Quantification of bacterial 16S rRNA gene copies as an indication of bacteria abundance in the seed inoculums

Total bacteria 16S rRNA gene copies present in the sources of inoculum (anoxic sediment and digested sludge) were quantified prior to being used in the reactors to determine the bacterial richness of the inoculums. Results of total bacterial 16S rRNA gene copies in the inoculum sources produced 2.54 x 10^8/ml for anoxic sediment while 1.82 x 10^8 copies/ml were recorded in the digested sludge indicating that both seed inoculum are rich in bacteria (Fig. 5.3).

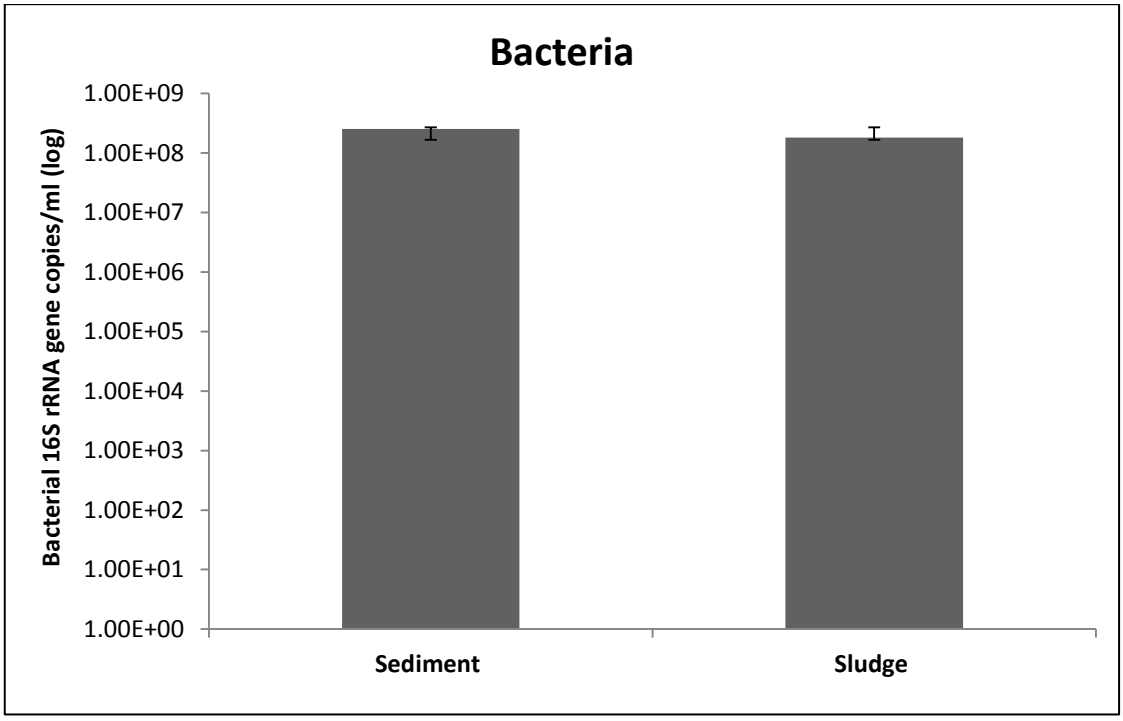


Fig 5.3. Bacterial (16S rRNA gene copies) abundance in the sources of inoculum for the anaerobic digestion of seaweeds in this study n=3

Although, the technique employed (q-PCR of 16S rRNA gene) does not distinguish between living and bacteria cells (one of the pitfalls of q-PCR) (J. Kim et al., 2013), the approach can yet provide vital information on the microbial richness of environmental samples (C. J. Smith & Osborn, 2009). Moreover, results of chemical and physical parameters obtained in this study (chapter 4) suggest that the bacterial community in the inoculum are mostly living rather than dead.

Many quantitative (PCR) studies of microbial communities within environmental samples including marine sediment and digested sludge have been reported. For instance, Schippers, et al., (2012) carried out q-PCR quantification of microbial communities of organic-rich marine sediments of the Black sea and the Benguela Upwelling in Namibia (both sediment similar to the one utilized in the current study), and recorded total bacteria 16S rRNA gene copies/ml of between 10^5-10^6 and 10^6-10^8 respectively within the first 10 m depth. The authors using a different technique (CARD-FISH; capturing only living bacteria cells) for the same samples recorded similar results of 10^5-10^6 and 10^6-10^7 for the Black sea and Benguela Upwelling (Namibia) organic-rich sediments, respectively. These results indicate that similar results of microbial (bacteria) abundance can be obtained with different techniques and that the results of q-PCR technique, which does not distinguish between dead and living cells, could be evaluated and validated. The results of bacteria abundance studies using the two techniques employed by Schippers et al., (2012) are similar to the values obtained for the organic-rich marine sediment utilized in the current study. A similar study which evaluated bacteria community structure in sediment beneath fish farms off the coast of Italy conducted by Vezzulli, et al. (2002), reported that microbial enrichment occurs in sediments as a result of fish farming activities. The authors recorded bacteria density in the top 1-3cm sediment collected in September (same month

sediment in this study was collected) in one of the stations as 8.70 x10^8 cells/g of sediment. This also is comparable to result of sediment inoculum in this study.

Conversely, digested sludge which is one of the most commonly used source of inoculum (Raposo, et al., 2012) has also been studied for microbial (including bacteria) community abundance (Duan et al., 2012; Steinberg & Regan, 2008; Yu et al., 2014). In one of the bacterial quantification studies, Dudley, et al., (1980), enumerated bacteria loads (living cells) in digested sludge to ascertain its associated potential health risks and found total bacteria counts in a two-stage anaerobic digestion system as 4.8 x 10^8cfu/g while sludge from a standard anaerobic digestion system operated for 30 days recorded 1.2 x 10^8cfu/g. In a similar studies, lower total bacteria counts were recorded in raw sludge and digested sludge of 2.6 x 10^6 and 1.3 x 10^5cfu/g from a sewage treatment plant in Sweden (Sahlström, et al., 2004). Results reported above are similar to the bacteria load in the digested sludge used as a source of inoculum in the current study.

5.3.1.2 Quantification of archaeal 16S rRNA gene copies as an indication of archaea abundance in the seed inoculums

Archaea components of the seed inoculum were analysed by quantifying the archaeal 16S rRNA gene using quantitative PCR approach. Amplification efficiency slopes and R^2 value obtained shown on Table 5.2 demonstrates the reliability of the amplification process. Efficiency and melt curve analysis obtained confirms the specificity of the primer pair utilised in this study (Fig. 5.4) and affirms the reliability of the results obtained.

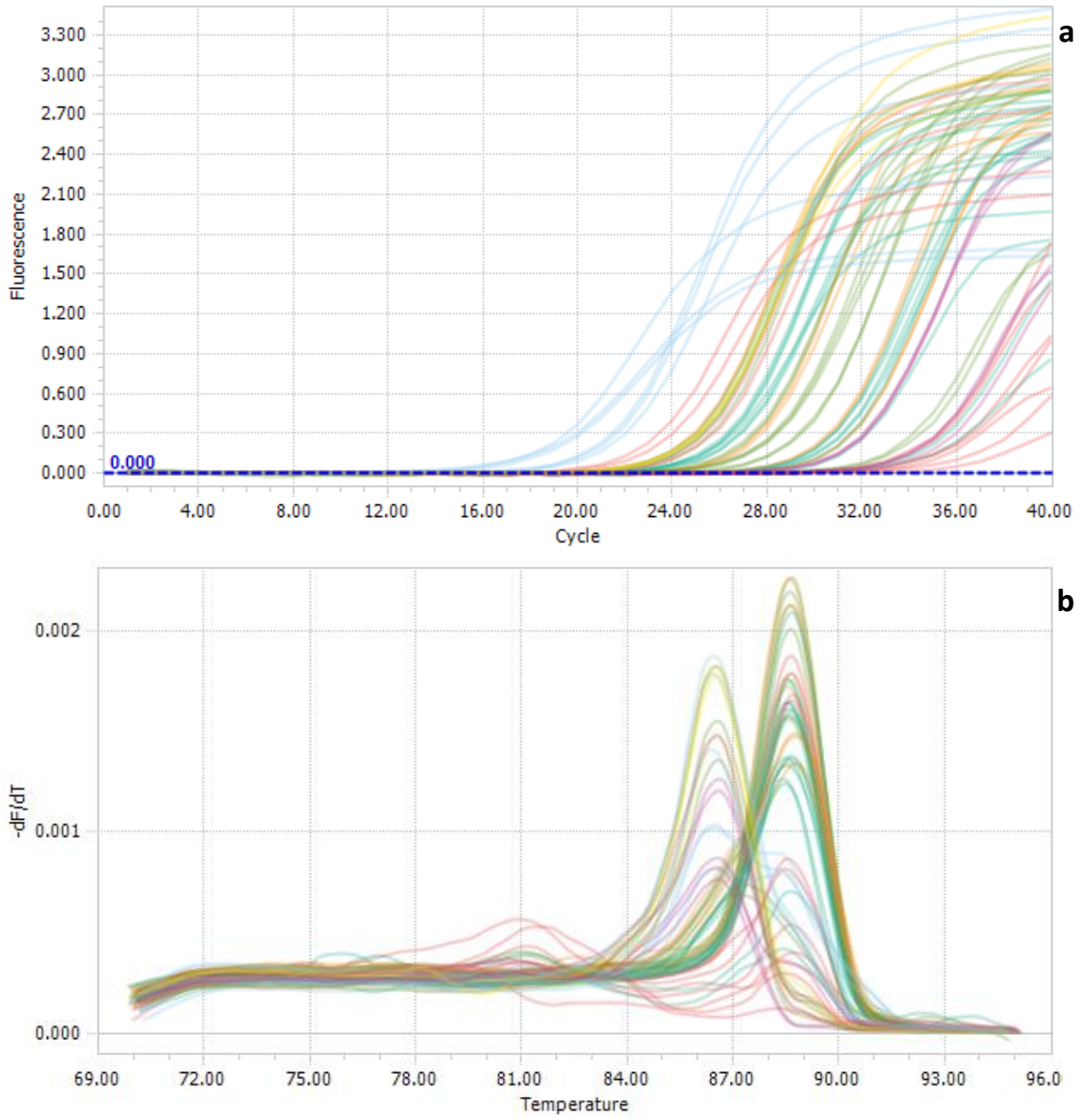


Fig. 5.4. Amplification curves (a) and melting curve analysis (b) of the melting peaks for all archaea q-PCR reactions in triplicates. Melt curve analysis was performed by heating the reactions to 95^{o}C (0.1^{o}C/s) and cooled to 55^{o}C while fluorescence was detected at 0.3^{o}C interval.

Result of quantitative PCR of archaea content of the anoxic sediment inoculum shows the prevalence of archaea populations (based on 16S rRNA gene copies) in the nutrient-rich sediment. Archaea 16S rRNA gene copies obtained in the anoxic sediment and digested sludge inoculum was 4.54 x10^{6} and 1.02 x10^{7} copies /ml, respectively (Fig. 5.5).

The result above confirms reports of archaea's prevalence in marine sediment (DeLong, 1992; Durbin & Teske, 2012; Kubo et al., 2012; Kumar, Dagar, & Puniya, 2012; Lloyd et al., 2013, 2010), although they are much more prevalent in organic-lean sediments than organic-rich ones (Breuker & Schippers, 2013).

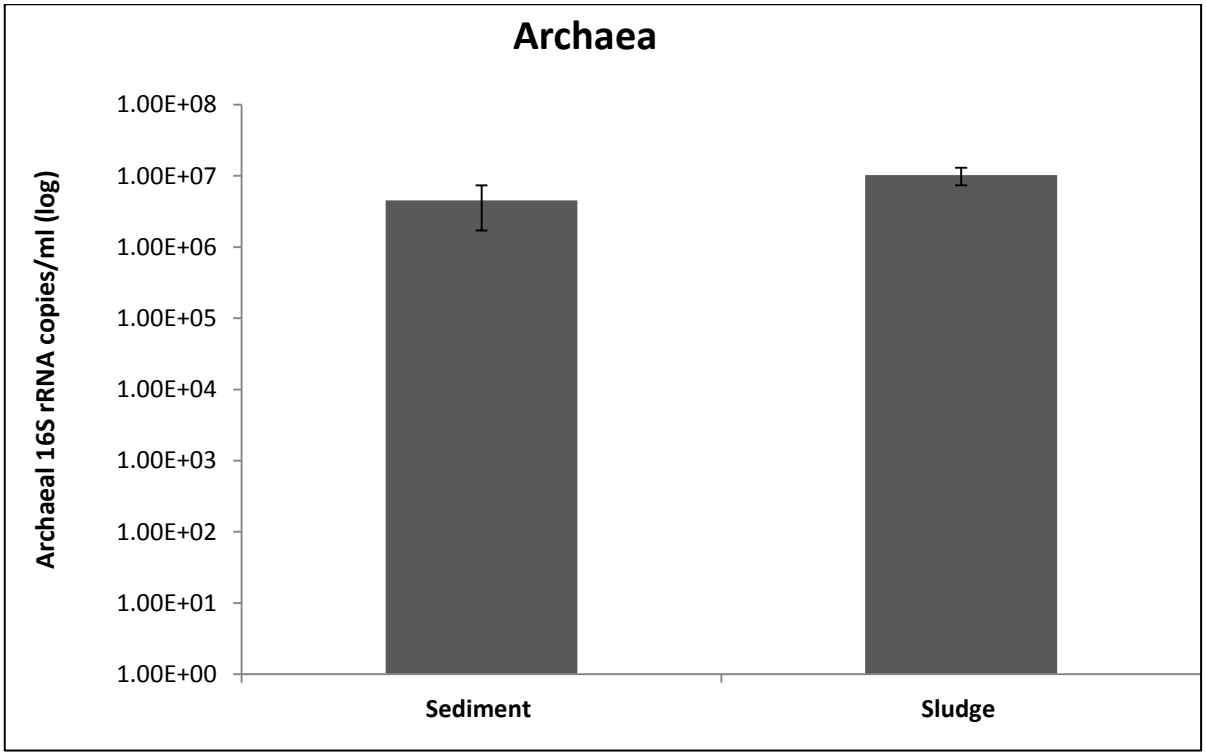


Fig 5.5. Archaeal (16S rRNA gene copies) abundance in the sources of inoculum for the anaerobic digestion of seaweeds in this study n=3

A number of studies have been carried to quantify archaea communities in environmental samples, with a view to understand their contribution to environmental process. An example is the quantitative microbial analysis of organic-rich sediments of the Black Sea and the Benguela Upwelling system of the coasts of Namibia carried out by Schippers et al., (2012). The authors, using a similar q-PCR protocol with the one in the current study reported archaea 16S rRNA gene copies ranging from 10^5-10^7 in the top layer of the organic-rich sediments of the Black Sea and 10^6 -10^8 organic-rich sediments of the Upwelling system of the coasts of Namibia. Using a different approach (CARD-FISH)

which stains and enumerate living cells, similar results were reported for the different but organic-rich sediments from the study areas. An indication that uncertainties associated with q-PCR amplification could be assessed and validated.

Similarly, quantitative (q-PCR) estimation of archaea content of depth profile of anoxic sediment of the Salton Sea by Swan, et al., (2010), recorded 8.5 x 10^6 archaeal gene copies /g in the top 2cm of sediment which declined steadily with depth.

The above studies confirm the variation in sediment archaea populations depending on locations while the results of archaea abundance in the sediments investigated were similar to results of the current study, which suggests that the archaea population in our sediment is comparable to other organic-rich sediments and could therefore be a suitable source of inoculum for anaerobic digestion of seaweeds.

There are not many reports of quantitative archaeal analysis of digested sludge in literature. Most of the currently available reports are on methane plants or anaerobic digesters under operations. One of the available reports of quantitative archaeal analysis carried out by Zhang et al., (2015) on effluent discharge (digested sludge) from wastewater treatment plant in Hangzhou Bay in China. The authors reported 8.5 x 10^7 gene copies/g for samples collected in summer and 4.3 x 10^7 gene copies/g for samples collected in winter. Those values are a little higher than archaea numbers in the sludge used in this study.

5.3.1.3 Quantification of mcrA gene copies as an indication of methanogen abundance in the seed inoculums

Methanogen community of the seed inoculum were analysed by quantifying the *mcrA* gene using quantitative PCR approach. Amplification efficiency slopes and R^2 value obtained shown on Table 5.2 demonstrates the reliability of the amplification process. Efficiency and melt curve analysis obtained confirms the specificity of the primer pair utilised in this study (Fig. 5.6) and affirms the reliability of the results obtained, with minimal nonspecific amplification.

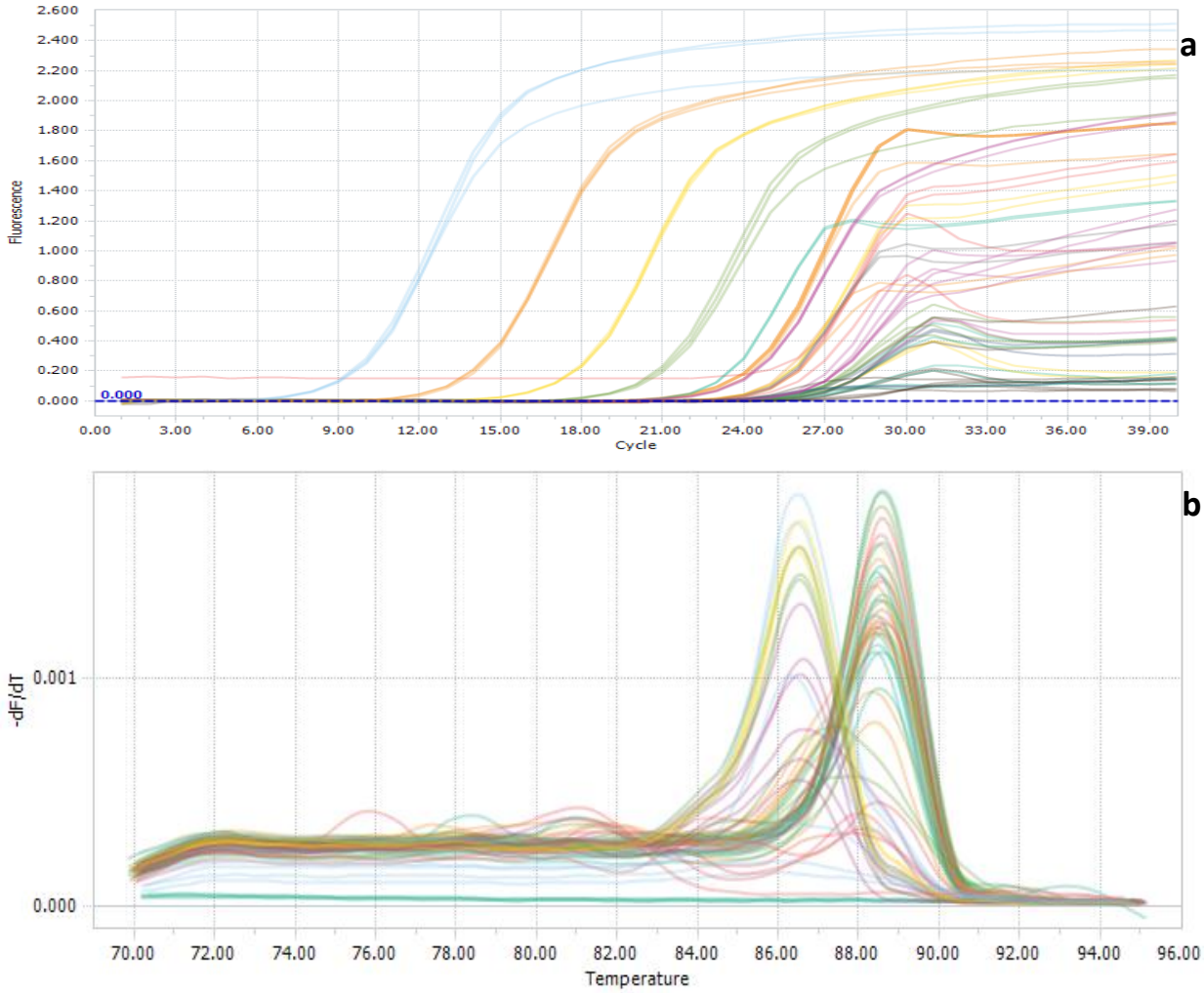


Fig. 5.6. Amplification curves (a) and melting curve analysis (b) of the melting peaks for methanogen (*mcrA* gene) q-PCR reactions in triplicates. Melt curve analysis was performed by heating the reactions to 95^{o}C (0.1^{o}C/s) and cooled to 55^{o}C while fluorescence was detected at 0.3^{o}C interval.

Despite the importance of 16S rRNA gene is molecular biology studies, especially in determining organisms' numbers during quantitative PCR; it cannot be used to determine metabolic activities of microorganisms as the expression of 16S rRNA gene is hardly influenced by changing growth conditions (Nettmann, et al., 2008). To obtain specific quantitative information about methanogen abundance in the current study, estimation of functional gene peculiar to methanogens was employed. Methyl-coenzyme M reductase subunit α gene (*mcr*A gene), is present in all methanogens and can therefore be used as a marker for methanogens studies (Luton, et al., 2002; Morris et al., 2014; Rastogi et al., 2008; Steinberg & Regan, 2009). Since methanogen are responsible for all methane production during anaerobic digestion processes (Morris et al., 2014), knowledge of methanogen numbers (which correlate with methanogenic activity (Traversi et al., 2012)) will be very important in the determination of process performance and functions.

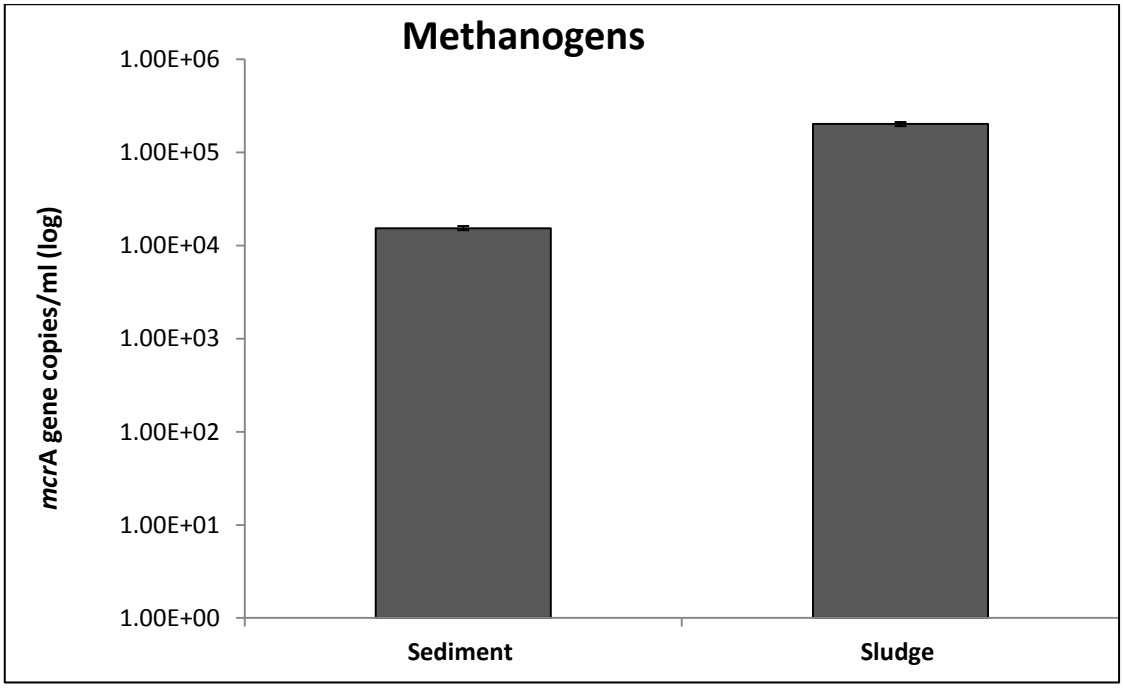


Fig 5.7. Methanogen (*mcrA* gene copies) abundance in the sources of inoculum for the anaerobic digestion of seaweeds in this study n=3

Results of methanogen q-PCR amplification of anoxic sediment used in this study, show methanogens presence, with 1.54 x 10^4 *mcr*A gene copies/ml recorded (Fig. 5.7). Similarly, quantitative PCR amplification of methanogens (*mcrA* gene copies) in the digested sludge to be used as the source of inoculums in the current study gave 2.02 x 10^5 *mcr*A gene copies /ml. This is significantly higher ($P<0.003$) than what was obtained from anoxic sediment *mcr*A genes amplification, although methanogen growth might have been affected by the low temperature and other inhibitory materials (e.g. salt, metals) in the sediment. A study involving q-PCR quantification of methanogens (*mcrA* gene copies) in nutrient-rich sediment of the Black Sea and Benguela upwelling system off the coasts of Namibia, Schippers et al., (2012) reported 5.0 x 10^5 *mcrA* gene copies/ml in the top 2cm of sediment and 1.02 x 10^6 *mcr*A gene copies /ml respectively. These reported *mcrA* gene copies are in the range of methanogens numbers obtained in the current study, especially as both sediments are nutrient rich. Conversely, methanogens number recorded in digested sludge used in this study is a little lower than the numbers obtained in digested sludge of anaerobic digesters treating dairy manure and brewery waste, reported by Steinberg & Regan, (2009), where 2.53 x 10^6 and 1.04 x 10^6 *mcr*A genes copies /ml were detected in both digesters, respectively.

5.3.2 Bacteria quantification in seaweeds anaerobic batch reactors under various inoculations

During the early stages (day 2) of the digestion process, results of total bacterial quantification produced 1.83, 1.34, 6.33, 7.54 and 4.95 x 10^9 gene copies /ml for Blank, Cellulose, *L. digitata, F. serratus, and S. latissima* reactors respectively (Fig. 5.8A). These results are approximately 1 order of magnitude higher than gene copies obtained from the anoxic sediment itself (Fig 5.3), an indication of early bacterial viability and growth in the reactors. The drop in pH in the first 48 hours of experiment in the three seaweeds' reactors (Fig. 4.10) is also an indication of high bacteria activities (acidogenesis). Comparison between seaweeds and cellulose reactors revealed early distinction between them. For example, as at day 2 there are 4 times more bacteria 16S rRNA gene copies in *S. latissima* reactors than present in cellulose reactors. Again, up to 5 times more bacteria gene copies were detected in *L. digitata* reactors than in cellulose reactors (Fig. 5.8A). These results attests to the results of chemical parameters (chapter 4) that bacteria in anoxic sediment might not be adapted to utilise cellulose, while they were better adapted to seaweeds substrates.

Similarly, results obtained in sludge-inoculated reactors at day 2 of the digestion process, indicates that bacteria gene copies ranged between 1.09 x 10^9 in *F. serratus* reactors to 3.06 x 10^9 /ml in cellulose reactors (Fig.5.8B). The results are approximately 1 order of magnitude greater than bacteria in the digested sludge inoculum, indicating increased bacteria growth at the start of the process in all reactors likely due to substrates availability. At this stage (day 2), bacterial gene copy numbers were similar (within 10^9) across all reactors; whether sediment or sludge inoculated (Fig. 5.8).

Bacteria growth in the 3 sediment-inoculated seaweeds' reactors were similar at the initial stages of the digestion process and did not vary as much (within the same order of magnitude) until after day 13 (Fig 5.8) confirming the results observed for VFAs (fig 4.5) and pH (fig. 4.10) production. However, there were significant drops in bacterial gene copies in the sediment-inoculated blank and cellulose reactors between day 2 and 13, an indication that the bacteria population is not yet adapted to cellulose degradation at that stage of the process (fig 5.8A).

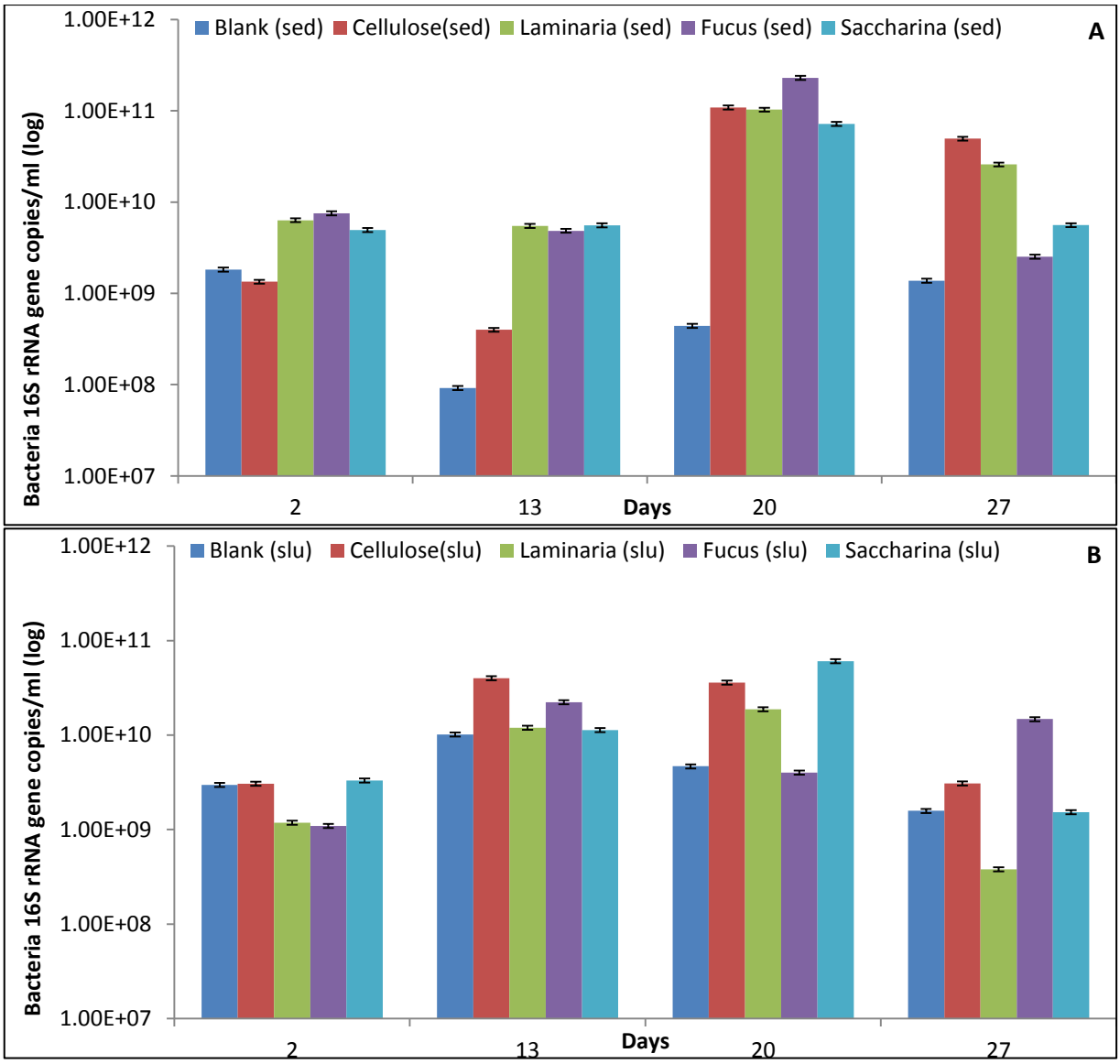


Fig. 5.8. Bacteria 16S rRNA gene copies during anaerobic digestion of seaweeds using (A) anoxic sediment and (B) digested sludge as the source of inoculum. Columns represent mean values while errors bars show standard deviation of the three replicates.

In contrast to sediment-inoculated reactors, significant increases in bacterial gene copies were recorded in all sludge-inoculated reactors between day 2 and 13 resulting in approximately 10 times increase in every reactor. The highest increase was recorded in *F. serratus* as bacteria gene copies increased 20 times to 2.23×10^{10} /ml compared to the blank reactors with only three times more bacteria gene copies at day 13 than at day 2 (Fig. 5.8B). Increases in bacteria gene copies at between day 2 and 13 correspond with the commencement of exponential increase in VFAs production in all sludge-inoculated reactors (Fig. 4.7).

Unlike sediment inoculated reactors, bacteria gene copies in sludge-inoculated seaweeds reactors differ considerably. For instance, at day 2, *S. latissima* reactors had the highest number of bacteria gene copies and *F. serratus* the least. The situation was reversed by day 13 at which point *F. serratus* reactors had the highest and *S. latissima* the least (Fig. 5.8B). This scenario might be due to bacteria population's differential attempt to adapt to the different composition of the various seaweeds.

After day 13, significant changes were observed in most of the sediment-inoculated reactors in relation to bacteria gene copies (population). For example, the initial drop in numbers of bacteria in the blank reactors, eased slightly as copy numbers/ml increased from 9.21×10^{7} on day 13, to 4.41×10^{8} on day 20. This might be due to increased bacteria growth/activity in an attempt to breakdown more recalcitrant materials in the organic-rich sediment inoculum. Further increase was recorded on day 27 to 1.38×10^{9} gene copy numbers/ml. However, bacterial gene copies on day 27 was less than the number on day 2, where most of the growth/activities might have occurred due to substrate availability.

Unlike the sediment-inoculated blank reactors where bacteria numbers never fully recovered, the situation in the sediment-inoculated cellulose reactors was different.

After the initial drop in bacteria numbers at day 13 (Fig. 5.8A), it appeared that the bacteria had become acclimatised to cellulose degradation as copy numbers jumped from 3.99 x 10^8 to 1.09 x 10^{11}/ml on day 20 (almost 10^3 increase). With that increase on day 20, bacteria numbers in cellulose reactors reached the same level with those of *L. digitata* (1.03 x 10^{11}) and *S. latissima* (7.18 x 10^{10}) and a little lower than that of *F. serratus* (2.30 x 10^{11}) (Fig. 5.6A). This increase in bacterial gene copies between day 13 and 20 sediment-inoculated reactors coincides with increases in volatile fatty acids (VFAs) production in all reactors (fig. 4.5). The growth of bacteria in all reactors appeared to peak around day 20 of the digestion process before the decline in gene copies recorded on day 27. The biggest decline in bacteria numbers was seen in *F. serratus* reactors where gene copies dropped from 2.30 x 10^{11}/ml on day 20 to 2.53 x 10^9. This was followed by *L. digitata* reactors, which recorded a drop from 1.03 x 10^{11} to 2.58 x 10^{10}/ml. In cellulose and *S. latissima* reactors bacteria gene copies dropped from 1.09 x 10^{11} and 7.18 x 10^{10} to 5.95 x 10^{10} and 5.59 x 10^9 /ml respectively. These results coincides with VFAs production activities in all seaweeds' reactors as VFAs production began to decline after day 27 (Fig. 4.7).

Likewise, increases in bacteria gene copies in sludge-inoculated reactors at day 13 correspond with the commencement of exponential increase in VFAs production in all reactors (Fig. 4.7). Bacteria numbers in cellulose reactors remains largely stable between day 13 and 20 corresponding to times of substantial increase in VFAs production, but decreased about 10-fold after day 27 which is simultaneously reflected in VFAs productions (Fig. 4.7).

A slight increase in bacteria gene copies was recorded in sludge-inoculated *L. digitata* reactors between day 13 and 20, but reduction in bacteria numbers occurred after day 27. A trend also recorded in VFAs production in sludge inoculated *L. digitata* reactors. By comparison, bacteria gene copies/ml in sludge-inoculated *F. serratus* reactors was about

the same between day 13 and day 27, resulting in peak VFAs formation at day 27. In sludge-inoculated *S. latissima* reactors, peak bacteria gene copies (6.07×10^{10} /ml; more than 10 times from day 13) was recorded on day 20 (Fig. 5.8B), again corresponding to peak VFAs formation (Fig. 4.7). A drop in bacteria number was also seen at day 27, indicating reduction in bacteria growth.

While there are currently no reports on bacterial quantification of sediment or sludge inoculated seaweeds reactors, a number of studies have looked at bacteria quantification within different anaerobic digesters. For instance, a study of microbial population dynamics of a maize silage-fed semi continuous methane plant inoculated with pig manure carried out by Blume et al., (2010), recorded 7.23×10^{8} bacteria 16S rRNA gene copies/ml on day 7 of the digestion process, the earliest time provided. In that study, bacteria gene copies increased to about 9.53×10^{10} on day 35 (peak), these values are lower than those obtained in the current study. In another study of the temporal changes in bacteria population in anaerobic lagoon treating swine waste, Cook et al., (2010) recorded the highest bacteria number (1.0×10^{9} cells/ml) in the summer months. These values are similar or lower than results from the current study, although processes and substrates differ.

5.3.2.1 Comparative studies of bacteria composition of anoxic sediment and digested sludge inoculated substrates

One-way analysis of variance method was used to determine differences in bacteria gene copies between sediment and sludge inoculated reactors at specific time points. Statistically significant interactions were further tested with Tuckey's pairwise post hoc analysis.

Bacteria gene copies were similar at day 2 in all reactors irrespective of the source of inoculums ranging from 1.09 x 10^9 to 7.54 x 10^9 copies /ml. By day 13, a shift in bacteria numbers patterns was recorded between sediment and sludge inoculated reactors. While there was a general decline in all sediment inoculated reactor between day 2 and day 13, the opposite occurred in sludge inoculated reactors. This scenario might be as a result of initial shock experienced by sediment bacteria coming from 8-9^{o}C sediment's temperature to 37^{o}C operational temperature. In contrast, sludge inoculum must have been adapted to the 37^{o}C operational temperature haven been drawn from mesophilic wastewater treatment plant. By day 20, bacteria in sediment inoculum appeared to have recovered from the initial temperature shock and became adapted to the substrates thereby outgrowing those in sludge-inoculated reactors. With the exception of the blank reactor, peak bacterial gene copies were recorded at day 20 in all sediment-inoculated reactors. However, records of peak bacterial gene copies in sludge-inoculated reactors varied considerably. For instance, while peak bacterial gene copies were recorded at day 13 in cellulose and *F. serratus* reactors, peak bacteria growth were recorded later at day 20 in *L. digitata* and *S. latissima* reactors.

One-way analysis of variance with Tuckey's pairwise post hoc comparison of mean peak bacteria gene copies (day 20) in all sediment inoculated reactors show that bacteria gene

copies are significantly higher in substrates containing reactors (Cellulose, *L. digitata*, *F. serratus* and *S. latissima*) than in no-substrate (blank) reactors ($P<0.0005$) (Fig.5.4). This is an indication that the substrates in this study provided carbon and energy source to support bacteria growth leading to biomass degradation and subsequent methane production. Statistical analysis revealed that there is no significant difference in bacterial gene copies between *L. digitata* and *S. latissima* reactors at day 20 ($P>0.08101$) which supports the similarity observed in VFAs production pattern in both reactors.

Although, the difference in bacteria numbers between sediment inoculated cellulose and *L. digitata* is not statistically significant ($P>0.9507$) (Fig. 5.9), the difference recorded in their VFAs production was significant (Fig. 4.7). This confirms that organism number may not always be a good indicator of activities and may reflect the growth of groups of bacteria not directly involved in VFAs production such as SRBs (sulphur reducing bacteria) commonly found in marine sediments which grow by oxidising organic compound and reducing sulphated compound to hydrogen sulphide (Hines & Buck, 1982; Mudryk, et al., 2000).

One-way analysis of variance and Tuckey's pairwise post hoc comparison of mean peak bacteria gene copies (day 13 in cellulose and *F. serratus*; day 20 in *L. digitata* and *S. latissima* reactors) in all sludge inoculated reactors showed that bacteria gene copies were significantly higher in cellulose reactors than all seaweeds reactors ($P<0.001$). This might contribute to the better performance of cellulose reactors than those of seaweeds, in terms of methane production. Statistical analysis also showed that there are no significant differences in bacteria gene copies between the three sludge inoculated seaweeds (*F. serratus*, *L. digitata* and *S. latissima*) reactors (Fig. 5.9).

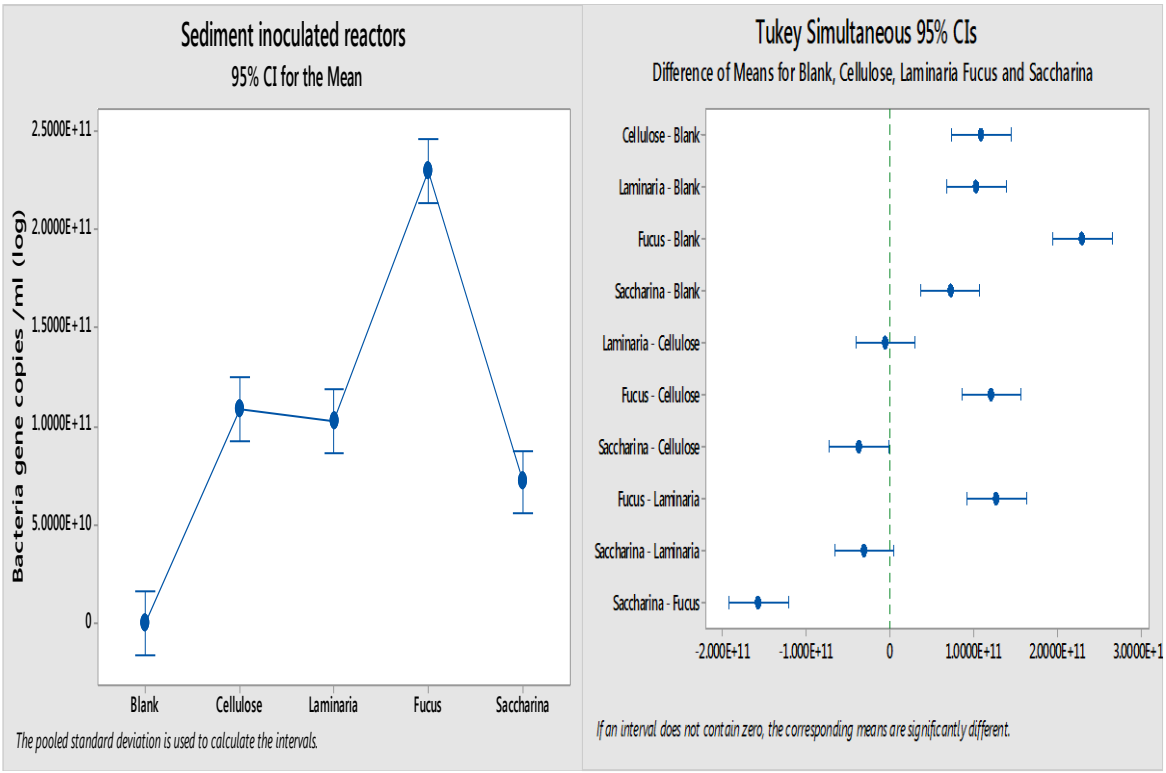


Fig. 5.9. One-way ANOVA and Tukey's pairwise post hoc analyis of differences in mean bacteria gene copies /ml between anoxic **sediment-inoculated** reactors. The test conducted at 95% confidence interval shows if there are differences in mean values recorded and determines if these differences are statistically significant (Significance level $\alpha = 0.05$).

One-way analysis of variance and Tuckey's pairwise post hoc comparison of peak bacteria numbers across the different sources of inoculum revealed that despite its poor performance in terms of VFAs and methane production, bacteria numbers were significantly higher in sediment inoculated cellulose reactors ($P<0.006$) than sludge inoculated ones (Fig.5.9). This unusual scenario may not be unconnected to the occurrence of SRBs in sediment inoculated cellulose reactors (Hines & Buck, 1982; Mudryk et al., 2000), or the presence other bacteria groups that are not involved in anaerobic digestion.

Statistical analysis of peak bacteria numbers for *L. digitata* showed that bacteria numbers were significantly higher ($P<0.003$) in sediment than sludge inoculated reactors (Fig.5.10). This suggests that the growth of other bacteria populations such as SRBs might be promoted in the sediment-inoculated reactors since there were no significant differences in VFAs and methane production between sediment and sludge inoculated *L. digitata*

reactors. Statistical analyses and comparison of bacteria numbers between sediment and sludge inoculated *F. serratus* and *S. latissima* also revealed a similar trend, where higher bacteria numbers were recorded in sediment inoculated reactors, despite the fact that differences in VFAs and methane production in the reactors were not statistically significant irrespective of source of inoculum.

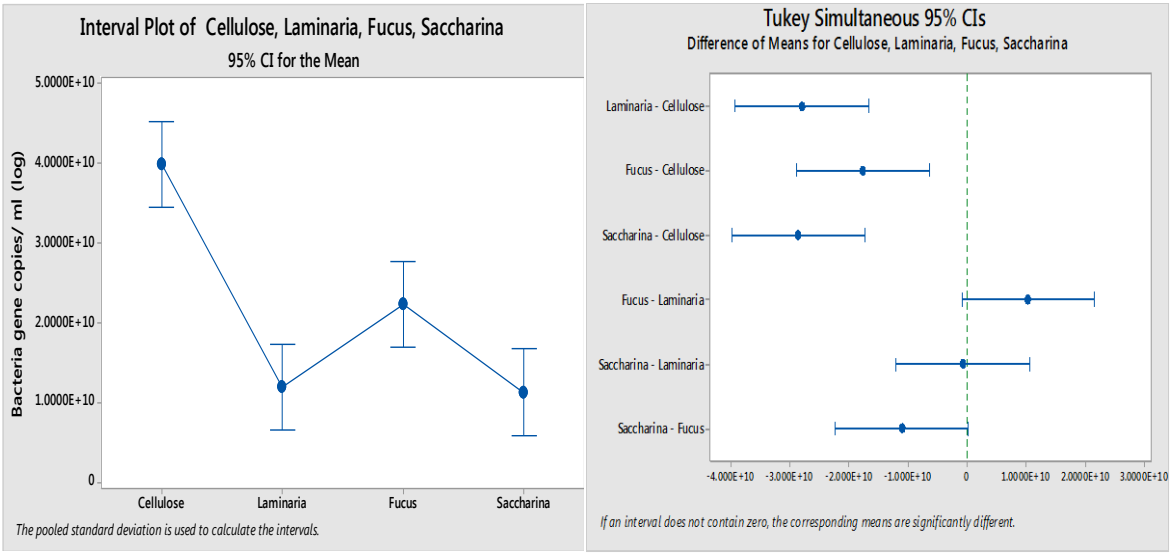


Fig. 5.10. One-way ANOVA and Tukey's pairwise post hoc analyis of differences in peak mean bacteria gene copies /ml between digesed **sludge-inoculated** reactors. The test conducted at 95% confidence interval shows if there are differences in mean values recorded and determines if these differences are statistically significant (Significance level $\alpha = 0.05$).

5.3.3 Archaea (16S rRNA gene) quantification in sediment and sludge-inoculated seaweeds batch reactors

Results of q-PCR at day 2 of the digestion process in sediment-inoculated reactors show increases (from numbers in the inoculum) in archaea numbers in all reactors including blanks. The growth recorded in blank reactors might be as a result of mesophilic temperature at which reactors were operated, which might be much more suitable for microbial growth than marine sediment temperatures (5-9^{o}C) (Khalid, et al., 2011; Raposo et al., 2012). Archaea numbers were similar in all reactors as at day 2 ranging from 5.07 x10^{6}/ ml in cellulose reactors to 1.07 x 10^{7}/ ml in blank rectors (Fig. 5.11a).

Conversely, at day 2, archaea numbers vary considerably amongst sludge-inoculated reactors. While the highest number (5.86 x 10^{7} gene copies/ml) was recorded in cellulose reactors, the least number of archaea (3.97 x 10^{6} gene copies/ml) was found in *F. serratus* reactors (Fig. 5.11b). At this stage, (day 2) archaea content of seaweeds' reactors was generally lower than in cellulose reactors. This is opposite of the result in sediment-inoculated reactors where archaea numbers in cellulose reactors were lowest (Fig.5.11).

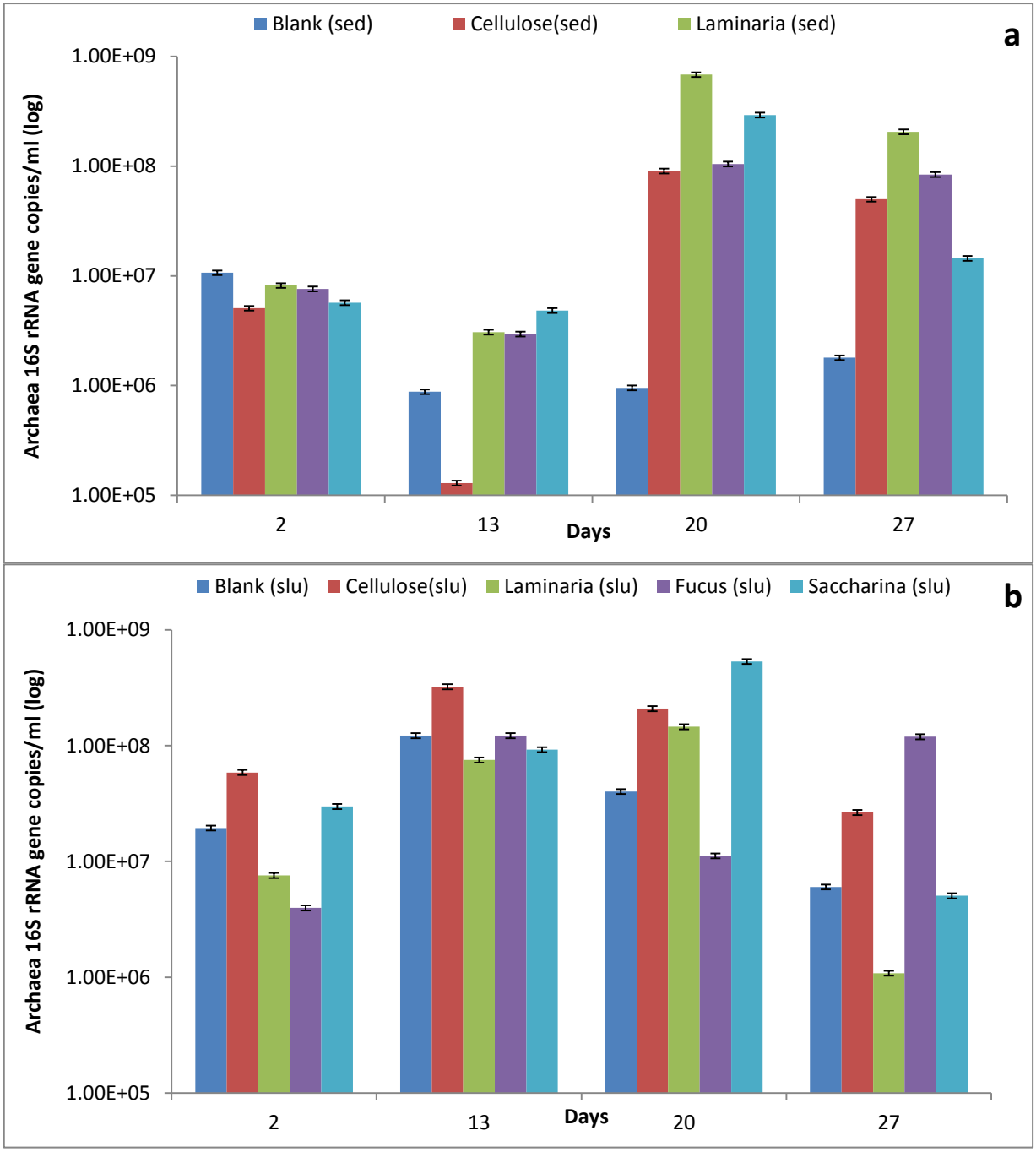


Fig. 5.11. Archaea (16S rRNA gene copies) number distribution during anaerobic digestion of seaweeds using (**a**) anoxic sediment and (**b**) digested sludge as the source of inoculum. Columns represent mean values while errors bars show standard deviation of the three replicates

After day 2, drops in archaea numbers were recorded in all sediment-inoculated reactors with the most significant drops seen in cellulose reactors at day 13 (Fig.5.11a). This suggest that archaea from marine sediment might not be able to optimally grow on the VFAs produced from cellulose or that suitable archaea substrates (VFAs) are not fully

available at that stage of the process. Archaea numbers in the three seaweeds' reactors remained fairly similar and stable at day 13 and rapidly increased by at least two orders of magnitude at day 20 (Fig.5.11a), suggesting that archaea community in the reactors are more likely used to marine biomass derivatives. It may also be that archaea substrates such as VFAs were produced earlier in seaweeds, due to the presence of readily hydrolysable sugars compared to cellulose, to sustain the archaea population. Peak archaea numbers were recorded in all reactors (except blanks) at day 20 corresponding to a time of increased microbial (bacterial and archaeal) activities in the sediment-inoculated reactors (Fig. 4.5 and 4.12), before a slight drop recorded on day 27 (Fig. 5.11a).

In comparison with sediment-inoculated reactors, where archaea population declined after day 2, there were increases in all sludge-inoculated reactors after day 2. Consequently, by day 13, archaea numbers in all sludge-inoculated reactors had increased by at least an order of magnitude with *F. serratus* accounting for the highest increase indicating archaea growth and availability of archaea substrates. Again, contrary to the observation in sediment-inoculated reactors, sludge inoculated cellulose reactors continued to have the highest numbers of archaea (3.22×10^8 gene copies/ml) up till day 13, suggesting the suitability of digested sludge as a source of inoculum for anaerobic digestion of cellulose unlike anoxic sediments.

Archaea numbers remained largely stable in all reactors between day 13 and 20 with a slight drop in cellulose and *F. serratus* but a slight increase in *L. digitata* and *S. latissima*. By day 27, general decline in archaea numbers was recorded in all reactors except *F. serratus* where archaea numbers had earlier decreased (Fig.5.11). Compared to sediment-inoculated seaweeds reactors, greater variations occurred in archaea number in sludge-inoculated reactors.

While there is scarcely any available, report on archaea, quantification of sediment inoculated seaweeds and cellulose reactors in literature, there are available reports on the use of sludge inoculum and other substrates (feedstock). For instance, a molecular ecology studies of a pilot-scale upward flow anaerobic sludge blanket treating swine wastewater operated for 382days carried out by Song et al., (2010) revealed archaea composition of between 5.3 - 8.2 x 10^7 gene copies /ml in the first 100 days of the process. The highest archaea number levels recorded in the entire 382 days process, a result similar to observation in this study. Estimating archaea component of a semi-continuous methane plant operated at mesophilic conditions, Bergmann & Naturwissenschaften, (2012) recorded the lowest number of archaea at day 7 (9.5 x10^6 gene copies/ ml) which increased to about 4.5 x10^8 gene copies/ ml by day 35 and then declined. Archaea growth occurred mainly during stages of suitable substrates availability (such as VFAs) for growth and development. These observations in archaea numbers are similar to what was obtained in all seaweeds reactors in this study, except that reactor parameters and experimental set-ups are different.

Generally, high archaea numbers were recorded in the middle of the study period (day 13-20), which corresponds to the time of increased microbial activities. However, the results indicate that archaea growth occurred earlier (from day 2-20) (Fig. 5.11b) in sludge-inoculated reactors than in sediment-inoculated ones (from day 13-27) (Fig. 5.11a).

5.3.4 Methanogens (*mcrA* gene) quantification in sediment and sludge inoculated seaweeds batch reactors

The *mcr*A gene copies are a measure of methanogens abundance in anaerobic digesters (Wilkins, et al., 2015) and have been shown to have significant correlation with methanogenesis or methanogen activity (Munk, et al., 2012; Traversi et al., 2012; Morris et al., 2014). Therefore, *mcrA* gene copies/methanogen numbers will sometimes be related or refer to activities.

Results of methanogens (*mcr*A) gene copies in sediment and sludge-inoculated reactors at different time points are shown in Figure 5.12. There was a general low methanogen numbers in all reactors at the start (day 2) of the process (compared to bacteria and archaea), as all reactors harboured 10^4 -10^5 *mcr*A gene copies /ml (Fig. 5.12). However, this stage of the process corresponds with the onset of methane production in all reactors (Fig. 4.12 and 4.14). After day 2, while there was general decline in methanogen numbers in most sediment-inoculated reactors (except in blank and *F. serratus* reactors where methanogen number remained stable until day 13); the opposite occurred in all sludge inoculated reactors. The most decline in methanogen number between day 2 and 13 was recorded in sediment-inoculated cellulose reactors (10^5 to 10^3), whereas there was a significant increase (from 10^5 to 10^7) in the sludge-inoculated cellulose reactors (fig. 5.12). This observation is consistent with the results of archaea numbers (Fig. 5.11) (expected as methanogens are archaea) and might be due to slow release of methanogenic substrates such as VFAs (Fig.4.5) by microbial (mainly bacteria) population in sediment inoculum which took longer to become adapted to utilising cellulose.

After day 13, methanogen numbers in the sediment-inoculated blank reactors went below the detection limit (Fig. 5.12a) suggesting a considerable decline in methanogen number

likely due to depletion of available methanogenic substrates. However, between day 13 and 20, methanogen number (*mcrA* gene copies) increased by at least 3 orders of magnitude in all the other sediment-inoculated reactors; with *mcrA* gene ranging from 4.74 x 10^7 copies /ml in cellulose to 1.53 x 10^8 copies/ml in *F. serratus* reactors (Fig. 5.12a).

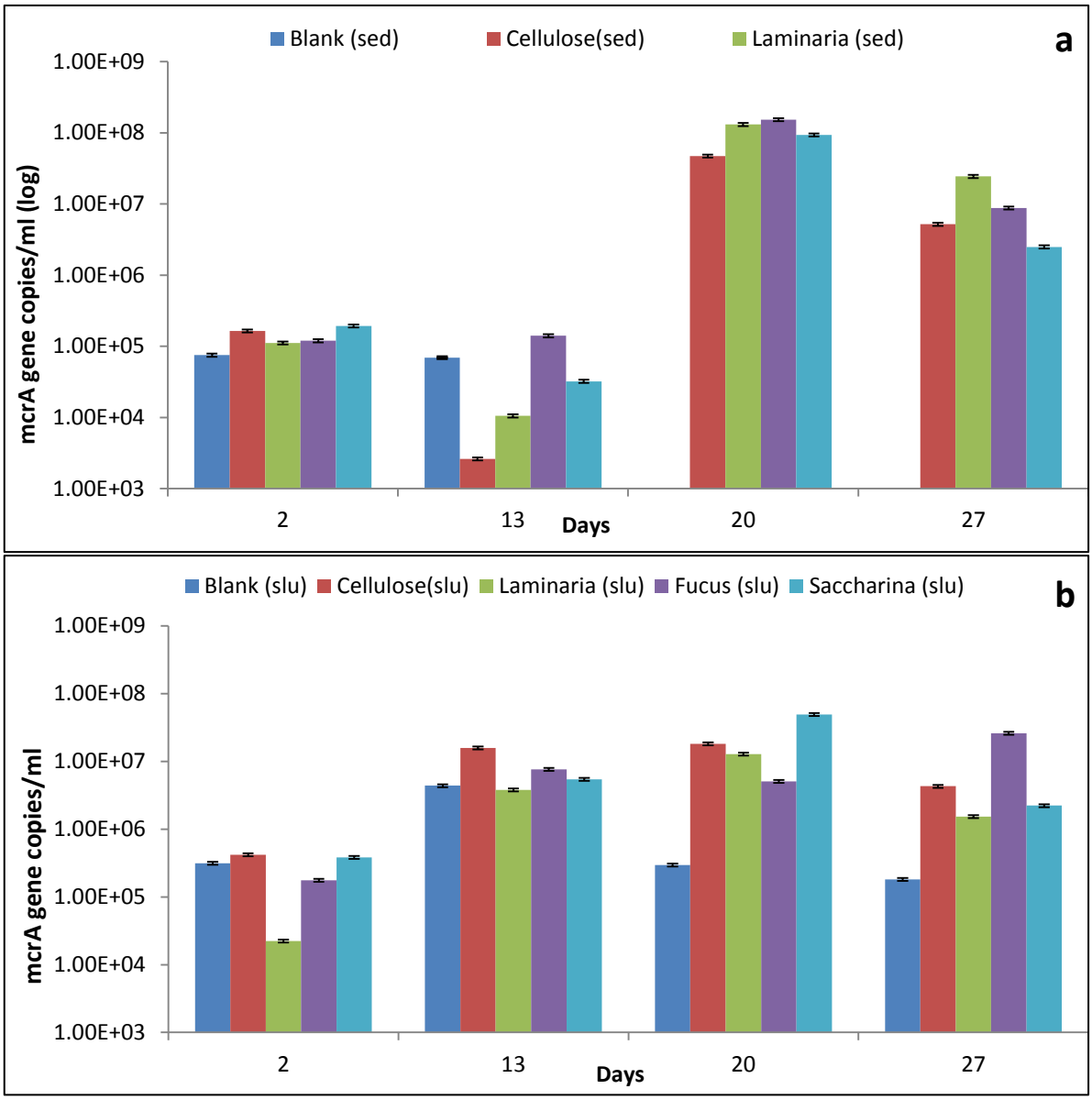


Fig. 5.12. Methanogen (*mcr*A gene copies) number during anaerobic digestion of seaweeds using digested sludge as the source of inoculum. Columns represent mean values while errors bars show standard deviation of the three replicates

Increases in methanogen numbers after day 13 coincides with significant methane production in all sediment inoculated seaweeds reactors (Fig. 4.12) and by day 20, exponential methane production had begun in all three seaweeds reactors, confirming that

the exponential increase (in methane production) is as a result of considerable increase in methanogen numbers (Fig. 5.12a). Although a slight drop in methanogen numbers was recorded at day 27 in all sediment-inoculated reactors, methane production continued due to the high numbers of methanogens still present.

In contrast to the sediment-inoculated reactors, methanogen numbers (*mcrA* gene copies) stayed largely stable between day 13 and 20 in sludge-inoculated reactors (Fig. 5.12b). This period coincided with, a time of suspected active methanogenic activities resulting in exponential methane production in all sludge-inoculated substrates' reactors (Fig. 4.14); the only exception was the blank reactor where methanogen numbers continue to decline after day 13. However, like sediment-inoculated reactors, a slight drop in methanogens number was recorded by day 27 in all sludge-inoculated reactors (except *F. serratus*); although steady methane production continued.

There are currently a limited number of reports on quantification of methanogens at different time points targeting the *mcrA* gene. In some of the available reports on methanogen (*mcrA* gene copies) numbers, results are provided on one or two samples taken from the reactors with no information about the stage of timing of sample collection e.g. (Steinberg & Regan, 2009; Morris et al., 2014). However, Morris et al., (2014) demonstrated that increases in *mcrA* gene copies has a concomitant increase in methane production over time. Another study of methanogens number during active methane production phase in maize-fed methane fermenter conducted by Munk et al., (2010) showed that methanogen numbers peaked at 7×10^7 cells /ml. This is similar to peak methanogens number recorded in sludge-inoculated reactors (Fig. 5.9) but lower than those of sediment-inoculated reactors in the current study (Fig. 5.8). Similarly, Traversi et al., (2012) conducted methanogens population studies of a pilot reactor fed with organic

fraction of municipal solid wastes and wastewater sludge to which two different pre-treatment techniques (turbo mixing and pressure extrusion) were applied. Quantitative PCR methanogen amplification revealed 6.3 x 10^7 and 5.4 x 10^9 *mcr*A genes copies/ml during peak methanogenesis for the two treatments, respectively. The authors concluded that pressure extrusion pre-treatment enhanced methane production and brought about higher methanogen population recorded.

5.3.4.1 Comparative studies of methanogen composition of anoxic sediment and digested sludge inoculated reactors

The first observable difference between the two sources of inoculum (anoxic sediment and digested sludge) was the total *mcr*A gene copies quantified by q-PCR. One-way ANOVA and Tukey's pairwise comparison of triplicate *mcr*A gene copies in both sources of inoculum show that methanogen content of digested sludge was significantly higher than anoxic sediment ($P<0.003$). The reason for this may be as a result of the mesophilic temperature of digested sludge which supports the growth of microorganism better than psychrophilic temperature of the sediment, assuming nutrient levels and anaerobic condition are similar.

Methanogen numbers were similar in all sediment inoculated reactors at the start of the digestion process (day 2) where *mcr*A gene ranges between 7.52 x 10^4 in blank to 1.93 x 10^5 copies /ml in *S. latissima* reactors (Fig. 5.8). But in sludge inoculated reactors, methanogen numbers differ considerably especially in seaweeds reactors at the start (day 2) of the digestion process (Fig. 5.9). This may be due to differential adaptation of methanogens in the digested sludge to the various components of the seaweeds or differences in availability of methanogenic substrates.

While considerable drop in methanogen numbers was recorded in all sediment inoculated reactors between day 2 and 13, the opposite was recorded in all sludge inoculated reactors as methanogen number increased significantly. Methanogenic growth (Fig. 5.12a) and activities (Fig. 4.12) appeared delayed in the sediment-inoculated reactors until after day 13. This observation may be due to time required by methanogens in the sediment (from psychrophilic) to adapt to mesophilic conditions, after which substantial methanogen increases were recorded, suggesting acclimatization (Fig. 5.12a). Peak methanogen numbers was recorded in all sediment-inoculated reactors at day 20 unlike in sludge-inoculated reactors where peak methanogen numbers were recorded at day 13 in some reactors and day 20 in others.

Comparison of peak methanogen numbers between sediment and sludge inoculated cellulose reactors revealed that despite the poor performance of sediment inoculated cellulose reactors, the methanogen population was significantly higher ($P<0.007$) than the numbers recorded in sludge inoculated reactors which produced 8 times more methane (Fig.4.16). The reason for this scenario is unclear, but might be due to delay in methanogen number increase in sediment inoculated rectors resulting in delayed methane production. Sustained high methanogen numbers for most part of the anaerobic digestion in sludge inoculated reactors may be responsible for better performance observed in cellulose reactors, unlike what happened in sediment reactors.

One-way ANOVA and Tukey's post hoc comparison of methanogens number between sediment and sludge inoculated *L. digitata* reactors on day 20 (peak), revealed that methanogen numbers were significantly higher ($P<0.003$) in sediment than sludge inoculated reactors which resulted in more methane production, although the difference in methane production was not statistically significant. This suggests greater efficiency in

methane production per capital in the sludge inoculated reactors. On the other hand, the relative stability of high methanogen numbers in sludge-inoculated reactors may be an important factor in overall methane production than higher peak methanogen numbers, which were not sustained in sediment-inoculated reactors.

Statistical analysis and Tukey's post hoc comparison of peak methanogens number between sediment and sludge inoculated *F. serratus* reactors highlighted that methanogen numbers were significantly higher ($P<0.004$) in sediment inoculated than sludge inoculated reactors. The fact that more methane was produced in the sludge *F. serratus* inoculated reactors suggests that peak methanogen numbers are not as important as sustained high numbers of methanogens over a considerable length of time, which was the situation in sludge inoculated reactors.

Statistical comparison of the peak methanogen numbers between sediment and sludge inoculated *S. latissima* reactors also shows that methanogen numbers in sediment inoculated reactors were significantly higher ($P<0.014$) than those in sludge inoculated reactors, although methane production between the two inoculations were not significantly different. This observation ties in with observations in other reactors showing the importance of stability in methanogen numbers during the process. Alternatively, since methanogenic activity vary across genera, reports suggest that some methanogens are much more active/productive than others; examples are those belonging to the genus *Methanosarcina* (Kendall & Boone, 2006; Ma et al., 2013; Von Klein, et al., 2002). Therefore, the predominant type of methanogen present in each reactor would likely also be a determinant of the productivity or amount of methane produced. Predominant methanogen groups would be identified using cloning and sequencing techniques subsequently (in chapter 7).

Generally, there was a trend of observable similarity between the performances of the two sources of inoculum whether in terms of microbial composition (numbers) or regarding methane production. The most noticeable exception to this is cellulose under both inoculations, where microbial numbers (gene copies) did not translate to productivity, especially in relation to methane production. In effect, even though methanogen numbers (gene copies) reached similar levels with those of the sludge-inoculated cellulose reactors (Fig. 5.12) (in the later stages of the process), methane produced was significantly lower in the sediment-inoculated cellulose reactors (Fig. 4.12) compared to sludge-inoculated cellulose (Fig. 4.14). The reasons for this disconnect between methanogen numbers (*mcrA* gene copies) and methane production is unclear. However, there are indications that the VFAs produced in sediment-inoculated cellulose reactor remained largely unutilized (Fig. 4.5). Accumulation of VFAs, although did not lead to substantial drop in pH (also shown by (Migliore et al., 2012)) (Fig. 4.10), might have inhibited methanogens present in the sediment-inoculated cellulose reactors. A similar scenario was reported by Miura et al., (2014), where accumulation of VFAs (mainly acetate and propionate) in the subculture (inoculated with anoxic sediment) resulted in inhibition and system failure. To unravel cause of the failure, the authors collected samples after systems failure and analysed the microbial composition using nextGen-sequencing approach (16S rRNA). They found that acetoclastic methanogen presence was very low (<2%), which resulted in failure of VFAs conversion (mainly acetate), leading to low methane production. The authors also reported that marine sediment showed low acetoclastic methanogenic activity and propionate oxidation. Research by Jeihanipour, et al., (2011) reported that acetate (or acetic acid) constitutes >70% of VFAs produced during hydrolysis of cellulose under various conditions. In contrast, acetate was reported to constitute ~20% of VFAs produced from seaweeds (*Saccharina japonica*) when inoculated with anoxic sediment. It is therefore

likely that the bulk of the VFAs produced during hydrolysis of cellulose in the current study were acetate (and propionate) for which marine sediment has been reported (Miura et al., 2014) to have low acetoclastic methanogenic activity.

5.3.5 Proportional representation and distribution of microorganisms during anaerobic digestion processes

Productivity of anaerobic digesters during methane production is dependent on a balanced interaction between the diverse microorganisms involved in the process (Williams, et al., 2013). The four-stage process culminating in methane production are so intricately linked that they are interdependent and the success or failure of the previous process determines what happens in the next (Ali Shah, et al., 2014). The first stage of the process termed hydrolysis is carried out by a group of hydrolytic bacteria which degrade polymeric materials by excreting hydrolytic enzymes. In the second step, fermentative bacteria produce volatile fatty acids, alcohols, CO_2 and H_2 from the products of the first step. In the next step called acetogenesis, products of the second step are converted to acetate and H_2 by acetogenic bacteria. A symbiotic relationship exists between members of acetogenic bacteria and those of hydrogenophilic methanogens which depends largely on H_2 concentration, because optimal performance of these organisms occurs at low H_2 partial pressure. During the last stage of the process: methanogenesis, acetate, methylated compounds, CO_2 and H_2 are converted to methane and CO_2 by three groups of methanogens (acetoclastic, hydrogenotrophic or methylotrophic methanogens) (Beckmann et al., 2011; Cardinali-Rezende et al., 2009; Mata-Alvarez, et al., 2000; Traversi et al., 2012; Velmurugan & Ramanujam, 2011; Vergara-Fernández, et al., 2008; Ward, et al., 2008; Weiland, 2010). The above shows that the bulk of the processes leading to methane production is carried out by different groups of bacteria whose activities are closely linked to those of archaea and methanogens at the later stages of the process (Klocke et al., 2008;

Nettmann et al., 2008). By nature, archaea and methanogens are slow growing and very sensitive to changes in environmental conditions but are usually efficient in the utilisation of substrates as a result, a lot of activities can be obtained from relatively low numbers of these organisms (Ali Shah et al., 2014).

5.3.5.1 Proportional representation and distribution of microorganisms during anaerobic digestion of seaweeds inoculated with anoxic sediment

Results of the microorganism's distribution during the 50-day anaerobic digestion of seaweeds inoculated with anoxic sediment showed the dominance of bacteria at every stage of the process in all reactors. At least 96% of the microorganisms present in the reactors at every point are bacteria (Fig 5.13); this highlights the contribution of bacteria to the anaerobic digestion process irrespective of the substrates being digested. The dominance of bacteria in anaerobic digestion of various substrates has been reported in literature (Cook et al., 2010; J. Guo et al., 2015; Hu et al., 2012; J. Kim et al., 2013; Tabatabaei et al., 2010). Reports suggest that the proportion of archaea versus bacteria community in anaerobic digestion processes ranges from 0.1 to 15% depending on the process parameters and the stage of the process (Ruiz-filippi & Pullammanappallil, 2014). By nature, bacteria are fast growing and very robust in their adaptive capacity to changing environmental conditions, the opposite is true for archaea and methanogens. It is therefore not surprising to see the dominance of bacteria in all anaerobic digestion processes especially as the bulk of the processes leading to methane production is carried out by bacteria (J. Williams et al., 2013).

Results of microbes' relative proportion in the sediment inoculated blank reactors shows that over 99% of the microorganisms are bacteria at day 2 of the digestion process, while only about 1% of the archaea present at this stage were methanogens. By day 13 about 99% of the microorganisms were bacteria but the methanogenic components of archaea

had increased to 7.5% and are responsible for the marginal methane produced in the blank reactors. After day 13, methanogen population has gone below the detection limit with archaea accounting for less than 0.5% of the microbes at day 20 and 27 (Fig.5.13).

In sediment inoculated cellulose reactors, bacteria continued to dominate the process, accounting for over 99% of all microorganisms at every stage of the process. At day 2, out of the tiny proportion (<1%) of archaea present, about 4% were methanogens and were responsible for the start of methane production at this point. Methanogen component of the archaea community decreased to about 3% by day 13 and then increased to 35% by day 20, the point at which most of the methane produced in these reactors were produced. Methanogen present in the archaea declined to 10% of the total archaea at day 27, yet methane production continued (Fig.5.13).

Similar to other reactors, sediment inoculated *L. digitata* reactors were dominated by bacteria (>99%) at every stage of the process as archaea and methanogens accounted for less than 1%. At day 2, about 2% of the archaea population were methanogens, which declined slightly at day 13, after which methanogens proportion began to increased and by day 20 the proportion of archaea that are methanogens had increased to 17%. The increase in methanogen proportion between day 13 and 20 coincides with the period of exponential methane production, which was sustained beyond day 27 despite the slight declined in the proportion of methanogens to about 11% of the archaea population (Fig.5.13).

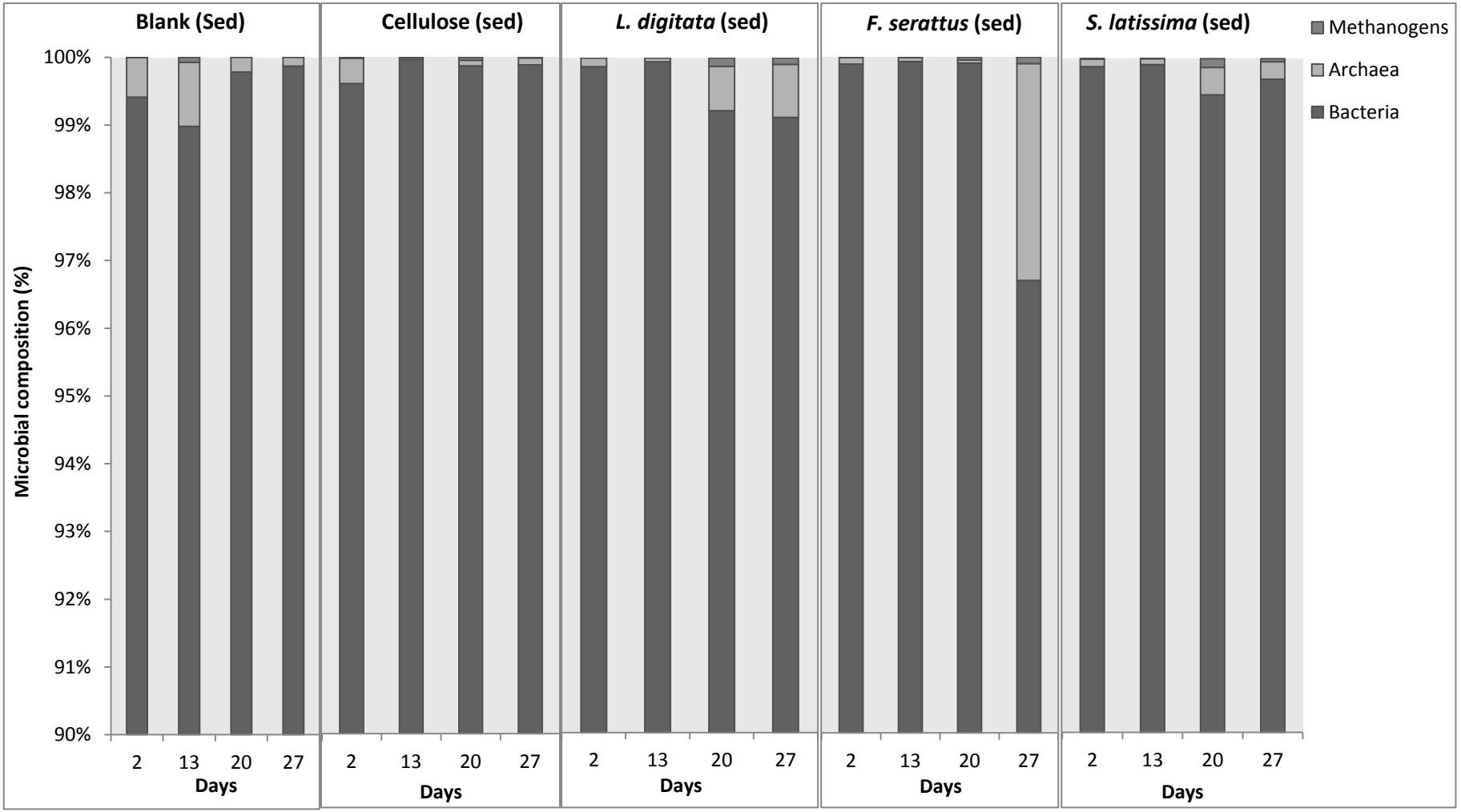


Fig. 5.13. Relative microbial composition (%) of various digesters inoculated with anoxic sediment. Percent contribution of various microorganisms involved in the AD processes is an indication of microbial activity and interactions.

Bacteria also dominated sediment inoculated *F. serattus* at every stage of the digestion process accounting for over 99% of the microorganisms present except at day 27 where bacteria population had declined to about 96.5%. Methanogens constituted only 2% of the archaea population at the start of the process (day 2) and are responsible for the commencement of methane production. Methanogen proportion of the archaea increased from 2% at day 2 to 5% by day 13 despite decline in numbers earlier recorded (Fig. 5.12a). Significant increase was also recorded in methanogens proportion between day 13 and 20 resulting in 47% of the archaea population recorded at day 20 being methanogens. That increase in methanogens ties in with the period of exponential increase in methane production. Between day 20 and 27, a gradual decline in percent methanogen was recorded resulting in only 5% of the archaea population at day 27, although methane production continued as a result of relative high methanogens numbers still present (Fig.5.13).

Similarly, bacteria also dominated sediment inoculated *S. latissima* reactors accounting for over 99% at every stage of the anaerobic digestion process. At day 2 of the process, bacteria constituted about 99.8% of the total microorganisms present, with archaea making up the balance. Within the archaea population, methanogens accounted for only 3% at day 2, which declined to about 1% at day 13. After day 13, the proportion of methanogens in the archaea population increased gradually reaching 25% at day 20, a time that coincided with the period of exponential methane production in the sediment inoculated *S. latissima* reactors (Fig.5.10).

5.2.1 Proportional representation and distribution of microorganisms during anaerobic digestion of seaweeds inoculated with digested sludge.

Similar to the observation in sediment inoculated reactors, bacteria dominated the microbial community in all sludge inoculated reactors accounting for at least 98% at every point during the digestion process. Since bacteria are responsible for three out of the four processes involved in methane production during anaerobic digestion, it is not surprising that they dominate every stage of the process. Additionally, the physiology, nutritional needs, growth kinetics and robustness of bacteria to environmental conditions puts them in better position to be able to dominate anaerobic digesters (Y. Chen et al., 2008). However, despite the dominance of bacteria in anaerobic digesters, a good balance between bacteria and archaea (methanogens) community is essential for optimum system performance, although this is influenced by operational and environmental conditions (Demirel & Scherer, 2008). Proportion of methanogen to bacteria in a continuous stirred tank reactor has been reported to be between 0.01 and 3% of the reactor's microbial community (Demirel & Yenigün, 2006), which is very similar to the observations in the current study.

In sludge inoculated blank reactors, the dominance of bacteria is evident from the start of the digestion process accounting for 99.3% at day 2 while archaea made up the balance. However, only 2% of the archaea community are methanogen at day 2 of the digestion process. By day 13, bacteria proportion had declined slightly below 99% while archaea formed the remaining 1.3%, even as methanogen component of archaea increased to 4% (Fig. 5.14). The proportion of bacteria increased slightly to 99% by day 20 as the proportion of archaea declined slightly; however by this time, methanogen component of the archaea community had increased to 8%.

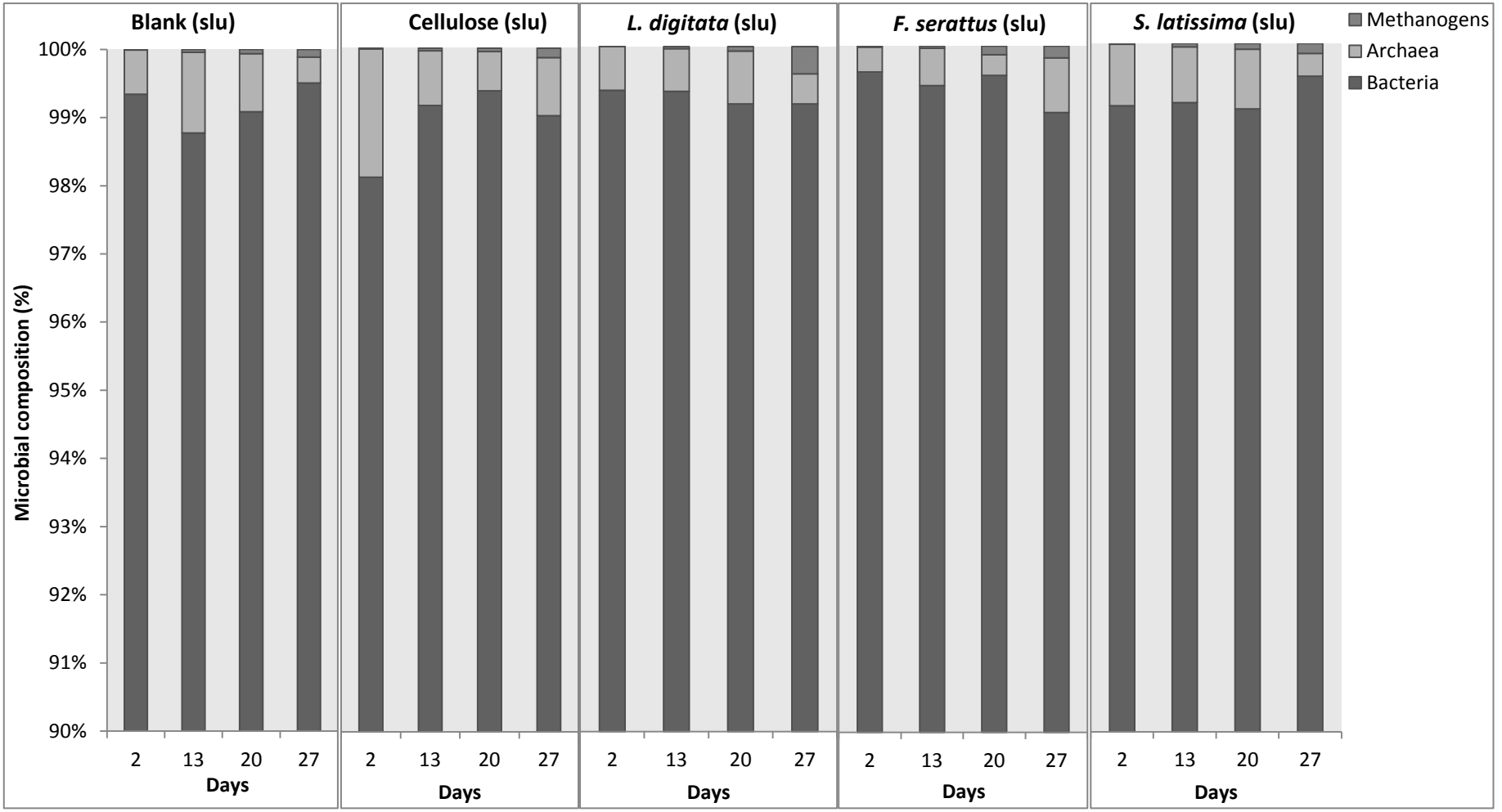


Fig. 5.14. Relative microbial composition (%) of various digesters inoculated with digested sludge. Percent contribution of various microorganisms involved in the AD processes is an indication of microbial activity and interactions.

By day 27, even though archaea component of the reactor's microbial community had declined slightly, methanogens proportion of archaea increased to 23%, which coincided with the peak of the marginal methane formation.

The dominance of bacteria was evident in sludge inoculated cellulose reactors making up to 98% of the microbial component of the reactors at the start of the process (day 2) even as archaea accounted for only 2%. Only about 1.5% of archaea community belong to methanogen at day 2, although some methanogenesis had begun at this time. Despite the decline in total archaeal number in relation to bacteria after day 2, methanogen component increased significantly to 5% at day 13. Increases in the proportion of methanogen component of archaea continued from 5% at day 13 to 8% at day 20 with a further increase to 14% by day 27 (Fig. 5.14). These increases correspond to exponential methane production in the reactors (Fig. 4.14).

Sludge inoculated *L. digitata* reactors were also dominated by bacteria during the digestion process making up over 99% at every stage. At day 2, archaea (including methanogens) accounted for less than 1% of the microbial community, even as methanogen made up only 1% of the archaea component. That 1% methanogen component of the archaea began the process of methane production recorded at day 2, an indication of high activities of methanogens even at low concentrations. Methanogen composition continued to increase as substrates became available and by day 13, the proportion of methanogens had increased to 5% resulting in the start of exponential methane production (fig.4.14). By day 20, methanogen component of archaea community had increased to 8%, which continued to increase until it reached 47% at day (Fig. 5.14) by the time substantial methane was being produced.

As in all reactors, bacteria dominated proceedings in sludge inoculated *F. serattus* reactors accounting for at least 99% throughout the digestion process. At the start of the digestion (day 2), archaea accounted for only 0.5% of the total microbial community in the reactors, out of which 5% were methanogens. Between day 2 and 13, the proportion of archaea increased slightly even as methanogen fraction increased to7%, and then to about 30% at day 20 before a slight decline after day 27 (Fig. 5.14). The period between day 13 and 27 coincided with significant increases in methane production (Fig. 4.14).

Microbial community analysis of sludge inoculated *S. latissima* reactors also revealed the dominance of bacteria (>99%) at every stage of the digestion process. At the start of the process, archaea made up roughly 1% of the total microbial population, which was stable till after day 20. However, methanogen component of the archaea community increased from 2 % at the start of the process (day 2) to 6 % by day 13, the time when the first 250 ml methane was produced (Fig. 4.14). Between day 20 and 27, methanogen component of the archaea community had increased from 9% (day 20) to 31 % (day 27), a period of sustained exponential methane production which continued from most of the 50 day process.

These results show considerable variability in microbial composition at different stages of the digestion process. The most stable group of microbes are the bacteria which are abundant at every stage of the process but gives way to some methanogen growth in other to achieve the intricate balance in microbial community structure needed to maintain process stability. Results above are in agreement with those obtained in other anaerobic digesters where bacteria population dominated every stage of the process even as the proportions of archaea (and methanogens) accounted for 0.01% to 16 % of the total microbial community (J. Williams et al., 2013). A study by Demirel & Yenigün, (2006),

to examine the behaviour of microbial community of anaerobic digester treating dairy wastewater in terms of total bacteria and methanogens using epifluorescence microscopy reported that autofluorescent methanogens accounted for 5-16% of the total microbial community, although not all methanogens exhibit autofluorescence. The proportion of methanogen in that study is higher than what was obtainable in the current study, although reactor type, study techniques and other operational parameters which are determinants of microbial community structure differ (Ma et al., 2013). A comprehensive dissection of microbial community structure in a full scale anaerobic reactor digesting activated sludge from wastewater, revealed the dominance of bacteria which accounted for 93% while archaea (including methanogens) made up 5.6% of the microbial community. In that study, clostridium accounted for the bulk of bacteria while *Methanosaeta* was the major archaea group recovered (J. Guo et al., 2015).

5.4 Conclusion

The current quest towards sustainable bioenergy, one without competition with food crops and agricultural lands has led to the consideration of various seaweeds materials as suitable substrates. Research has shown that biofuels production via anaerobic digestion is the most efficient way of marine biomass utilisation for bioenergy production. Many researchers have considered process optimization as a means of enhancing the productivity of the process leading to methane production such as pre-treatment of substrates, co-digestion and process separation. However, the biological nature of the process necessitates deeper and better understanding of the microbial ecology and community structure in the systems. This will among others provide information about microbial interactions and process kinetics, which are important for process monitoring and maintenance.

This chapter has given insights into numerical distributions of the 2 (3) main groups of microorganisms involved in anaerobic digestion processes. It has shown a correlation between organisms number as in indication of process productivity. To achieve process stability and productivity, the intricate balance between organisms at different stages of the process has been demonstrated.

Although bacteria appeared very dominant during anaerobic digestion processes, the contribution of the methanogenic archaea cannot be over-emphasized, as no methane is produced without them. Methanogens' efficiency was highlighted in this study, indicated by large quantities of methane produced by relatively low numbers of methanogens.

Although the initial distribution of microorganisms changes over time depending on operational conditions, the current study has shown that variations in the proportion of microorganisms present in the reactors were geared towards the attainment of the intricate

balance required to achieve and maintain optimum system performance at various stages of the process- irrespective of the source of inoculums.

The current study has also demonstrated that time (stage of the process) and substrate types are the major factors that determine organism numbers, distribution and activities during anaerobic digestion and that the source of inoculum employed played a minor role.

Now that organisms' numbers and proportional variation have been demonstrated, the next chapter of this study will look at the distribution and diversity of the various microbial components of the anaerobic reactors at different time points using denaturing gradient gel electrophoresis (DGGE) technique. Impacts of the source of inoculum on diversity will be assessed.

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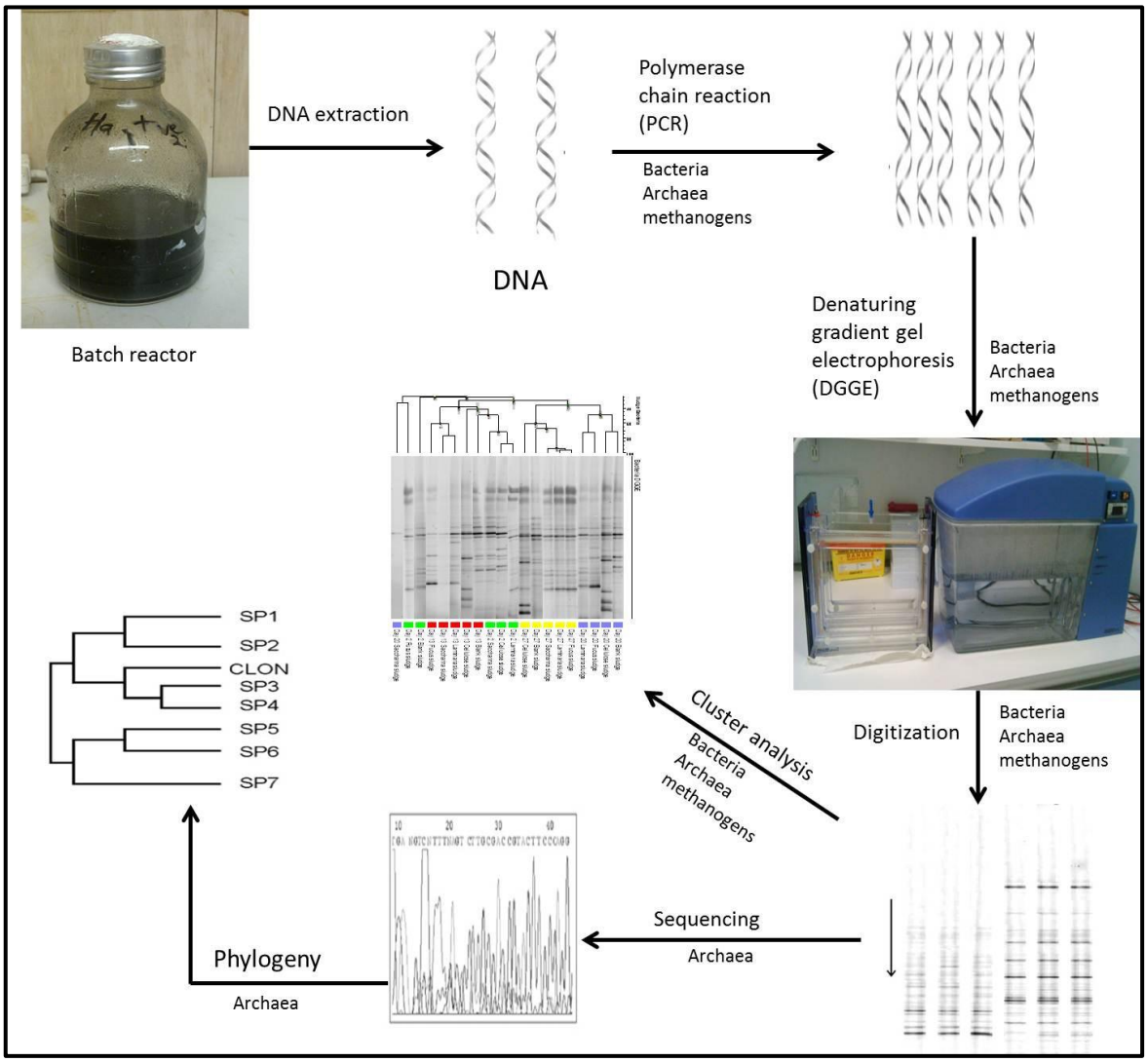
6. Chapter 6

"*In the attempt to make scientific discoveries, every problem is an opportunity – and the more difficult the problem, the greater will be the importance of its solution*"

- E.O Wilson

6 Denaturing gradient gel electrophoresis (DGGE) fingerprinting approaches to study the microbial diversity and composition of sediment and sludge inoculated seaweeds reactors.

In this chapter, composition and diversity of the microbial ecology of microorganisms involved in the anaerobic digestion process were monitored over time using a fingerprint technique; denaturing gradient gel electrophoresis (DGGE). This distribution will be viewed as an indication of growth and responses of organisms to changing conditions during the process.



Schematic representation of the process and techniques used in this chapter

6.1 Introduction

The desire to gain an in-depth understanding of the microbial ecology of anaerobic digesters necessitates the need for a multi-dimensional approach involving the use of various molecular techniques to obtain information on various parameters involved. This multi-dimensional approach not only provides additional information, and validates the results generated via the other approaches (Muyzer, 1999). While q-PCR (chapter 5) may provide important numerical data of the various microbes present in the reactors and information on the types of processes occurring over time, it does not provide sufficient insights into the diversity of the different groups of microorganisms involved. To be able to determine the genetic diversity of the complex microbial populations in the anaerobic reactors, a fingerprinting technique, that allows processing of many samples simultaneously, is vital (Díez, et al., 2001). Denaturing gradient gel electrophoresis (DGGE), offers the possibility of rapidly observing microbial community diversity over time based on DNA separation due to differences in sequence (Valášková & Baldrian, 2009. It is therefore useful for the monitoring of dynamic changes in microbial communities due to changing environmental conditions (Sanz & Köchling, 2007). In theory, each DGGE band represents a single operational taxonomic unit (OTU), a species or a group of very closely related organisms. Therefore DGGE can be applied to analyse organism's communities in complex environments to obtain species diversity and relatedness (Cho et al., 2013; Demirel & Scherer, 2008; Hwang, et al., 2010; Keyser et al., 2006; Kim, et al., 2013; Munk, et al., 2010). DGGE has the advantage of allowing selected bands to be sequenced to provide additional information about the specific phylogenetic composition of the microbial community. However, sequences used in DGGE are usually short (less than one-third of the total length of small subunit rRNA) and of variable quality.

The shorter the sequence derived from DGGE, the less refined the phylogenetic inference (Díez, et al., 2001).

DGGE has been used in a number of microbial ecology applications to study diversity in a range of environments including urban river (Araya, et al., 2003), agricultural soils (Garbeva, et al., 2003), animal guts (Liu, et al., 2012; Regensbogenowa et al., 2004), paddy field soils (Watanabe et al., 2004), deep-sea sediment (Fry, et al., 2006) and food (Ercolini, 2004). Under anaerobic conditions, DGGE has been employed to determine microbial species richness and diversity in wastewater treatment plants (Boon, et al., 2002), abandoned coal mines (Beckmann et al., 2011), UASB anaerobic reactors (Keyser, et al., 2006) and overloaded anaerobic digesters (Tale, et al., 2011). The above research was carried out in order to relate microbial community composition and diversity to process functions and performances.

In the current study, the biomethane production potentials of common seaweeds in west coast of Scotland were assessed, when inoculated with different types of inoculums during a 50-day anaerobic digestion process. Results obtained from volatile acids production as well as cumulative methane productions (chapter 4) have shown that there were no significant differences in the process productivity between the two types of inoculums. However, differences exist between reactors both within (over time) and across inoculation (based on source of inoculum) during the course of the digestion process. Results of quantitative PCR (chapter 5) also showed that organism numbers vary significantly with time depending on the source of inoculation as well as substrate types.

This chapter presents results obtained using DGGE techniques on samples obtained from the anaerobic digestion from seaweed process study outlined in chapter 4, employed to enhance our understanding of the structural diversity of microbial communities over time

during the digestion process. This is the first time DGGE is applied to study microbial diversity and community richness of seaweeds anaerobic reactors to the best of our knowledge. However, as in many microbial mediated processes changes in microbial community structure do not occur concurrently with experimental parameters such as VFAs or methane production, as such it is somewhat difficult to directly match community structure profile with other process functions (Malin & Illmer, 2008). This necessitates the use of multiple molecular techniques to obtain more rounded information as a basis for monitoring process parameters, (Tabatabaei et al., 2010).

6.2 Methods

Replicate samples were collected from the sediment and sludge inoculated reactors on days 2, 13, 20 and 27 and targeted for comparison of different microbial groups using DGGE. These were bacteria (targeting the 16S rRNA gene), archaea (targeting the 16S rRNA gene) and methanogens (targeting the *mcr*A gene). The experimental procedure used depended on the targeted group. Reproducibility of replicates were checked by running individual replicate on a DGGE gel (Fig 6.1), before replicate samples were pooled to capture all possible groups of microorganisms. Primers specific to each group were employed. At the 5' end of each forward primer, an additional 40-nucleotide GC-rich sequence (GC-clamp) was added to achieve a stable melting point for the DNA fragments in the DGGE according to Muyzer, et al., (1993). DGGE analysis of the bacteria community was also carried out on the inoculum sources prior to the digestion process.

DGGE was carried out using an INGENYphorU-2 system (Ingeny, Netherlands). PCR products and loading buffer (40% [wt/vol] sucrose, 60% [wt/vol] 1 x Tris-acetate-EDTA [TAE], and bromphenol blue) were mixed in a 1:1 ratio. The mixture of PCR amplicons and loading buffer were applied directly to 10% (wt/vol) polyacrylamide gels with a linear

gradient of 40 to 60% denaturant for bacteria and archaeal PCR products (<200bp) and 8% (wt/vol) polyacrylamide gels 40 to 80% denaturant for methanogens PCR products (~500bp) (100% denaturant corresponds to 7M urea and 40% [vol/vol] formamide). Electrophoresis was carried out in 1x TAE buffer (40 mM Tris-acetate [pH 7.4], 20 mM sodium acetate, 1 mM sodium EDTA) at a constant voltage of 100 V and at 60°C for 19 h. After electrophoresis, gels were stained for 30min in 1 x SYBR Gold solution (Molecular Probes, Eugene, OR) in 1xTAE and washed with distilled water. The gel was digitized using a digital imaging system (Alpha Innotech Alphaimager) with UV transillumination (Beckmann et al., 2011).

Gel analysis was carried out with the software GelCompare II version 6.6 (Applied Maths, Belgium). Comparison was performed using a similarity coefficient Dice with 0.5% optimisation band matching tolerance of 0.5%. Uncertain bands were ignored. DGGE gels were compared through Cluster analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The evolutionary history of selected archaea bands were inferred using the Neighbour-Joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) was shown next to the branches (Felsenstein 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method, (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved five nucleotide sequences. Codon positions included were 1st+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary phylogenetic analysis was conducted in MEGA6 (Tamura et al., 2013).

6.2.1 DGGE procedure for the analysis of bacteria community structure in seaweeds anaerobic reactors

Polymerase chain reaction (PCR) was carried out to obtain a small DNA fragment (<200bp) suitable for DGGE using the bacterial specific primer pair primer 2/3 (Muyzer, et al., 1993). The PCR program for bacterial DNA included an initial denaturation step for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. Primer extension was carried out for 5 min at 72°C. Aliquots (5 µl) of the PCR products were analysed by agarose gel electrophoresis in 1.5% (wt/vol) agarose gels and ethidium bromide (0.8 ng/ml) staining for 20 min before viewing on a UV transilluminator as previously described (chapter 2).

6.2.2 DGGE procedure for the analysis of archaea community structure in seaweeds anaerobic digesters

PCR amplification of general archaea requires nested PCR in other to obtain specific product for DGGE analysis. The first round of PCR amplification was carried out to obtain a larger size fragment using archaea specific primer pair PRA46/1017 (Øvreås, et al., 1997), which is specific for archaea. The second round was performed using the primer pair 344fgc/Parch519r (Banning et al., 2005) giving a product internal to the first round product. Additional 1 µl of 15mM $MgCl_2$ was added to reaction mix to (improve *Taq* efficiency) enhance sediment samples' archaeal amplification (Schmidt, et al., 2014). The PCR program for archaea DNA included an initial denaturation step for 5 min at 94°C, followed by 30 cycles of 30 s at 95°C, 30 s at 40°C (55^{o}C for second round), and 1 min at 72°C, primer extension was carried out for 5 min at 72°C (Øvreås et al., 1997).

6.2.3 DGGE procedure for the analysis of methanogens community structure in seaweeds anaerobic digesters

To determine methanogen community diversity in the batch reactor using DGGE, the α-subunit of the methyl coenzyme M reductase (*mcr*A) gene which is conserved and exclusive to all methanogen (except methane oxidizing archaea) was targeted in a PCR using methanogen specific primer pair mlasgc/m-rev following the procedure described by (Steinberg & Regan, 2009). The PCR program for methanogen DNA amplification included an initial denaturation step at 95 °C for 3mins, followed by 5 cycles at 95°C for 30secs; 48°C for 45secs and 72°C for 30secs. This was followed by 30 cycles at 95°C for 30secs, annealing at 55°C for 45secs, extension at 72°C for 30secs and final extension at 72°C for 10mins (Steinberg & Regan, 2008).

All PCR runs were carried out in a total 25µl volume containing 0.5µl of forward primer, 0.5 µl of reverse primer (10ρmoles/ µl), 0.1 µl of MyTaq polymerase (5u/ µl). Other components of the mix are 5µl of PCR Buffer (comprising 5mM dNTPs, 15mM $MgCl_2$, stabilizers and enhancers), 18.4 µl of molecular grade water (17.4 µl for sediment archaeal and methanogen amplification) and 0.5 µl of DNA extract (1:10, 1:100 dilutions). Additional 1 µl of 15mM $MgCl_2$ was added to reaction mix to (improve *Taq* efficiency) enhance sediment samples' archaeal and methanogen amplification (Schmidt, et al., 2014). Negative controls containing 0.5µl of sterile molecular grade water were included in all cases. Different dilutions were tested in chapter 3 while 1:10, 1:100 dilutions were subsequently used for archaea (and methanogen) and bacteria respectively. All primers were obtained from Integrated DNA Technologies, (Belgium) while other reagents were obtained from Bioline Reagents Ltd, (London, UK).

Methodology employed in this chapter is fully described in chapter 2.

6.3 Results and discussion

Previous results obtained from volatile acids production as well as cumulative methane productions (in chapter 4) showed that there were no significant overall differences in the process productivity between the two types of inoculums. However, differences exist between reactors both within (over time) and across inoculation (based on inoculum source) during the course of the digestion process. Results of quantitative PCR (in chapter) also showed that organism numbers vary significantly with time, depending on the source of inoculation as well as substrate types.

In this chapter, DGGE technique is employed to monitor changes in microbial communities in different reactors over time in relation to the source of inoculums. Results obtained are related to other general spatial and temporal process performances and functions.

6.3.1 DGGE analysis of bacteria community structure in the two sources of inoculum prior to anaerobic digestion

Results of bacteria community composition and diversity present in the inoculums indicated that both sources of inoculum harbour diverse groups of bacteria, although the bacterial community in the sludge inoculum appeared more diverse. Multi-dimensional scaling (MDS) plot and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analyses performed on the DGGE gels clearly showed that different groups of bacteria were present in the two sources prior to the digestion process (Fig. 6.1). This result is an indication of the ability of different ecosystem to select for microbes that thrive in various environments based on environmental conditions and substrate availability (Guo et al., 2012; Guo et al., 2015; Liu, et al., 2012).

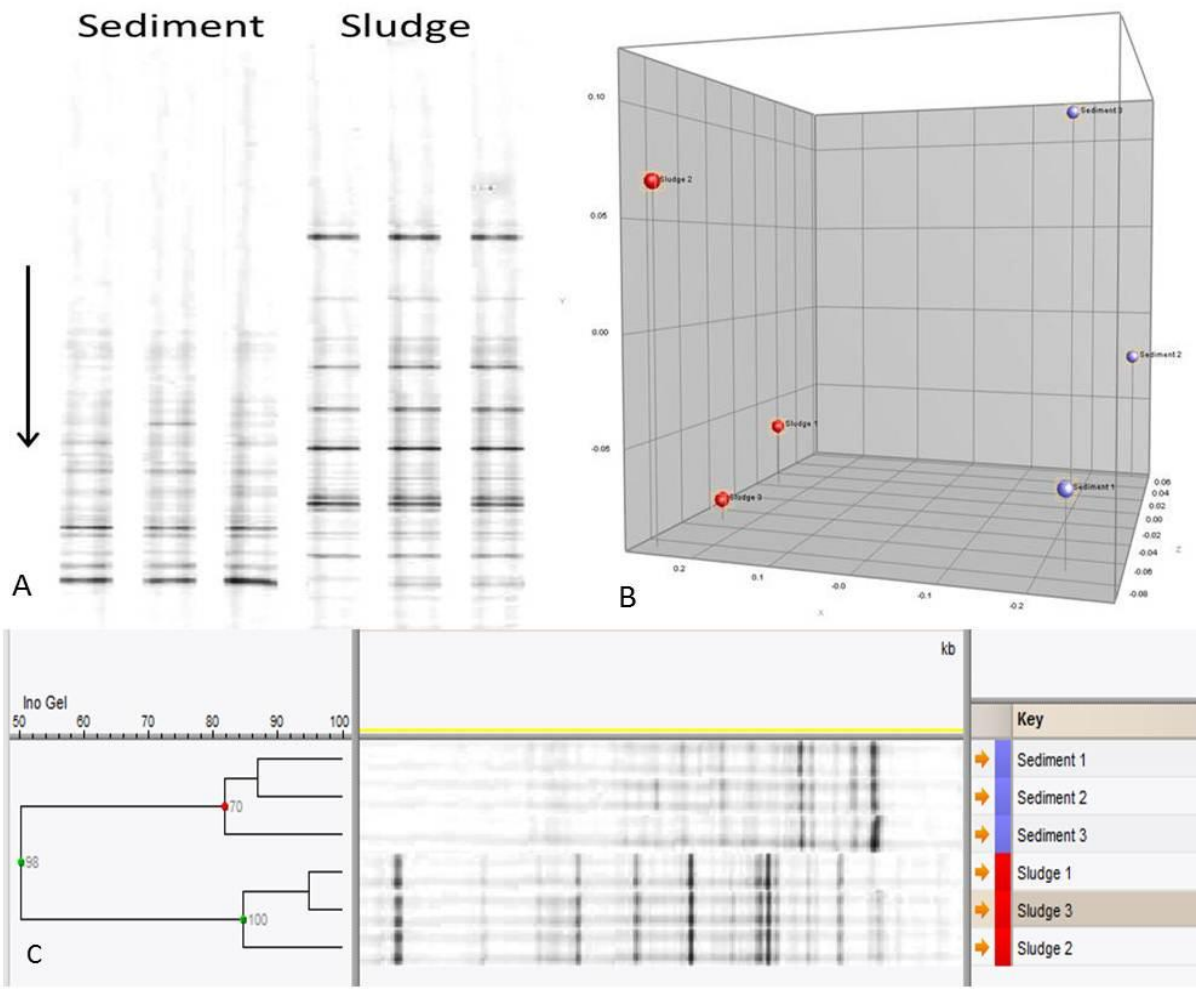


Fig. 6.1. Inoculums bacterial composition analysis using (A) denaturing gradient gel electrophoresis (DGGE), (B) multi-dimetional scaling (MDS) plot of sediment (blue) and sludge (red) bacteria and (C) Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis. DGGE was run in a 10% acrylamide with 30-60% denaturant gradient at 100V and 60^{o}C for 19 hours. Analysis was performed on triplicate samples.

6.3.1.1 DGGE analysis of bacteria community structure in seaweeds anaerobic reactors inoculated with anoxic sediments

The result of DGGE analysis of sediment inoculum and the sediment inoculated reactors suggests that the introduction of substrates into the reactors altered the bacterial population slightly at the start (day 2), with greater fluctuations observed later in the process (Fig 6.1 and 6.2)

Analysis of the bacteria DGGE pattern of sediment-inoculated batch reactors indicates broad similarities in all reactors at the start of the digestion process (day 2) (Fig 6.2). The result shows that the bacteria communities at the start were fairly diverse with at least five distinct bands (OTUs) in each of the reactors at the start of the hydrolysis when polymeric materials are broken down. The initial similarities observed might be due to similar process taking place in the reactors at the start of the anaerobic digestion process. Bacteria community present at this stage might also be those carried over from the sediment inoculum.

However, a form of succession was observed between day 2 and 13 across the reactors resulting in a shift in bacterial community from GC-rich to a less GC-rich sequence types (Fig 6.2) as indicated by the position of DGGE bands on the gel. AT rich sequences were at the upper part of the gel, while GC rich moved further to the bottom of the gel at day 13. Most of the bacteria community present at day 13 are different from the start (day 2) of the process. The reason for this shift might be as a result of changes in the predominant process from hydrolysis to acidogenesis and acetogenesis. It might also be due to a succession process where bacteria community from psychrophilic sediment inoculum gives way to a more mature community adapted to mesophilic reactor conditions. Apart from a shift in the bacterial community between day 2 and day 13, there was also an increase in bacterial composition (band intensity) and richness (number of bands) in most reactors. Low initial microbial community diversity in anaerobic rectors has been previously reported (Malin & Illmer, 2008). However, variation in microbial community over time, reflecting greater diversity in microbial community at the middle of the process has also been reported (O'Reilly et al., 2010).

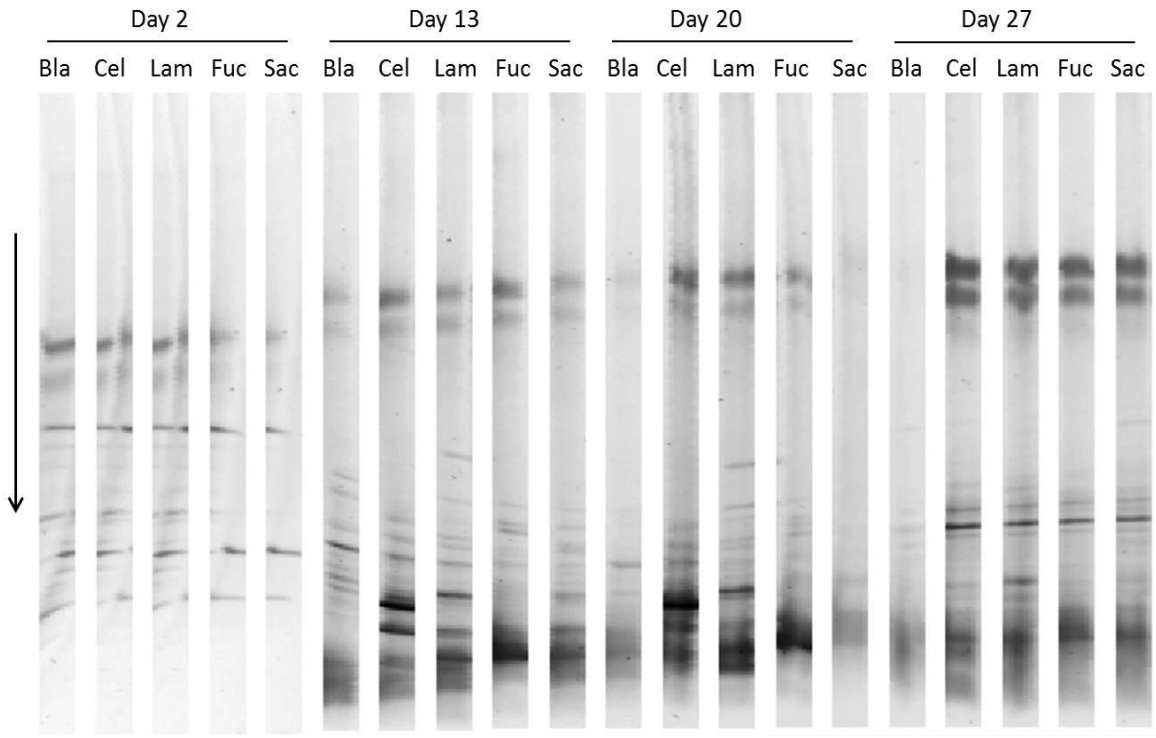


Fig. 6.2. Bacteria DGGE profile of anoxic sediment inoculated reactors during anaerobic digestion on seaweeds. DGGE was run in a 10% acrylamide with 40-60% denaturant gradient at 100V and 60^{o}C for 19 hours. Bla: blank, Cel: cellulose, Lam: *L. digitata*, Fuc: *F. serattus*, Sac: *S. latissima*. Arrow indicates direction of increasing denaturant and acrylamide gradient from lower to higher concentration.

Across reactors, bacteria community structure varied considerably as different groups of bacteria adapt differentially to different substrates and changing conditions in the reactors. By day 13, the least diversity was observed in *F. serattus* while *L. digitata* reactors harboured the most diverse bacteria community. The high bacteria diversity in *L. digitata* reactors at the peak of bacteria activities between day 13-20 (hydrolysis, acidogenesis and acetogenesis) might contribute significantly to its overall performance of all the sediment-inoculated reactors. However, research by Malin & Illmer, (2008), suggests that the presence of multiple bands on a DGGE gel is not a direct indication of microbial activity.

After the initial diversity and band intensity observed in blank reactors, bacteria diversity diminished after day 13, an indication of reduced activity occasioned by depletion of available biomass in the sediment inoculum. High bacteria diversity was observed in

cellulose reactors between day 13 and 20, which was also reflected on bacteria numbers quantified by q-PCR in the previous chapter. Similar diversity and intensity patterns were observed across reactors between days 13 and 20, except in blank and *S. latissima* reactors. A slight shift in community structure was observed in all reactors at day 27, suggesting the dominance of different groups of bacteria at this stage of the process. Although bacterial community was diverse toward the end of the process (day 27), there were similarities across reactors indicating similarity in functions and maturity of the community succession (Fig. 6.2).

Cluster analysis of the sediment inoculated reactors shows a clear disticntion between highly intensive and semi-intensive bands in relation to time and stage of the digestion process. Although fairly diverse, all day 2, day 27 blank and day 20 *S. lattisima* reactors were clustered due to reduced microbial community diversity at the stage of the process revealed by faintly but distinct band intensities (Fig. 6.3). Increased dominance of specific groups of bacteria resulted in the clustering of all reactors at day 13, suggesting similar activities (e.g. acidogenesis) occuring in the reactors at the time. A shift in bacteria community structure after day 13 resulted in the clustering at day 20 in most of the reactors likely based on different dominant bacteria community composition.

Generally, the results of the bacteria community structure and variations suggest that different bacteria groups dominate different stages of anaerobic digestion process. This scenario is likely driven by availability of suitable substrates to the respective groups of dominant bacteria population during the process.

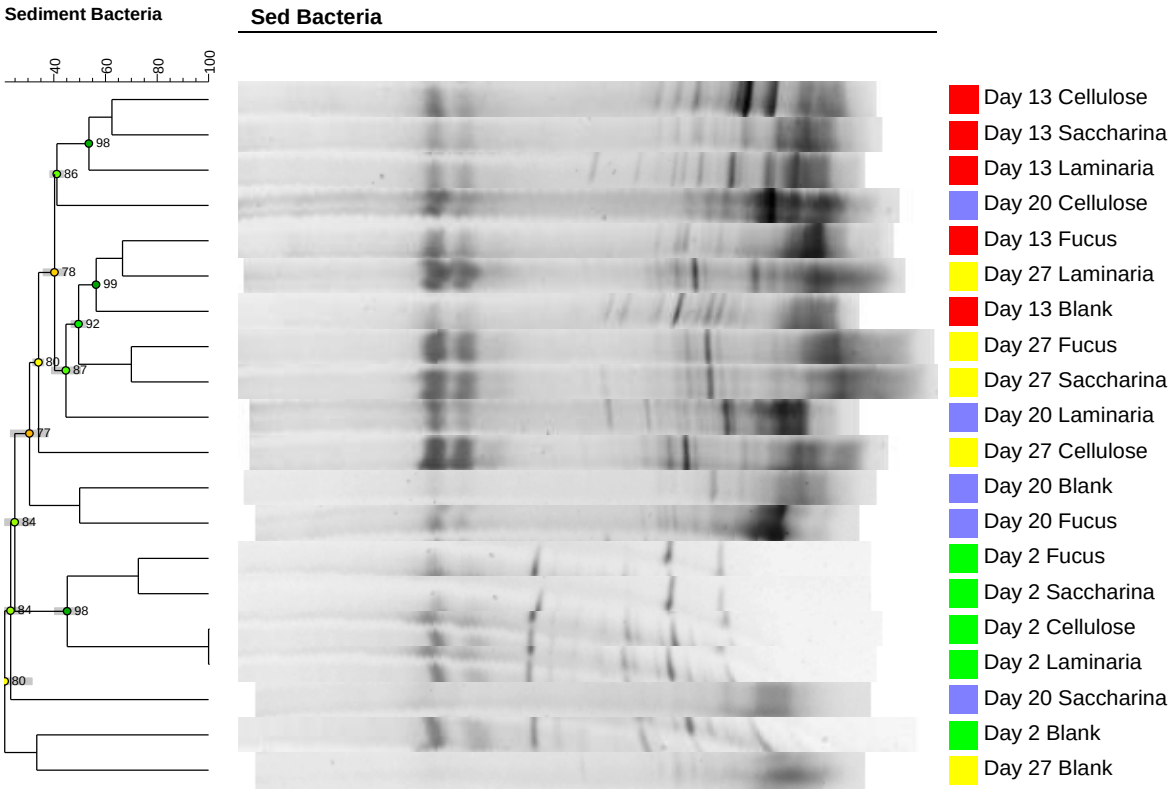


Fig. 6.3. Clustrering analysis of DGGE pattern of bacterial population structure in anoxic sediment inoculated reactors at different time points during anaerobic digestion of different substrates using gel analysis software GelCompare® II. Cluster analysis was performed using the unweighted pairwise grouping method with mathematical averages (UPGMA)

6.3.1.2 DGGE analysis of bacteria community structure in seaweeds anaerobic reactors inoculated with digested sludge

DGGE analysis was carried out to reveal the diversity and distribution of the bacteria community present in the sludge and sludge-inoculated reactors over time. Results obtained showed that the introduction of substrates and saltwater altered the bacteria community in the reactors, resulting in different community structure at day 2 of the process (Fig. 6.1 and 6.4). The start of the process (day 2), shows a general similarity in bacteria distribution and diversity between blank and cellulose reactors as well as amongst seaweeds reactors. There are a few visible bands in the seaweeds reactors at day 2 which are not visible in the blank and cellulose reactors, especially at the upper part of the gel (Fig. 6.4). Higher bacteria diversity in the seaweeds reactors at this stage of the process

suggests that the seaweeds biomass might harbour some inherent bacteria community in its biomass carried on into the reactors, although this difference was not observed when anoxic sediment was the source of inoculum. It might also be suggestive of increased bacterial growth (band brightness) resulting from readily degradable components of the seaweeds.

However, after the initial diversity observed across reactors at the start of the digestion process, by day 13, only few of the reactors retained their bacteria diversity. For instance, much of the diversity observed in the blank reactor at the start of the process had disappeared by day 13, with only a single distinct band observed. The situation was similar in *S. latissima* reactors where a couple of bands are visible at day 13. In contrast, bacteria diversity increased in cellulose reactors after day 2 with at least 13 visible bands at day 13. This was followed by *L. digitata* reactors, with at least 11 visible bands at day 13 and *F. serattus* with about 9 visible bands (Fig. 6.4).

By day 20, a general increase in bacteria community richness was observed in all reactors, with cellulose reactors harbouring the most diverse bacteria community with about 14 visible bands. The loss of diversity observed in blank, *S. latissima* and *F. serattus* was mostly restored by day 20 with the emergence of previously non-existing bands in most of the reactors. This is an indication of slight change in the process and types of bacteria present at the stage of anaerobic digestion process. From day 13, cellulose reactors harboured the most diverse groups of bacteria throughout the process. For instance, there were at least three visible bands (towards the bottom of the gel) from day 13 until day 27, which occurred only in the cellulose reactors. Although research has shown that there is little correlation between microbial diversity and activity (Malin & Illmer, 2008), these additional bacterial communities (bands) might have contributed to the VFAs formation and other process functions, resulting in the highest methane production from these

reactors (recorded in chapter 4). The bacteria diversity and distribution observed between day 20 and 27 (Fig. 6.4) remained largely stable, marking the peak of bacteria activities resulting in considerable VFAs formation and its concomitant conversion to methane.

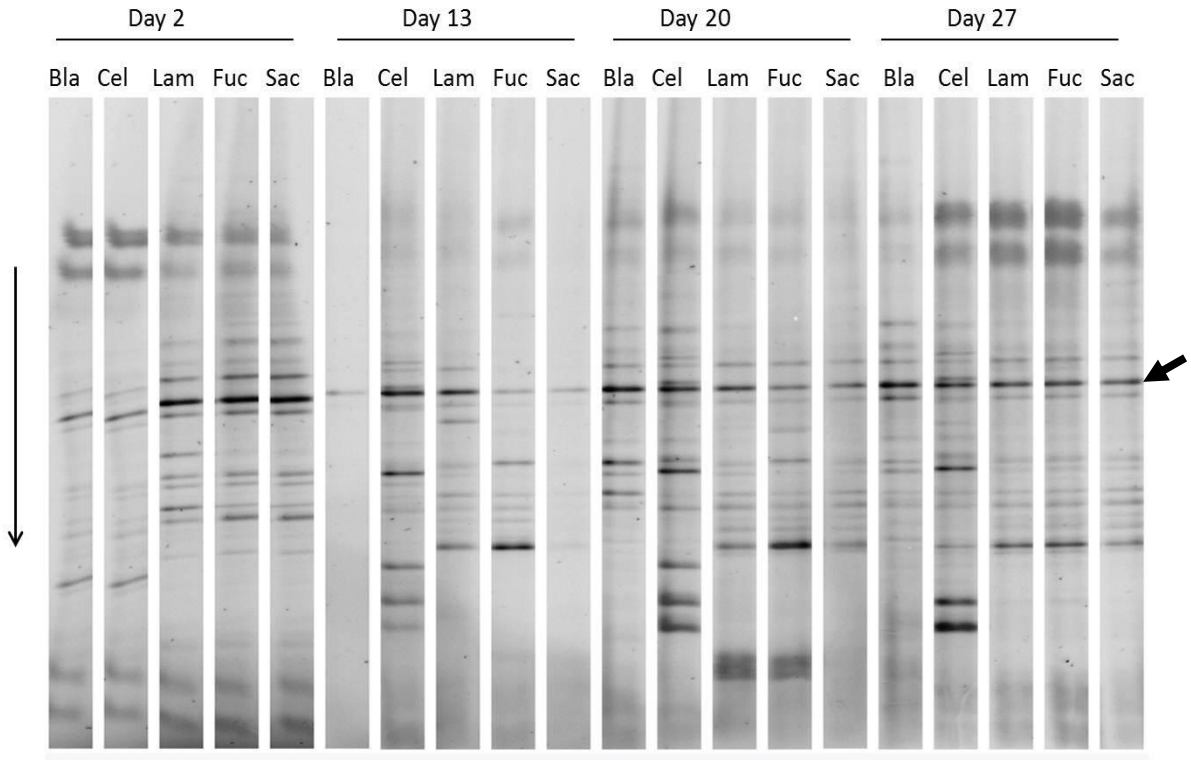


Fig. 6.4. Bacteria DGGE profile of digested sludge inoculated reactors during anaerobic digestion on seaweeds. DGGE was run in a 10% acrylamide with 40-60% denaturant gradient at 100V and 60^{o}C for 19 hours. Bla: blank, Cel: cellulose Lam: *L. digitata*, Fuc: *F. serattus*, Sac: *S. latissima*. Arrow indicates direction of increasing denaturant and acrylamide gradient from lower to higher concentration. Bold arrow represents a consistent band throughout the process in all reactors.

Unlike sediment inoculated reactors, bacteria distribution and diversity were largely sustained (apart from day 13 blank and *S. latissima* reactors) during the entire AD process. In fact, one of the bands (Fig 6.4 bold arrow) was present at every point in all reactors throughout the process, possibly indicating the importance of this organism (represented by the band) to the digestion process. This observation is consistent with the reports by

Salvador, et al., (2013), on the endurance and importance of certain microbial community during anaerobic digestion processes.

Cluster analysis of the bacteria community in sludge inoculated reactors reveals the aggregation of reactors with substantial bacteria diversity and composition mainly between day 20 and 27 (Fig. 6.5).

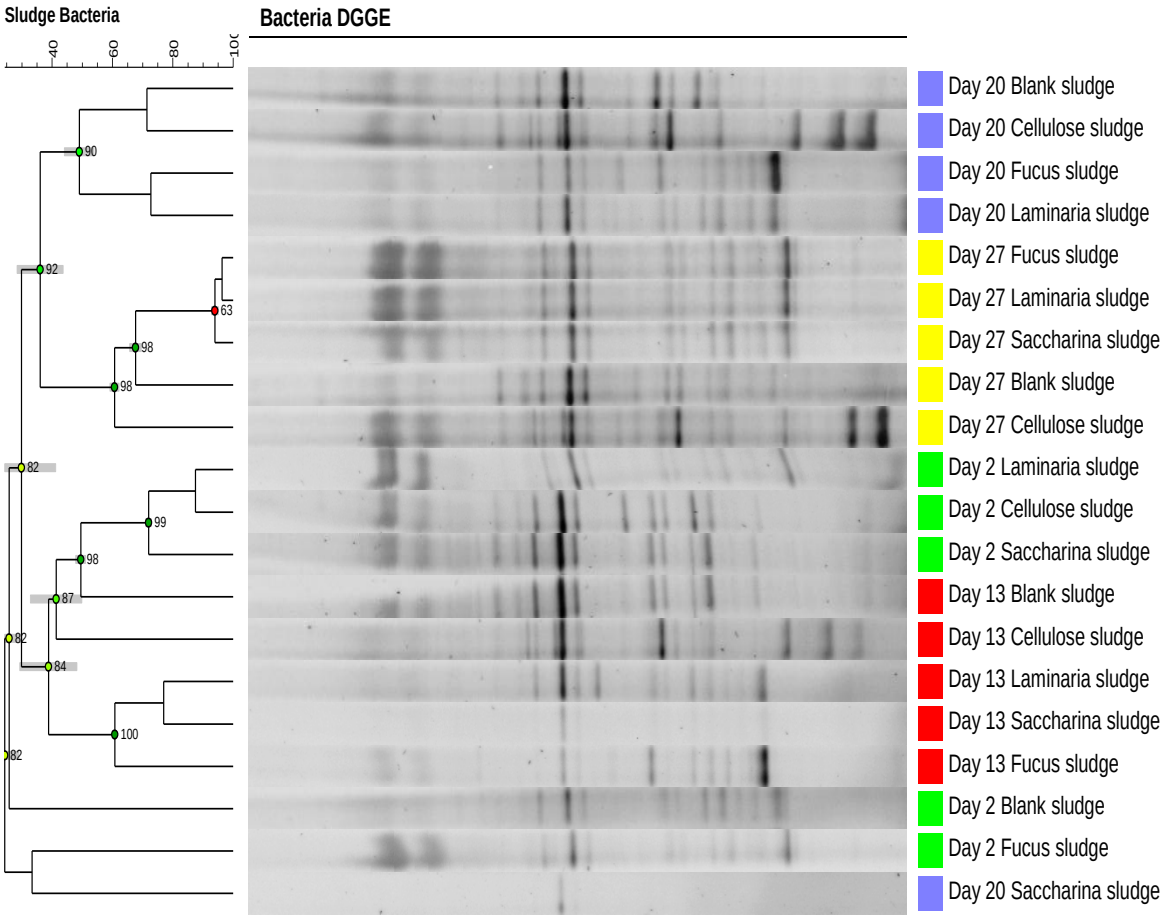


Fig. 6.5. Clustrering analysis of DGGE pattern of bacterial population structure in digested sludge inoculated reactors at different time points during anaerobic digestion of different substrates. Cluster analysis was performed using the unweighted pairwise grouping method with mathematical averages (UPGMA)

Day 2 and 13 reactors with lower bacteria diversity, which marked the stages of minimal (day 2) and the onset of exponential VFAs formation (day 13) (Fig.4.7) were clustered towards the bottom of the cluster analysis. Greater species richness and diversity observed at the later part of the process (day 20 and 27) coincides with stages of exponential and

consideable VFAs formation recorded in chapter 4, were clustered towards the top of the cluster analysis. At the later stage of the process, there was a high degree of similarities in bacterial communities across reactors, with ~50% at day 20 and > 60% at day 27 (Fig. 6.5), indicating that similar reactions and processes maybe carried out by the dominant bacteria communities in the different reactors at that point in time (Fig. 6.5).

Microbial community structure analysis revealed temporal shifts in microbial population irrespective of substrates in the reactors, an indication that the stage of the process is a strong determinant of the microbial community profile. It also suggests a succession process where immature (unstable) communities give way to more mature ones as well as the emergence of certain microbial groups depending on the stage of the process involved. The variation in bacteria community in different reactors over time, suggests that there might be differences in the (rate of) degradation of various feedstock biomass, leading to differences in subtrates availability per time, as well as the concomitant bacterial community. This agrees with some published reports on the importance of time as the main driver for changes in microbial community structure. For instance, in a study of the microbial community dynamics of lab-scale solid waste bioreactor in the presence or absence of biosolids, Nayak, et al., (2009) reported that time rather than substrates drives the shift in community structure. Temporal shifts in microbial community structure during anaerobic digestion process have also been demonstrated by Yu et al., (2014), who used 454 Pyrosequencing procedure to evaluate temporal variations in microbial communities of waste activated sludge digesters. The authors reported that microbial (bacteria and archaea) communities clusters occur in relation to time, as different clusters represented different stages during the process. Similarly, the fluctuations in microbial communities over time was also reported by Cook, et al., (2010), who investigated spatial and temporation changes in microbial community in anaerobic swine wastewater treatment lagoon. In that

study, the authors reported changes in microbial communites based on season; even as different clusters were recorded in summer, winter and fall.

Analysis of bacteria community structure revelaed significantly more diversity in the sludge inoculated than sediment inoculated reactors, this is an indication that source of inoculum plays a vital role in the determination of the richness of the microbial community during anaerobic digestion processes. Although, microbial community diversity may not result into greater microbial activities (Malin & Illmer, 2008), it appears that there are some correlations between bacterial community diversity (espeacially at the later stages of the process) and other process functions recorded in chapter 4.

Generally, the observed differences in bacterial community between the two sources of inoculum did not appear to affect process functions in relation to the bacterial activities and VFAs formation (Fig 4.5, 4.7). This suggest that the sources of inoculum employed in this study, though different, harbour hydrolytic, acidogenic and acetogenic bacteria to support efficient bioconversion of the algal biomass into volatile fatty acids.

6.3.2 DGGE analysis of archaea community structure in seaweeds anaerobic reactors under two distinct inoculations

Following the successful numerical quantification of archaea population of the reactors in the previous chapter, DGGE techniques was employed to determine the diversity of the different archaea groups or species present in each of the reactors at different stages of the process. To investigate the archaea community structure and diversity in anaerobic reactors in the current study, a nested PCR was conducted with the second round targeting the V3 region of the 16S rRNA gene resulting in 191bp amplicon length. Suitability of this primer pair was attested to by Yu, et al., (2008).

6.3.2.1 DGGE analysis of archaea community structure in seaweeds anaerobic reactors inoculated with anoxic sediment

Results of DGGE analysis of archaea population in the sediment-inoculated reactors show that the archaeal community were fairly diverse are the start of the digestion process (day 2) with at least 3 visible bands in each of the reactors. At this stage, *S. latissima* reactors haboured the most diverse archaeal community while *F. serattus* reactors contained the least diverse groups (Fig. 6.6). Archaea composition in blank and cellulose reactors were similar at this stage. By day 13, a slight shift in archaea community structure was observed, resulting in increased band intensity in some reactors as well as emergence of new bands in others especially in cellulose, *L. digitata* and *F. serattus* reactors. This scenario might be as a result of gradual emergence of methanogenic archaea to initiate methane production as potential methanogenic substrates became available.

About two (2) conspicous new bands emerged in the cellulose reactors gel at day 13 which were not detected in any other reactors at this stage. Archaea represented by these bands might be important for the utilization of cellulose derivatives which are absent in other

reactors at the stage of the process. Although the initial (day 2) diversity observed in *S. latissima* reactors was sustained, band intensity diminished considerably by day 13, suggesting a possible reduction in number of archaea groups as a result of changes in reactor conditions (Fig. 6.6).

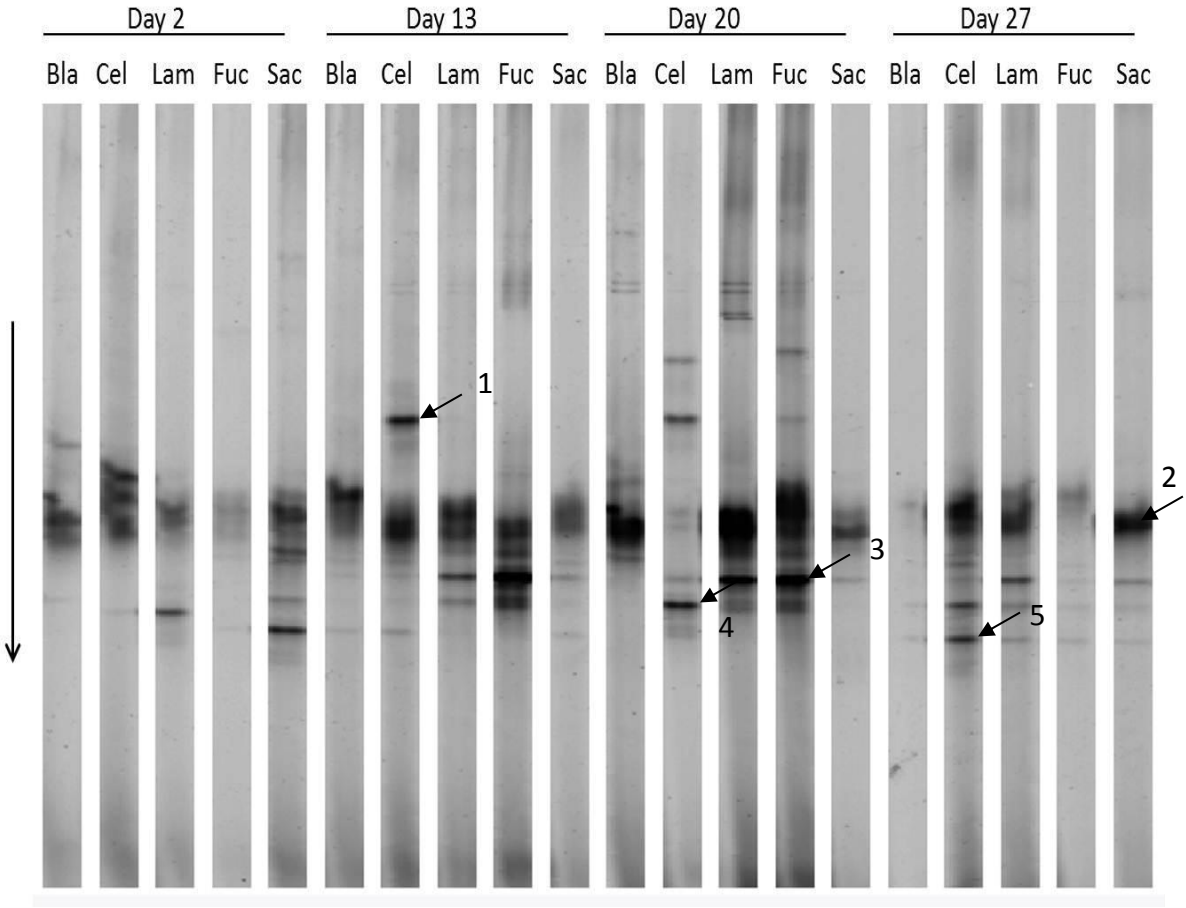


Fig. 6.6. General archaea DGGE profile of seaweeds and cellulose fed anaerobic bacth reactors inoculated with anoxic sediment. DGGE was run in a 10% acrylamide with 40-60% denaturant gradient at 60^{o}C for 19 hours. Bla: blank, Cel: cellulose Lam: *L. digitata*, Fuc: *F. serattus*, Sac: *S. latissima*. Arrow indicates direction of increasing denaturant and acrylamide gradient from lower to higher concentration, arrows within the gel represent bands excised for sequencing, starting with the code BSA (band sediment archaea).

A further shift in archaea community was observed in all reactors between day 13 and 20 with the emergence of new bands as well as increase in bands intensity especially in *L. digitata* and *F. serattus* reactors.

This stage (day 13-20) of the process coincided with the period of exponential production methane production in most of the reactors (Fig. 4.14). By day 27, a form of stability was attained in all substrate reactors, leading to the observed similarity in the diversity of the archaeal community. There were however less diversity and reduced bands intensity at this stage (Fig. 6.6), suggesting that methanogenic archaea may have been the dominante archaeal group at this phase of active methanogenesis (observed in chapter 4). Despite the observed variation in archaeal community (bands), one of the bands appeared consistent throughtout the process (Fig 6.6 side arrow 2) in most of the reactors and its thought to represent an important species or groups of archaea in the reactors.

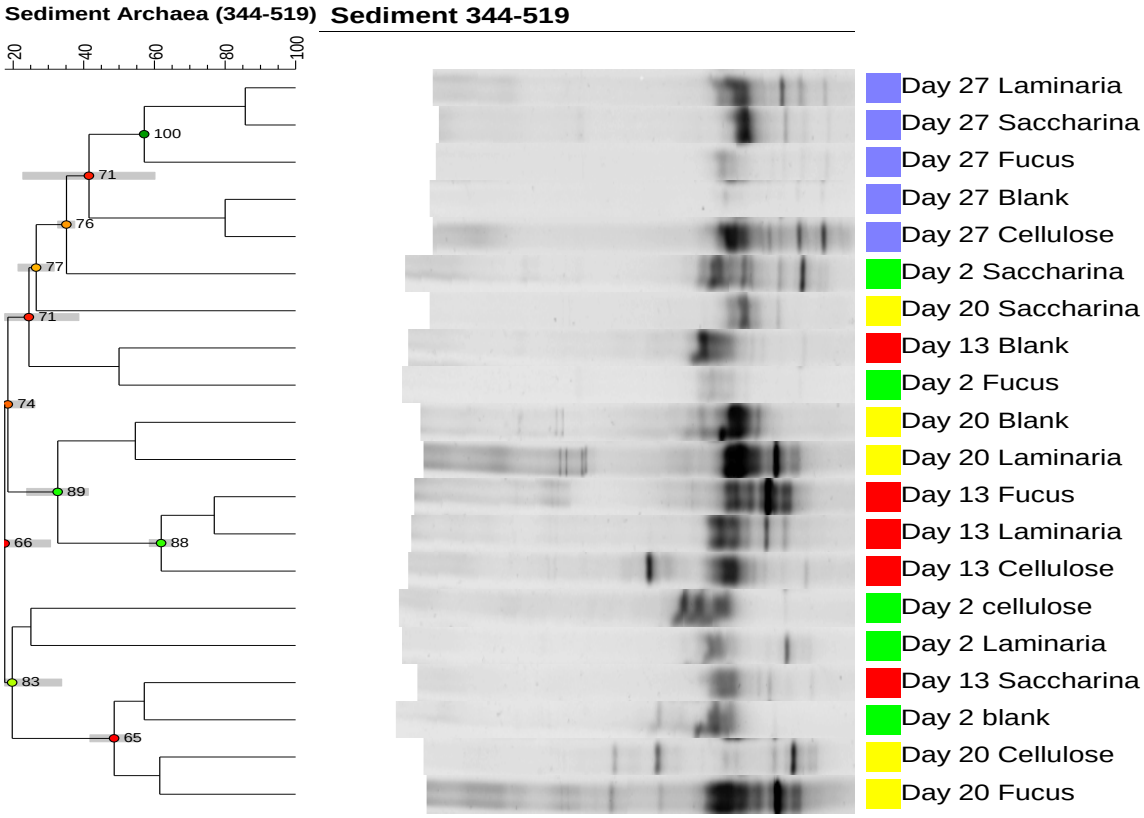


Fig. 6.7. Clustrering analysis of DGGE pattern of archaea population structure in anoxic sediment inoculated reactors at different time points during anaerobic digestion of different substrates. Cluster analysis was performed using the unweighted pairwise grouping method with mathematical averages (UPGMA)

Cluster analysis of the DGGE profile of archaea community structure highlighted similarities and disimilarities between intensive and semi-intensive archaea DGGE bands in relation to the stage of anaerobic digestion process. It also shows diversity clusters of DGGE profiles across reactors in relation to time as an indication of microbial activities at different stages of the process. Similarities between archaea community DGGE profile between day 13 and 20 was highlighted by the cluster analysis as the habouring the most diversed are archaea community (Fig. 6.7). This result is largely consistent with the q-PCR results previously reported.

6.3.2.2 DGGE analysis of archaea community structure in seaweeds anaerobic reactors inoculated with digested sludge

Result for DGGE analysis of sludge-inoculated reactors revealed a different archaeal community profile compared to sediment inoculated ones. The first noticeable difference is the lack of discernible bands at the start of the process (day 2) across all reactors, which was not the case in sediment inoculated reactors (Fig 6.8). The reason for the lack of clear bands at the start of the process (day 2) is unclear. It is possible that archaea community might be present at this stage, but was below the detection limit of the DGGE technique. Additionally, the lack of visible bands at the start of the process might be an indication of supression of archaea growth occasioned by exposure of archaea community in sewage sludge to high level of salt in the reactors mix. It might also be due to lack of suitable nutrient source for the archaea community at this stage of the digestion process. By day 13, when substantial VFAs have been produced (Fig. 4.7), possibly suitable for archaeal utilization, the DGGE profile highlighted a very diverse and rich archaea community in all reactors with most of the diversity occuring in the seaweeds reactors. At at this stage, a noticeable similarity was observed in the DGGE profile of the archaea community between blank and cellulose reactors, and amongst the three seaweeds' reactors (Fig 6.8).

Most of the diversity and band intensity observed at day 13 was retained through day 20 till the end (day 27) of the process with only slight variations in bands intensity and emergence. For instance, additional bands emerged in seaweeds reactors between day 13 and 27 suggesting that different groups of archaea (methanogens) might be present in seaweeds reactors and not in the cellulose ones.

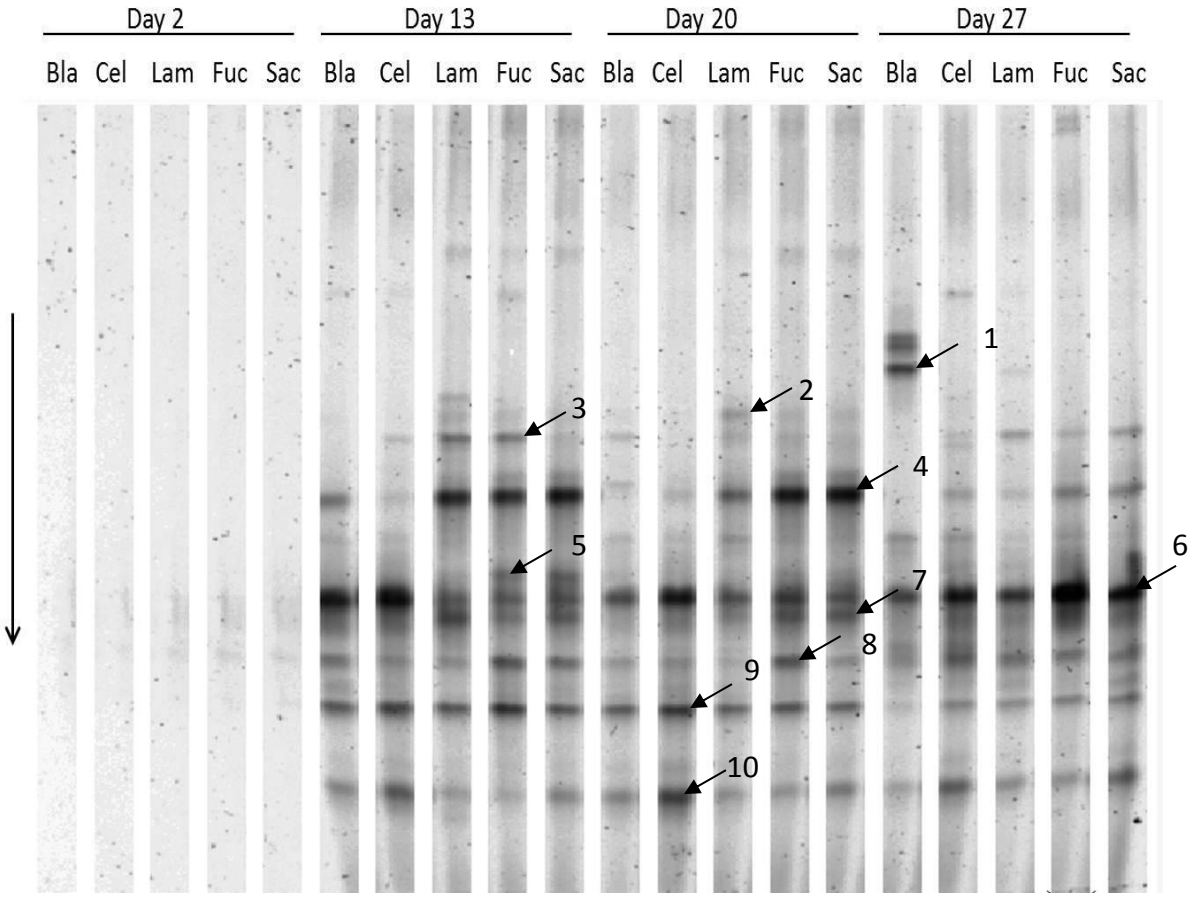


Fig. 6.8. General archaea (344f/519r) DGGE profile of seaweeds and cellulose fed anaerobic bacth reactors inoculated with digested sludge. DGGE was run in a 10% acrylamide with 40-60% denaturant gradient, at 100V and 60^{o}C for 19 hours. Bla: blank, Cel: cellulose Lam: *L. digitata*, Fuc: *F. serattus*, Sac: *S. latissima*. Arrow indicates direction of increasing denaturant and acrylamide gradient from lower to higher concentration, arrows within the gel represent bands excised for sequencing starting with the code BHA (band Hatton-sludge archaea).

Differences in microbial community structure of reactors inoculated with the same material is an indication of the ability of substrates to selectively determine microbial growth based on substrates composition (Ali Shah, et al., 2014). However, the stage or phase of the

anaerobic digestion process appears to be the main determinant of fluctuations in microbial community structure in AD reactors.

Cluster analysis of the archaea DGGE profile shows grouping of various reactors archaea composition based on prevalence (band brightness) and diversity (number of bands) over time (Fig. 6.9). It shows a distinct segregation between diverse archaea communities and less diverse ones.

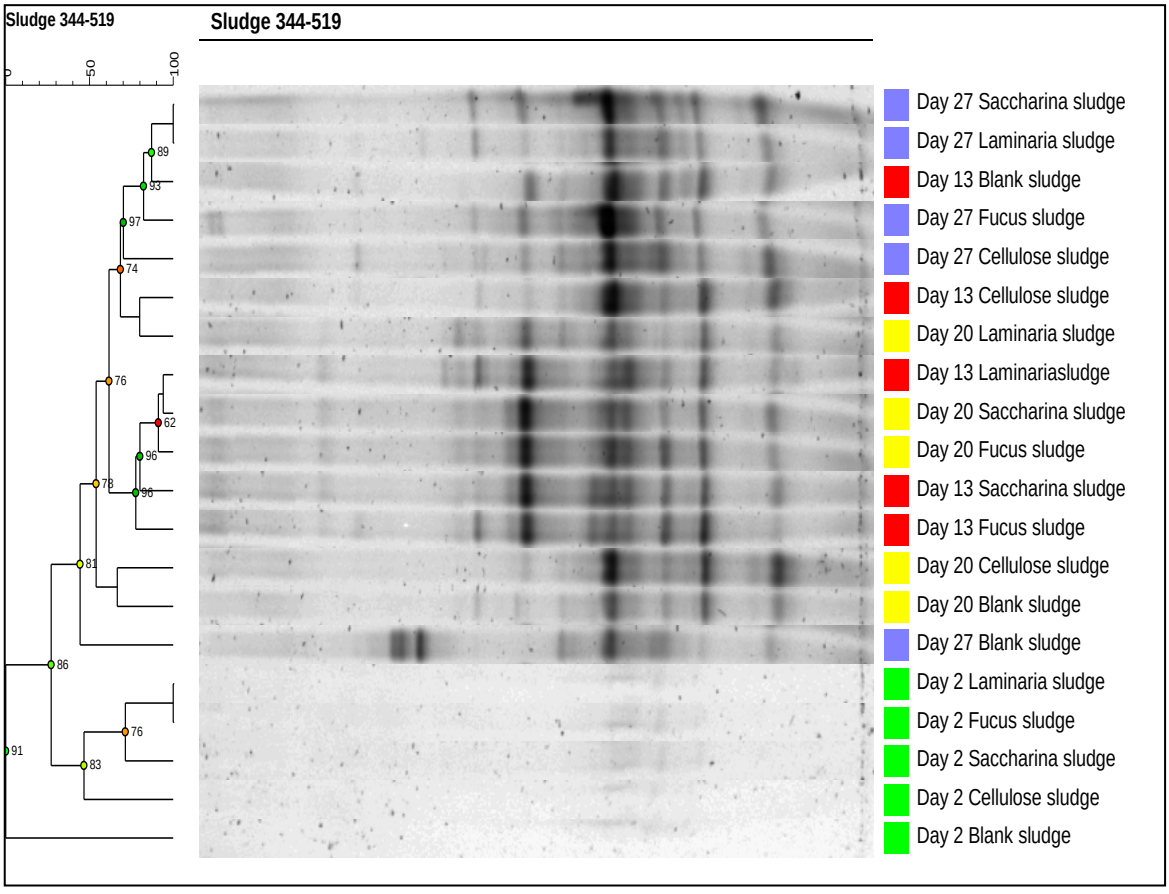


Fig. 6.9. Clustrering analysis of DGGE pattern of archaea population structure in digested sludge inoculated reactors at different time points during anaerobic digestion of different substrates. Cluster analysis was performed using the unweighted pairwise grouping method with mathematical averages (UPGMA).

All the reactors at day 2 were clustered as a result of similarities ocassioned by lack of intensive bands, which denotes lack of archaea presence (below detection) at this stage of the process. It is difficult to determine the cause of the observed result at day 2, as archaea

community are expected to be present, at least those carried over from the sludge inoculum. As earlier proposed, a number of factors might have been responsible for this result. Reactors containing several archaea DGGE bands (mostly at day 13 and 20) as well as intense bands (some day 13 and 27) were clustered (Fig. 6.9). While band intensity is an indication of numbers of the species or OTUs present, presence of several bands shows diversity of the microbial community. The period between day 13 and 27 coincides with the stage of considerable microbial activities recorded in chapter 4. This activites are thought to be driven by different groups of the microbial (including archaea) community. The phase (time) of the digestion process appears to be the main driver of shifts or fluctuations in the microbial community structure across reactors. However, the source of inoculum, availability of suitable susbtrates (food source) as well as type of substrates also appear to play some roles in the determination of the microbial community structure. Nayak, et al., (2009) using cluster and PCA analysis of DGGE from lab-scale reactors, demostrated that the shift in microbial community structure was time related. In that study, the authors reported 3 distinct microbial clusters based on the stage of the anaerobic digstion process. Shifts in anaerobic digester's microbial community structure as a result of time or stage of the digestion process has also been demostrated by Malin & Illmer, (2008), who observed the appearance and disappearance of certain bands at different times during the process. The authors reported a distinct shift in archaea community at times of substantial methane production. That report is similar to the observations in the current study.

Although microbial community structure changes over time during anaerobic digestion processes, certain species or groups of organisms endure all or most part of the process. This might be an indication of their importance to the entire process in other to sustain system stability. This phenomenon was observed in the DGGE gels of both bacteria and

archaea community in this study whether inoculated with sediment or sludge. This observation agrees with reports of a study on the endurance of methanogenic archaea in anaerobic bioreactors treating oleate-based wastewater over time. In that report Salvador, et al., (2013), observed the endurance of certain groups of archaea for over 300 days and reported that those groups of archaea were very important to the sustainance of the process.

Comparison of archaea DGGE community profile between sediment and sludge inoculated reactors highlighted some unique distinctions. For instance, while there were discernible bands at the start of the process (day 2) in all sediment inoculated reactors, the opposite was the case in all sludge inoculated reactors. However, after day 2, archaea presence and diversity in all sludge inoculated reactors increased, with at least 7 distinct bands observed at day 13, and till the end of the process, resulting in a more diversed archaea community than in the sediment inoculated reactors.

Comparison between bacteria and archaea community structure also revealed a significant difference in both community composition and structure, especially in the first half of the process irrespective of the source of inoculum. For instance, bacteria diversity decreased slightly between day 2 and 13 of the process across reactors while the opposite holds for the archaea community structure as there was a significant increase in archaea composition and diversity. A similar phenomenom was reported by Yu et al., (2014), during a study of temporal variation in microbial community of waste activated sludge digester. They reported that bacteria community was fairly stable while there was a significant increase in the number of archaea OTUs during that start up phase of the process.

6.3.2.3 Phylogenetic analysis of the archaea communities retrieved from DGGE bands in both sediment and sludge inoculated reactors

To determine the specific archaea species present at different stanges of the process, selected bands (from Fig. 6.6 and 6.8) were excised, cleaned and PCR-ed before sending for sequencing. Bands were selected based on prevalence through the process as well as new emergence at specific time points. A total of 5 and 10 bands were excised for sediment and sludge inoculated reactors respectively. Due to the small fragment length (191bp) produced from the nested PCR, sequences length for the phylogenetic analysis ranged between 120-163bp. The short length of sequences affected the quality and reliability of the phylogenetic relationships of band sequences with the database (Nettmann, et al., 2008; Sanz & Köchling, 2007) resulting in low sequences similarity of 80-95% to the associated entries in the GenBank database (Table 6.1).

6.3.2.3.1 Phylogenetic analysis of the archaea communities retrieved from DGGE bands of sediment inoculated reactors

Five DGGE bands were selected based on prevalence and distinctiveness from the sediment-inoculated reactors and excised. These excised bands were sequenced to obtain qualitative information and confirm the identity of the dominant archaea species. Results obtained from NCBI database BLAST search confirmed that all five sequenced bands represent organisms in the archaea domain with at least two archaea Orders represented. Band '1' (BSA1) closely related to *Methanosarcina baltica* was found only in cellulose (at day 13, 20) and *F. serattus* (day 20) reactors. *M. baltica* belongs to archaea order *Methanosarcinales*, which reduces methyl compounds such as acetate, methanol, methylated amines to produce methane. It cannot utilise formate and H_2/CO_2 and are therefore termed acetoclastic (sometimes methylotrophic) methanogens (Kendall & Boone, 2006; Von Klein, et al., 2002). Sediment has been reported to be dominated by these

groups of methanogens especially at low temperatures obtainable in sediments (Glissmann, et al., 2004). However, the report suggested that as temperature increases from psychrophilic to mesophilic conditions, there is a shift from acetoclastic to hydrogenotrophic methanogenesis (Glissmann, et al., 2004).

The poor performance of sediment inoculated cellulose and *F. serattus* reactors which temporarily harboured *M. baltica* strain DSM 14042, suggests that the archaea may not be involved in methane production and this may account for its lack of detection after day 20 (Fig. 6.6). Bands BSA2 and BSA3 were similar to uncultured archaea, one of which (BSA2) was originally excised from DGGE band, as is the case in this study. Band BSA2 represent the most prevalent and consistent archaea groups and may play an important role in process stability and methane production. Band BSA3 representing an uncultured archaea is also mostly present in seaweed reactors and may have contributed to the methane production in those reactors.

Table 6.1. Similarity of archaeal sequences excised from DGGE gels being amplified with archaeal primers A344f and A519r-GC – based on sequence alignments of partial 16S rRNA gene sequences for sediment and sludge inoculated reactors.

Bands*	Closest relative (GenBank)	GenBank Accession number	Order	Source of closest relative	% Sequence homology	Functional group[a]	Occurrence in reactors
BSA1	*Methanosarcina baltica* strain DSM 14042	AB973356	*Methanosarcinales*	Baltic Sea sediment	88	A/M	D13 Cel, Day 20 Cel & Fuc
BSA2	Uncultured archaeon isolate DGGE gel band	KJ402286	-	Sewage sludge	80	-	All except D27 Bla
BSA3	Uncultured archaeon clone	JQ738683	-	Lonar crater basalts	82	-	All D13, D20 &27 Cel, Lam, Fuc,Sac
BSA4	*Methanosarcina baltica* NR_041986	AY663809	*Methanosarcinales*	Sediments in Skan Bay, Alaska	83	A/M	D2 Sac, D13 Cel, Lam, Fuc, D20 Cel,Lam, Fuc, All D27
BSA5	*Methanospirillum stamsii* strain ps	NR117705	*Methanomicrobiales*	Anaerobic bioreactor	83	H	D2 L,F,S, D13 B,C,S, D20 C, 27 All
BHA1	*Methanospirillum hungatei* strain JF	KM408634	*Methanomicrobiales*	anaerobic Reactor	82	H	D27 Bla only
BHA2	Uncultured *Methanomicrobiales* archaeon	AM998457	*Methanomicrobiales*	Marmara Sea Sediments	85	H	D13,20 Lam, Fuc, Sac
BHA3	Uncultured archaeon clone CD_69	KM036415	-	mudflat Sediment	95		D13 C,L,F,S; D20 B,L,F,S D27 C,L,F,S
BHA4	*Methanosarcina baltica* strain DSM 14042	AB973356	*Methanosarcinales*	Baltic Sea sediment	88	A/M	All D13,20, D27C,L,F,S

Bands*	Closest relative (GenBank)	GenBank Accession number	Order	Source of closest relative	% Sequence homology	Functional group[a]	Occurrence in reactors
BHA5	Uncultured archaeon clone 24Earc92	JN605035	-	Marine Sediment	85	-	D13L,F,S; D20 C,S, D27 C,L
BHA6	Uncultured archaeon isolate DGGE gel band	KJ402286	-	Sewage sludge	80	-	ALL D,13,20,27
BHA7	*Methanosarcina baltica* NR_041986	AY663809	*Methanosarcinales*	Sediments in Skan Bay, Alaska	83	A/M	D13L,F,S,D20C,L,F,S, D27CLFS
BHA8	Uncultured archaeon clone	JQ738683	-	Lonar crater basalts	82	-	ALL D2-D27
BHA9	*Methanospirillum stamsii* strain ps	NR117705	*Methanomicrobiales*	Anaerobic bioreactor	83	H	All D13,20,27 faint D27B
BHA10	Uncultured *Methanosaeta* sp. clone arc I20	KC769086	*Methanosarcinales*	Anaerobic sludge	93	A	All D13,20,27

*BSA: Band sediment archaea, BHA: band sludge(Hatton) archaea. [a] A: acetoclastic, M: methylotrophic H: hydrogenotrophic

Another strain of *M. baltica* (strain NR_041986), represented by band BSA4 was present mostly in reactors containing substrates, an indication that it might be involved in catabolic activities leading to methane production (Table 6.1). Band BSA5 relates closely to *Methanospirillum stamsii* strain PS which occur at every stage of the process across reactors. *M. stamsii* strain PS was found in all seaweeds reactors at the start of the process (Day 2), and then appeared in different reactors at different times. This is probably in reaction to the availabilty of H_2/CO_2 on which they thrive. It belongs to the order of methanogen (*Methanomicrobiales*) that produce methane from H_2/CO_2 called hydrogenotrophic methanogens. They are very important to the stability of the digestion process as they maintain the critical partial pressure of hydrogen in reactors (Ali Shah et al., 2014).

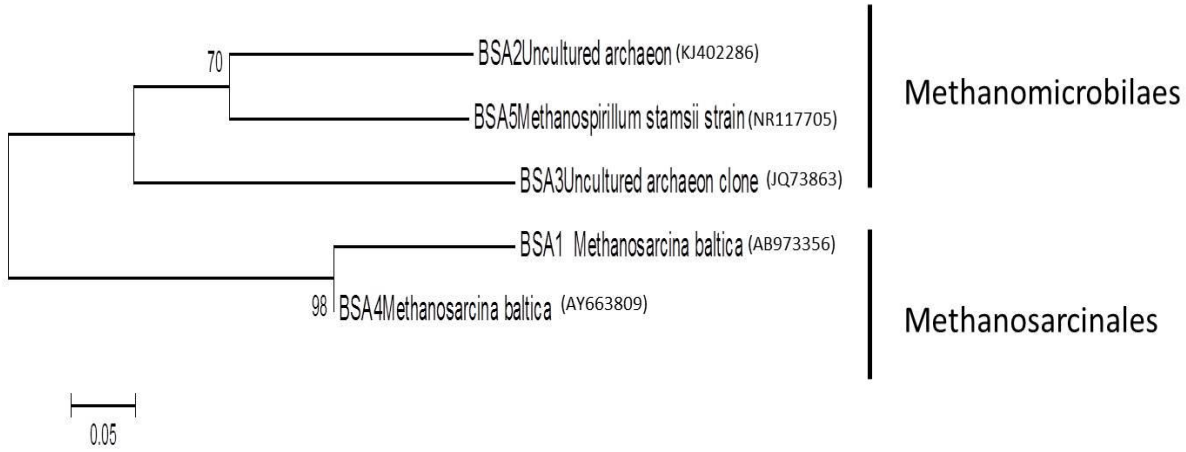


Fig. 6.10 Evolutionary relationships of five archaea taxa found in the sediment inoculated reactors. The evolutionary history inferred using the Neighbour-Joining method, (Saitou and Nei 1987). The optimal tree with the sum of branch length = 1.20797676 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004), and are in the units of the number of base substitutions per site. The analysis involved five nucleotide sequences. Codon positions included were 1st+Noncoding. There were a total of 31 positions in the final dataset All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Phylogenetic relationship of the DGGE bands shows that the retrieved archaea community in the digestion are somewhat similar (Fig. 6.10). Bands BSA2, BSA3 and BSA5 were grouped together and are thought to belong to the same order i.e. *Methanomicrobiales*, which are mostly hydrogenotrophic. Bands BSA1 and BSA4 are both related to methanogens from the genus *Methanosarcina*, which are known to produce methane via acetoclastic methanogenesis (Beckmann et al., 2011; Kendall & Boone, 2006; Ma et al., 2013; Rastogi, et al., 2008; Von Klein et al., 2002). Based on the results of this section, it could be hypothesised that there may be more archaea related to the order *Methanomicrobiales* in the sediment-inoculated reactors than there are those related to the order *Methanosarcinales*. Further work would however be required to confirm this assumption.

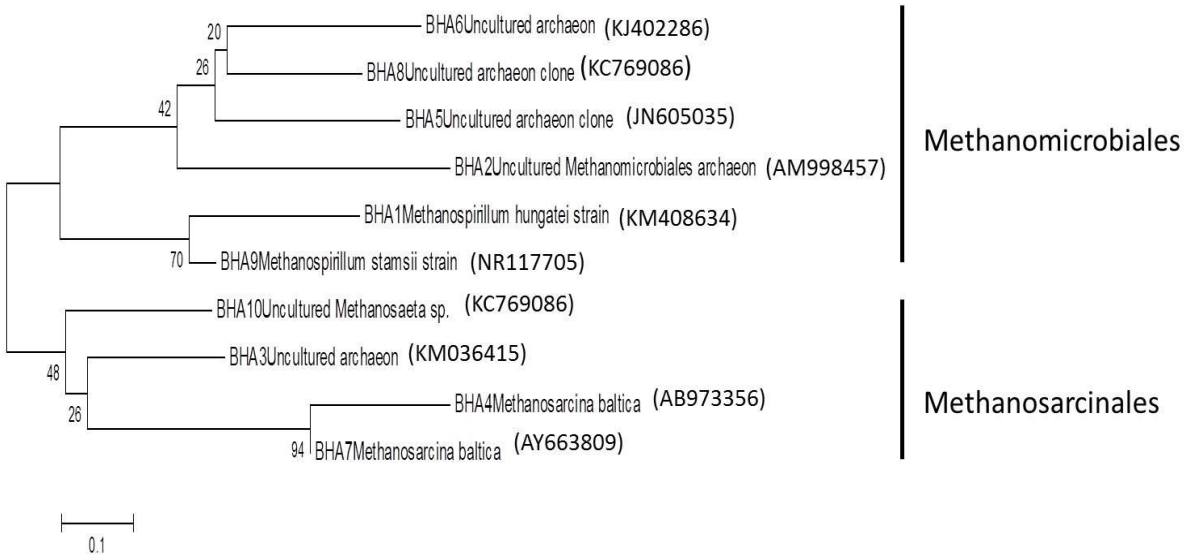


Fig. 6.11. Evolutionary relationships of 10 archaea taxa present in the sludge inoculated reactors. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 2.80245900 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, et al., 2004), and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+Noncoding. There were 27 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

6.3.2.3.2 Phylogenetic analysis of the archaea communities retrieved from DGGE bands of sludge inoculated reactors

Phylogenetic analysis of 10 archaea sequences of sludge-inoculated reactors retrieved from the NCBI database revealed that they belong to different groups of the domain Euryarchaeota. Band BHA1 is closely related to *Methanospirillum hungatei* which is a hydrogenotrophic methanogen belonging to the order *Methanomicrobiales*. Five other bands (BHA2, BHA5, BHA6, BHA8 and BHA9) were also found to be related to archaea belonging to the order *Methanomicrobiales* based on phylogenetic tree analysis (Fig. 6.11). *M. hungatei* has been shown to be distinctly important during anaerobic digestion for the rapid consumption of VFAs especially, propionate (Tale et al., 2011). This methanogen would be important to methane production in reactors containing high levels of propionate, but strangely was found only in blank reactors at day 27.

The other four bands (BHA3, BHA4, BHA7 and BHA10) were found to be closely related to uncultured archaeon, *M. baltica* (strain DSM), *M. baltica* and Uncultured *Methanosaeta* sp respectively, all belonging to the order *Methanosarcinales*. Like in sediment-inoculated reactors, sequence results suggest that more of the archaea in the sludge-inoculated reactors belongs to the order *Methanomicrobiales* than are *Methanosarcinales*. Although these results agree with previous reports of the dominance of *Methanomicrobiales* in bioreactors operated under mesophilic conditions, further research is needed to substantiate the observation. Nettmann et al., (2008) while studying archaea diversity within commercial methane plant fed with cow manure and maize silage operated under mesophilic conditions reported the dominance of archaea belonging to the order *Methanomicrobiales* which, accounted for at least 85% of the total archaea population. However, the dominance of archaea of the order *Methanosarcinales* was reported in reactors operated at low temperature (Zhang et al., 2012), in abandoned coal mines

(Beckmann et al., 2011) and anaerobic digesters operated at short retention times (Ma et al., 2013).

6.3.3 DGGE analysis of methanogen community in seaweeds anaerobic reactors under two distinct inoculations using *mcrA* gene marker

Methane production during anaerobic digestion of biomass is made possible by the interaction and cooperation of four different groups of microorganisms namely: fermentative bacteria, acidogenic bacteria, acetogenic bacteria and methanogenic archaea. The activities and contribution of these groups of diverse microorganisms are dependent on the stage of the process (Cook et al., 2010; Demirel & Yenigün, 2006; Dhaked, et al., 2010; Durbin & Teske, 2012; Sanz & Köchling, 2007; Song, et al., 2010; Tabassum & Rajoka, 2000; Tabatabaei et al., 2010; Wilkins, et al., 2015). The final phase of anaerobic digestion process termed methanogenesis is carried out by methanogens, which are physiologically different from other microorganisms involved in the process. Methanogens are slow growing and highly susceptible to inhibition, and therefore might not be able to respond promptly to high productivity of the others microorganisms involved in the process. As a result, methanogenesis is suspected to be the rate limiting step of the anaerobic digestion process (Shah et al., 2014; Banning et al., 2005; Biddle, 2006; Von Klein et al., 2002; Yu, et al., 2005; Zhang et al., 2011). Of all the microorganisms involved in the process of anaerobic digestion leading to methane production, methanogens are considered most important not only because they carry out the final stage of the process, but also because they are involved in the rate-limiting step (Malin & Illmer, 2008). Any conditions that influence the activities of these methanogens therefore, could inhibit efficient methane production. To study methanogen distribution and diversity within the reactors, DGGE technique was employed after PCR amplification of a section of

the *mcrA* gene, which is conserved and exclusive to methanogens. This could help to differentiate general archaea from those potentially involved in actual methane production.

6.3.3.1 DGGE analysis of methanogens community of anoxic sediment inoculated anaerobic reactors

Results of methanogenic DGGE profile analysis revealed no methanogen presence (band) in any of the sediment inoculated reactors during the first 13 days of the digestion process (Fig. 6.12). The failure of the DGGE to detect methanogens in sediment inoculated reactors at the initial stages of the process was suprising because, some methane production occurred during this time. Despite the observed marginal methanogenic activities during the first 13 days of AD process, the number of the *mcr*A gene present appears to be below DGGE detection limit. However, research has shown that only organisms or species that constitute more than 1% of the total microbial community can be detected by DGGE (Malin & Illmer, 2008). Results obtained from q-PCR experiments previously reported (chapter 5) have shown that methanogens (in sediment inoculated reactors) constituted less than 1% of the total microbial community at the initial stages of the digestion process. This might be why DGGE failed to pick up the methanogens community at this stage, although some methane was produced by their small population.

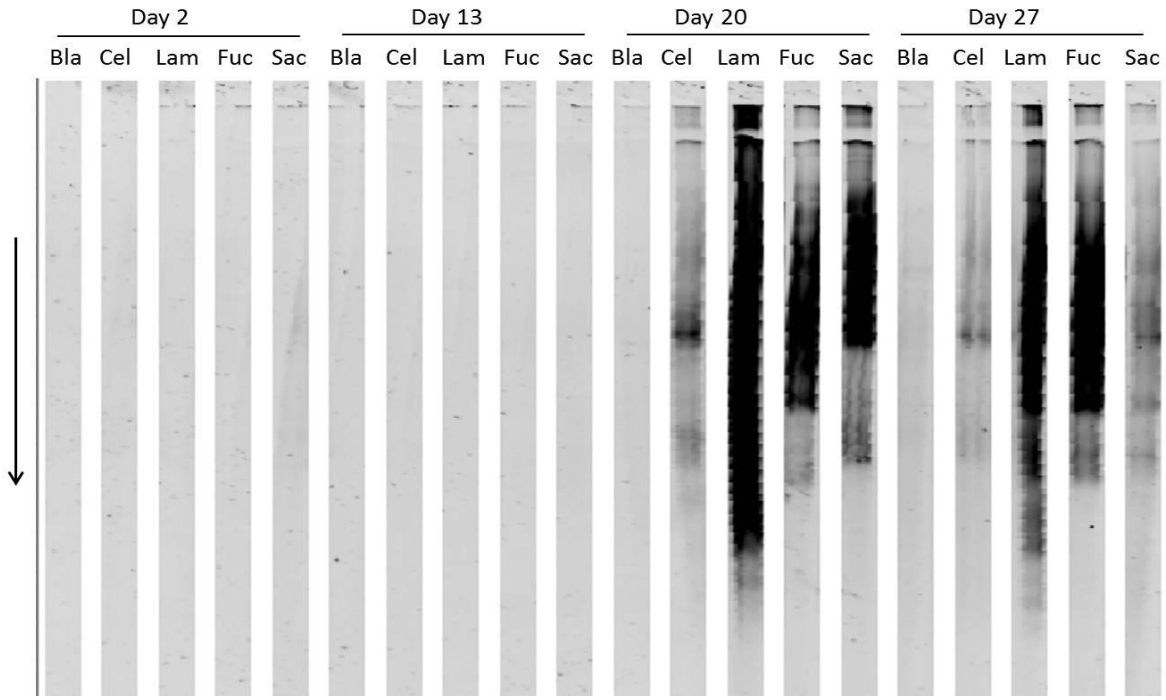


Fig. 6.12. Methanogens (*mcr*A gene) DGGE profile of seaweeds and cellulose fed anaerobic bacth reactors inoculated with anoxic sediment. DGGE was run in a 8% acrylamide with 40-80% denaturant gradient, at 100V and 60^{o}C for 19 hours. Bla: blank, Cel: cellulose, Lam: *L. digitata*, Fuc: *F. serattus*, Sac: *S. latissima*. Arrow indicates direction of increasing denaturant and acrylamide gradient from lower to higher concentration.

Increases in methanogen numbers after day 13 recorded in q-PCR results (Fig. 5.8) were reflected in the DGGE profile. Methanogens presence, though not refined into distinct bands was observed in all seaweeds and cellulose reactors after day 13, which corresponds to the onset of cumulative methane production (Fig 4.14). Lack of band resolution observed might be because of the fairly large DNA fragment (~500bp) used for the methanogen DGGE analysis. Research has shown that DNA fragments of >500bp are difficult to separate by electrophoresis (Zhang & Fang, 2000). Other issues that may have led to the observed result (lack of bands at the start and poor band delineation at later stages) might be as a result of many uncertainties and biases associated with PCR-based method such as polymerase inhibition by humic materials and preferential amplification as a result of differences in priming and elongation rates between amplicons. These potential

biases carried on from PCR can change the relative concentration of PCR products so that the resulting profile of phylotypes no longer reflects the composition of the microbial community (Díez et al., 2001).

Methanogen DGGE band absence in all blank reactors (Fig. 6.12), suggest that there might be little methanogen presence (and perhaps little methanogenic activities) in those reactors resulting in low methane production (recorded in chapter 4). Methanogenic composition and diversity is difficult to assess from the DGGE profile because of poor bands delineation. However, results of methane production recorded in the later stages of the process (Fig. 4.12) provide evidences to suggest that substantial methanogenic activities occurred in seaweeds reactors in the latter stages (day 20-27) of the process and that, although DGGE bands were not clearly delineated, those bands represent active methanogen community. The presence of only a couple of methanogen DGGE bands in the sediment inoculated cellulose reactors confirms the poor methanogenic activities resulting in very low methane production earlier reported (Fig 4.12). It also substantiates the assumption that sediment might not be a suitable source of inoculum for anaerobic digestion of cellulosic materials.

Generally, DGGE profile of the methanogen community shows that there was a delay in the growth (and resultant activities) of the methanogens and that methanogens community present in the sediment-inoculated reactors were not diverse (Fig. 6.12).

As expected, cluster analysis of the DGGE profile revealed two main clusters based on composition and diversity in the gel (Fig. 6.13). The first cluster represents the initial stages (day 2-13) of the digestion process where little methanogenic presence occurred resulting in lack of DGGE bands. The second cluster, which represents poorly delineated methanogen community (bands), corresponds to the time of considerable methanogenic

activity (resulting in considerable methane production Fig 4.12) revealed by the presence of intense bands at the later stages (day 20-27) of the digestion process. This result confirms earlier reports by Tale et al., (2011) that bands intensity of methanogenic community profile has a direct relationship with specific methanogenic activity (SMA). The authors using a principal component approach also reported that gel lanes containing minimal bands clustered around stages of very low methanogenic activity.

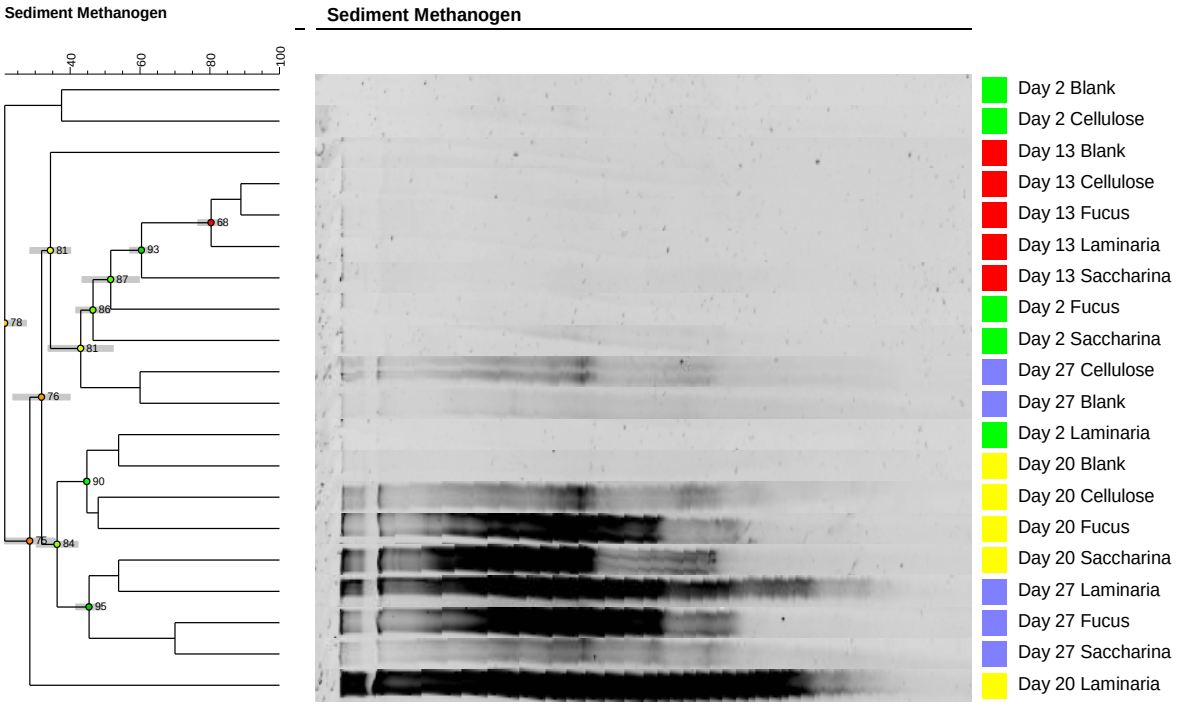


Fig. 6.13. Clustrering analysis of DGGE pattern of archaea population structure in anoxic sediment inoculated reactors at different time points during anaerobic digestion of different substrates. Cluster analysis was performed using the unweighted pairwise grouping method with mathematical averages (UPGMA).

6.3.3.2 DGGE analysis of methanogen community of sludge inoculated anaerobic reactors

Results of methanogen DGGE profile analysis of sludge-inoculated reactors shows that unlike sediment-inoculated reactors, there was methanogen presence (bands) in all reactors right from the initial stages (day 2) of the process (Fig. 6.14). It also highlights the difference in methanogen community diversity between the two sources of inoculum.

Methanogen community of sludge inoculated cellulose reactors were the most abundant and diverse for most part of the digestion process. These results support high methane production from the cellulose reactors, an indication of the suitability of sludge as the source of inoculum for AD of cellulosic materials. It also suggests that process functions could be assessed from the microbial point of view.

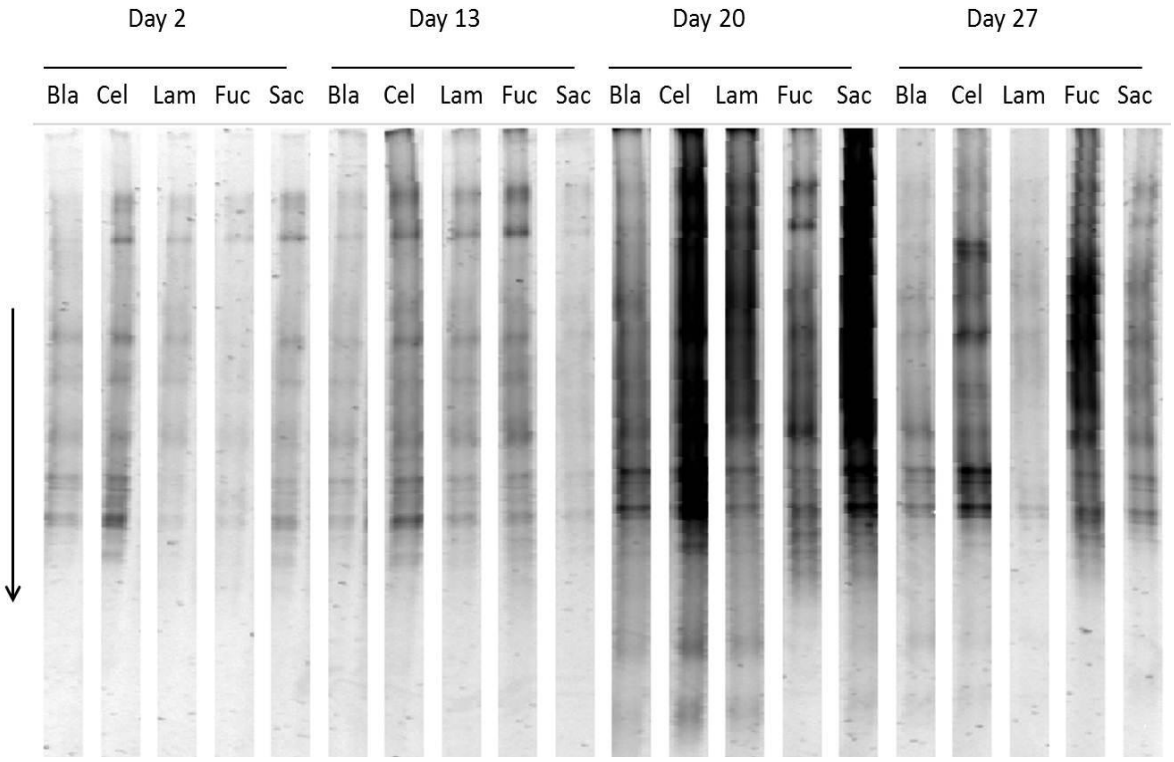


Fig. 6.14. Methanogens (*mcr*A gene) DGGE profile of seaweeds and cellulose fed anaerobic bacth reactors inoculated with digested sludge. DGGE was run in a 8% acrylamide with 40-80% denaturant gradient, at 60^{o}C for 19 hours. Bla: blank, Cel: cellulose, Lam: *L. digitata*, Fuc: *F. serattus*, Sac: *S. latissima*. Arrow indicates direction of increasing denaturant and acrylamide gradient from lower to higher concentration.

Methanogenic diversity and community structure shifts over time reflect the results obtained from quantitative PCR reported earlier (Fig. 5.9). There was a general increase in methanogens number and diversity in all reactors from day 2 through day 13 to day 20, after which a decline occurred (Fig. 6.14). For instance, at day 2 and 13, methanogen (*mcrA*) gene copies were highest in the cellulose reactors, but by day 20, highest *mcrA*

gene copies were recorded in the *S. latissima* reactors, followed by cellulose, and by day 27, *F serattus* reactors had the highest *mcrA* gene copies (Fig 5.12). These observations were reflected in the DGGE profile (Fig. 6.14).

Most of the methanogen OTUs or species present in the reactors were preserved and endured throughout the process even as more diversity and band intensity occurred at the later stages of the process. This observation attests to the suitability of digested sludge as a choice source of inoculum during anaerobic digestion of a wide range of biomass materials (Khalid, et al., 2011; Raposo, et al., 2012), including seaweeds.

Methanogen community DGGE profile of sludge-inoculated reactors gave a reflection of methane production pattern earlier recorded in chapter 4, where increases in bands intensity (Fig. 6.13) over time, appears to coincide with increased methane production after day 13 (Fig 4.14). These results suggest that the physico-chemical parameters of anaerobic reactors could be assessed and understood with other microbial parameters of the reactors over time. It also highlights the linkages and responses of microbial community to changes in operational and environmental conditions within the reactors. This understanding would not only be important for process design and monitoring purposes, but also for a rapid diagnosis of the reactors status per time (Bernhard Munk et al., 2010).

Cluster analysis to determine the similarities (or dissimilarities) of methanogen DGGE profile in sludge-inoculated reactors brought about a few clusters based on gel composition and diversity over time during the digestion process (Fig. 6.15). The first cluster (top) contains bands from day 2 reactors when methanogenic activity was minimal, based on results of methane production (Chapter 4). The second cluster represents methanogen community structure within reactors during phases of active methanogenesis (day 13) which marked the onset of substantial methane production.

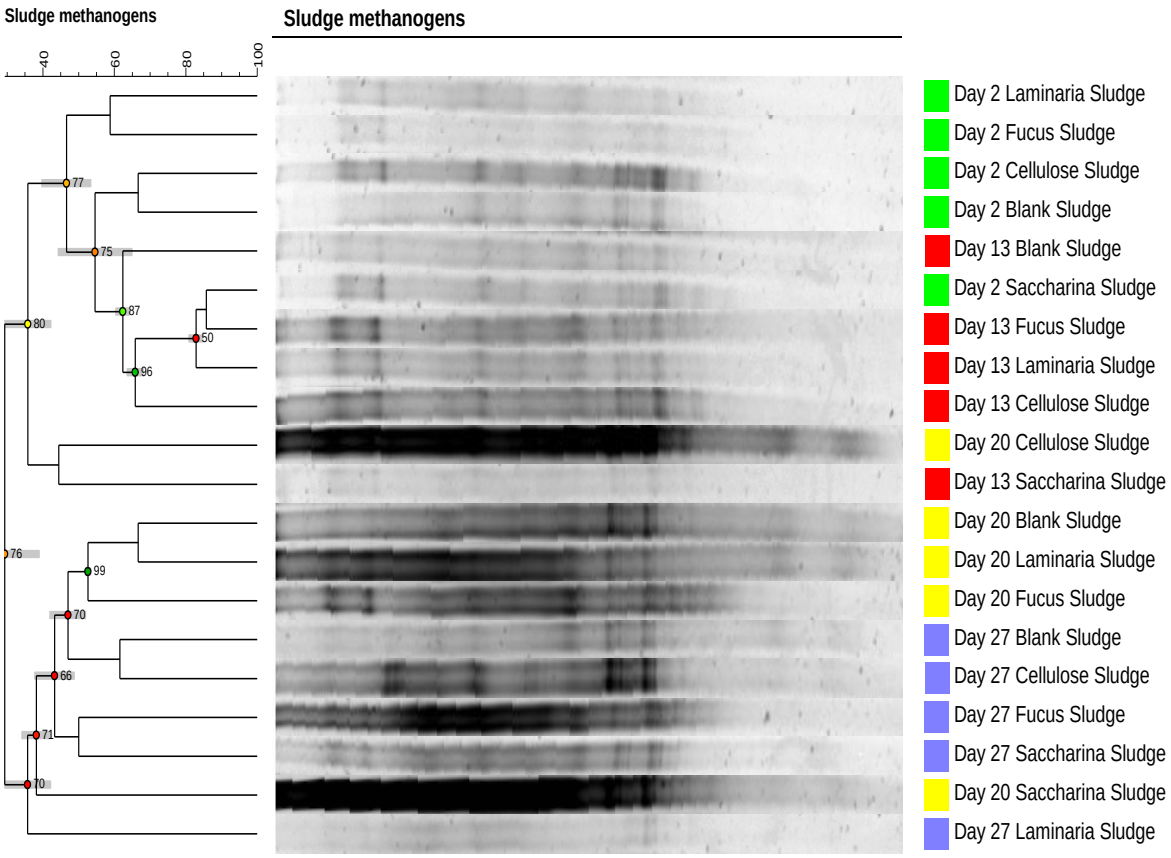


Fig. 6.15. Analysis of the composition of methanogenic community in seaweeds anaerobic reactors, inoculated with digested sludge showing similarities in population at different time points across reactors. Cluster analysis was performed using the unweighted pairwise grouping method with mathematical averages (UPGMA).

The third cluster brings together DGGE profile of methanogens in reactors undergoing extensive methanogenic activities (day 20-27). It corresponds to the times of sustained exponential methane production in all substrates' reactors.

Research has shown that microbial cluster analysis of DGGE from environmental samples occur as a result of the stage or in relation to time. For instance, Salvador et al., (2013), while studying the endurance of methanogenic archaea in anaerobic bioreactors found that methanogen community profile was influenced by the time or duration of the anaerobic digestion process. In that study, the authors reported most methanogen diversity at the latter half of the process with three clusters produced from the similarity analysis based mainly on time. Similar result was reported by Nayak et al., (2009), who studied the

microbial population dynamics of lab-scale solid waste bioreactor. Archaea DGGE cluster analysis in that study resulted in the formation of two main clusters based on number of days of anaerobic digestion.

Generally, there are more methanogens in the sludge-inoculated reactors in terms of abundance (band thickness/brightness) and diversity (number of bands) than in sediment-inoculated reactors for most part of the digestion process. Additionally, the delay in methanogens increase in sediment-inoculated reactors reported earlier in q-PCR results was reflected in the DGGE community profile analysis. However, methanogenesis appeared more efficient in sediment-inoculated seaweeds reactors than sludge inoculated ones due to production of substantial amount of methane by relatively low numbers of (and less diverse) methanogen over a shorter period of time. This observation confirms the report during band pattern analysis of DGGE profile of anaerobic waste reactor by Malin & Illmer, (2008), that the number of bands which is a function of diversity does not have direct correlation with process performance.

6.4 Conclusion

Denaturing gradient gel electrophoresis (DGGE) has become a powerful tool in molecular biology as it relates microbial community variations with changing physical and environmental conditions. It provides relatively quickly, a broad snapshot of changes in microbial community in relation to factors such as time, depth and treatments. There are however, a number of potential biases associated with PCR and DGGE, which might have influenced the results in this chapter and its interpretations.

The current study has demonstrated the ability of fingerprint technique (DGGE) to provide a time series microbial community responses to changing anaerobic conditions in different reactors. It has highlighted the interactions between microbial community composition and diversity and systems functions and performance.

DGGE profile analysis of bacteria, archaea and methanogenic component of sediment and sludge inoculated reactors showed more microbial (richness) diversity in the sludge-inoculated reactors. However, despite the greater microbial diversity in sludge-inoculated reactors, the similarity recorded in process performances alludes to the efficiency of the microbial communities in the sediment-inoculated reactors. Cluster analysis of DGGE profiles highlights the importance of time as the main determinant of the microbial community structure. Nonetheless, substrates composition is also an important determinant of microbial community structure.

Phylogenetic analysis of selected archaea DGGE bands indicated the dominance of two main methanogen orders *Methanomicrobiales* and *Methanosarcinales*, which are mainly hydrogenotrophic and acetoclastic/methylotrphic, respectively. However, a much more detailed study is required to identify the dominant archaea (including methanogens) in seaweeds anaerobic reactors and the favoured route towards methanogenesis.

Although the source of inoculum determined the microbial composition and diversity, it did not determine the productivity of the process during anaerobic digestion of seaweeds. The opposite is however the case with anaerobic digestion of cellulose.

Generally, the results obtained suggest that shifts in microbial community structure occur as a form of response to the predominant process (es) (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) taking place in the reactors at specific times during the process.

To date, this is the first time a fingerprinting technique such as DGGE is used to elucidate microbial community structure of seaweeds reactors; whether inoculated with anoxic sediment or digested sludge.

Now that microbial community profile has been established, although attempt to sequence selected bands did not provide sufficient information, the next chapter will report the actual cloning and sequencing of larger fragment size DNA for both archaea and methanogens under the two inoculations. This will provide more information about the diversity and identity of various dominant archaea and methanogens involved in the process. Emphasis is on archaea (and methanogens) because of their involvement in the rate-limiting step of the AD process.

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7 Chapter 7

"*Now, a living organism is nothing but a wonderful machine endowed with the most marvellous properties and set going by means of the most complex and delicate mechanism*"

- Claude Bernard

7. Phylogenetic identification of dominant archaea (and methanogen) population within seaweeds anaerobic reactors

7.1 Introduction

The biological nature of anaerobic digestion confers some uniqueness on the process. These include differential responses by different microbial communities involved to, changes in environmental and operational conditions. The digestion process can be influenced by the response of the microbial community to changes in environmental and operational conditions. This response depends largely on the types and scale of the changing conditions and the adaptive capacity of the organisms involved. Therefore any inhibition of microbial activities, will impede the functionality of the processes and as such the potential for methane production (Sundberg et al., 2013). It is important to study and understand the microbial responses to these changes in order to monitor and optimise process performances. Conventional techniques aimed at studying microbial ecology have been based on morphological features, which are problematic in terms of distinguishing organism to class level. Therefore, a number of high throughput molecular techniques have been applied to unravel microbial interactions during anaerobic digestion processes. Some of these techniques include denaturing gradient gel electrophoresis (DGGE) and (Calli, et al., 2006; Keyser, et al., 2006), fluorescent in situ hybridization (FISH) (Calli et al., 2006; Narihiro & Sekiguchi, 2011; Tabatabaei et al., 2009). Others are quantitative PCR (Chen, et al., 2014; Munk, et al., 2012; Munk, et al., 2010; Steinberg & Regan, 2009; Traversi, et al, 2012; Williams, et al., 2013) and sequencing (Calli et al., 2006; Guo et al., 2015; Narihiro & Sekiguchi, 2011; Sundberg et al., 2013; Wilkins, et al., 2015; Williams, et al., 2013; Wirth et al., 2012; Zhu, et al., 2011). Cloning and sequencing of 16S rRNA genes fragments obtained from anaerobic reactors enables in-depth characterization of active

microorganisms and provide better insights into microbial diversity in nature (Díez, et al., 2001). The use of a large fragment sized DNA (>500bp) sequences can provides more refined phylogenetic information about the organism under investigation (Sanz & Köchling, 2007). The importance of methanogenic archaea to anaerobic digestion necessitates the need for its in-depth study especially when the process involves unusual substrates like seaweeds. Despite their slow growth and susceptibility to inhibitions (Marquez, et al., 2013; Zhang et al., 2011), methanogens carry out the final phase of anaerobic digestion and are the sole producers of methane during the process (Morris et al., 2014). This project is therefore, largely focused on the activities of archaea (especially methanogens) during anaerobic digestion of seaweeds under mesophilic conditions, because the operational efficiency of the process is ultimately dependent on viable and active archaea (including methanogen) community. Methanogens are very important during anaerobic digestion processes as they are responsible for all methane produced during the process. It is therefore essential to study their activities and how they cope with changing conditions in the reactors, which has been demonstrated throughout this study. This will provide valuable information on process stability, control and monitoring as the process is ultimately dependent on active microbial populations (Tabatabaei et al., 2010).

To date, no studies have been carried out to identify the dominant archaea and methanogen involved in anaerobic digestion of seaweeds. Therefore, the main objectives of this chapter are to identify the main methanogenic groups involved in the anaerobic digestion of marine materials under different inoculations and possibly relate the types of methanogens present to process functionalities in the anaerobic bioreactors.

7.2 Methods

The current chapter is focused on archaea and methanogen because of their importance in methane production during anaerobic digestion processes. For the purpose of the phylogenetic study, digestate samples were taken from Day 20 *L. digitata* (as a representative of the seaweeds) sediment and sludge inoculated reactors. To characterize the dominant archaea and methanogens present in the reactors, PCR was conducted using appropriate primers targeting most of the archaea and methanogen community as described below.

7.2.1 Cloning and sequencing techniques for archaea and methanogens present in sediment and sludge inoculated *L. digitata* reactors

A large DNA fragment size (>500pb) is important during cloning and sequencing experiments to obtain sufficient phylogenetic information (Nettmann, et al., 2008; Sanz & Köchling, 2007). To obtain a large DNA fragment (~971bp), primer pair PRA46 (5'-YTAAGCCATGCRAGT-3')/Arch1017 (5'-GGCCATGCACCWCCTCTC-3') which is specific for archaea 16S rRNA gene was used in a nested PCR reaction as described by Øvreås, et al., (1997). The product of the first round PCR was then used as a template for a second round with the same primer pairs to achieve sufficient amplification. Duplicate PCR products from the two sets of reaction were pooled and purified prior to being used in the cloning reaction (Ciotola, et al., 2013).

For specific methanogens studies, primer pair targeting the *mcr*A gene: mlas (5'-GGTGGTGTMGGDTTCACMCARTA-3') and m-rev (5'-CGTTCATBGCGTAGTTVGGRTAGT-3') which produces ~500bp DNA fragment were employed as previously described (Steinberg & Regan, 2008). Reaction conditions were as described in section 2.4.2.3. PCR products were loaded in 1.5% agarose gel to ensure the

PCR products were the correct sizes. A single round of application produced sufficient band brightness (PCR product) when checked on agarose gel. Duplicate PCR products from the two sets of reaction were pooled and purified with the QiaQuick PCR Purification Kit (Qiagen, USA) prior to being used in the cloning reaction (Ciotola, et al., 2013).

7.2.2 Clone Library Construction

Clone libraries were constructed by ligating the 16S rRNA fragment (archaea) and *mcrA* gene fragment (methanogen) PCR products into pCR 2.1-TOPO® vector and transformed into One Shot TOP10 chemically competent *Escherichia coli* using the TOPO TA® cloning kit according to the manufacturer's instructions (Invitrogen, CA, USA). Transformed clone were screened using LB plates containing Ampicillin (50mg/ml) (Nayak, et al., 2009). Randomly selected colonies were re-streaked onto new LB plates overnight at 37°C. Selected clones were used directly in PCR reactions using vector-specific primers M13F (5'-TGTAAAACGACGGCCAGT - 3') and M13R (5'-CAGGAAACAGCTATGACC -3') (Invitrogen) as previously described (Cardinali-Rezende et al., 2009; Rastogi, et al., 2008). PCR product (5 µl) were visualised by agarose gel electrophoresis to check if the sequences were the right sizes as described above. Clones of the correct size were purified (QIAquick spin columns, Qiagen, Crawley, UK) and sequenced using the primer M13F by Source Bioscience (Glasgow, UK). Five (5) µl of each reaction normalised to 1ng/µl per 100bp and primer (3.2pmol/µl) were used for the sequencing reactions.

7.2.3 Phylogenetic analyses of archaea and methanogen sequences

All sequences were viewed and corrected using FinchTV Version 1.4.0 (Geospiza Inc.). Sequences were aligned using Bioedit Sequence Alignment Editor (Hall, 1999). Nucleotide sequences were determined for each clone type from the clone library and were compared

to the GenBank database using FASTA (Pearson & Lipman, 1988). BLAST (blastn) searches were conducted with the 16S rRNA (archaea) and *mcrA* (methanogens) sequences to determine their relationship to reference sequences in GenBank® database. The acceptable percentage of identity was set at ≥70% (for *mcr*A gene fragments) and ≥90% (for 16S rRNA fragments), minimum nucleotide length was 196bp (Wirth et al., 2012). Phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Confidence in the inferred relationships was assessed using Bootstrap analysis (100 replicates) (Tamura, et al., 2007).

A detailed description of methodology is available in chapter 2 of this thesis.

7.3 Results and discussion

The main objective of this chapter is to identify the dominant archaea and methanogens present in seaweeds reactors at the stage of active methanogenesis. In this study, both 16S rRNA and functional (*mcrA*) gene clone libraries were used. The use of functional gene marker could provide information on the archaea community potentially involved in actual methane production. The results presented are from a limited number of clones and as such may only be representative of a small component of the microbial community.

7.3.1 Composition and phylogenetic analysis of archaeal community in sediment inoculated seaweed reactors

Twenty-six (26) selected archaea clone sequences from sediment-inoculated reactors were analysed for community composition and diversity. All the clones were at least 90% related to known archaea in the GenBank database and are affiliated to three different orders of the domain archaea (Table 7.1). *Methanomicrobiales*-like archaea constitute the largest proportion of the archaea community accounting for 73% of the total archaea in the sediment inoculated seaweed reactors. Twenty-three (23%) are related to the order *Methanobacteriales* while *Methanosarcinales*-like archaea made up only 4% of the archaea population (Fig. 7.1). These results suggest the dominance of hydrogenotrophic archaea, which produce methane by reducing CO_2 with H_2. Most member of this order can also utilise formate while many species use alcohols. They however, cannot utilise acetate and C-1 compounds such as methanol (Garcia, et al., 2006). Like other members of the archaea domain, *Methanomicrobiales* inhabit diverse anaerobic environments. They are found in marine and fresh water sediment, anaerobic digesters as well as rumen of animals. In addition, they thrive best under mesophilic conditions (Garcia et al., 2006; Khalid, et al, 2011; Klocke et al., 2008), which may aid its dominance of reactors in the current study.

Methanobacteriales on the other hand are distinguished from other archaea by their predominant use of CO_2 reduction to produce methane (Bonin & Boone, 2006), and this may account for its low presence in the reactors in this study.

Results of sequence analysis from sediment-inoculated reactors to genus level indicate that archaea related to the order *Methanomicrobiales* is made up of three distinct genera. The genus *Methanoculleus*-like archaea dominates, accounting for 58% of the order, while those related to *Methanofolis* and *Methanosphaerula* accounted for 38% and 5% respectively (Fig.7.1). Archaea related to the order *Methanobacteriales* in the sediment-inoculated reactors are made up entirely of the genus *Methanobacterium* while the order *Methanosarcinales* is constituted entirely of those related to the genus *Methanosaeta*.

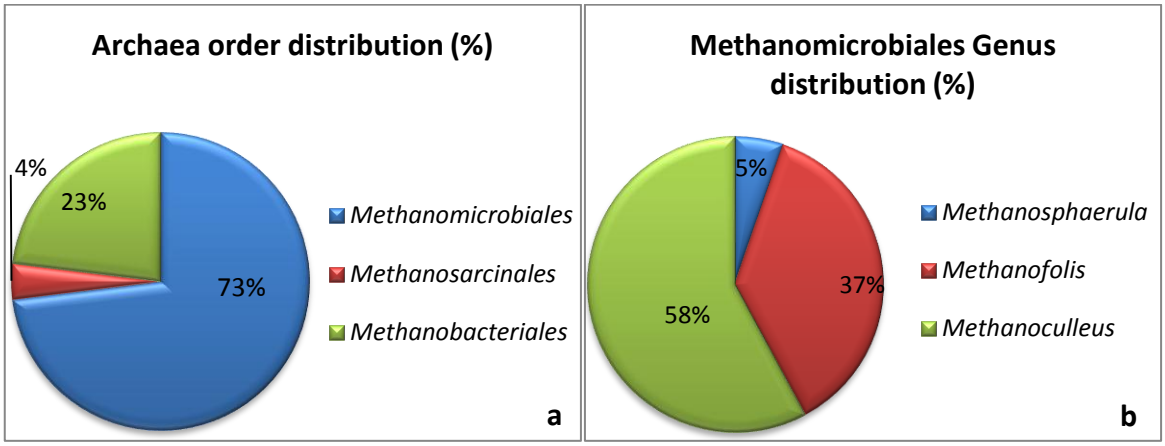

Fig. 7.1. Archaea (n=26) order distribution (a) and order *Methanomicrobiales* genus distribution (b), of clones retrieved from sediment inoculated reactors. *Methanomicrobiales* (n=19), *Methanobacteriales* (n=6), *Methanosarcinales* (n=1).

Interestingly, archaea related to the order *Methanosarcinales* was made up entirely of the genus *Methanosaeta*, which thrive mainly on acetate. A few studies have examined dominant archaea population within anaerobic reactors. For instance, a study of the diversity of archaea in wastewater treatment plant carried out by Fredriksson, et al., (2012), found that 63% (82 clones) of the total archaea population belong to the genus *Methanosaeta*, an indication of its importance during methane production. Similarly, a

study by Narihiro et al., (2009) found that *Methanosaeta* constitute 51-70% of the total archaea (49 clones) population of anaerobic digestion treatment plants studied using sequence-specific rRNA cleavage method. Reports suggest that the dominance of *Methanosaeta* is dependent on low acetate concentration for which it has high affinity, but at high acetate concentrations, it is outcompeted by the genus *Methanosarcina* (Kendall & Boone, 2006). Therefore, the detection of few *Methanosaeta*-like archaea in sediment-inoculated reactors might be an indication of high acetate concentration in the volatile fatty acids.

Analysis of the origin of the closest relatives of sediment-inoculated archaea clones retrieved from the NCBI database reveals the widespread of archaea community in natural anaerobic environments (Fig. 7.2).

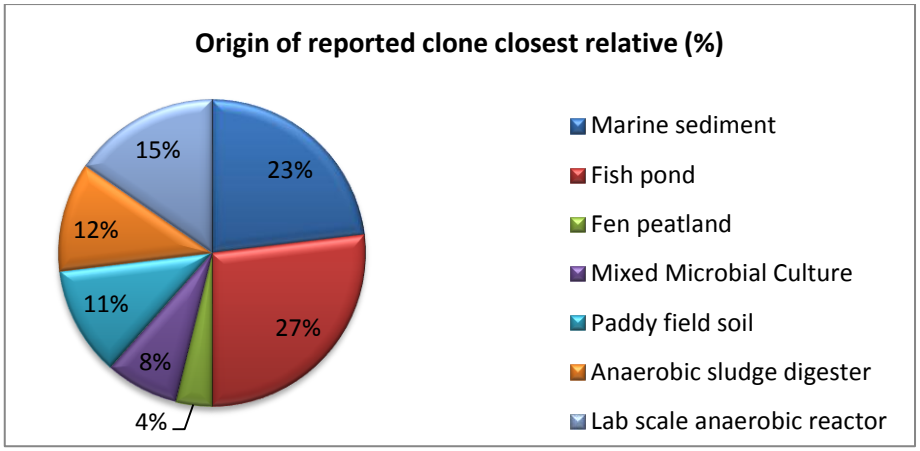

Fig. 7.2. Origin of closest relatives of archaea clones from sediment inoculated reactors. n=26.

About 27% of the archaea closest relatives were reported from fishpond while the origin of another 23% was reportedly traceable to marine sediment. A little over 27% of the nearest neighbours originated from anaerobic digester/reactors; whether lab-scale or full scale while paddy field, mixed culture and fen peatland accounted for 11, 8, and 4% respectively (Fig.7.2). Coincidentally, a bulk of the archaea community (65%, 18 clones) is traceable to soil or sediment based habitat. This result ties in with the source of the sediment inoculum employed in this study, which was drawn from sediment beneath a fish farm.

Table 7.1. Relationship of archaeal nucleotide sequences from **sediment** inoculated methane reactor compared with GenBank database

Clone[a]	**No of clones**	**Most closely related organisms**	**Accession number**	**Mean Sequence similarity (%)**	**Order**	**Functional group**[b]	**Source of closest relative**
SA2,SA3,SA12, SA19,SA24, SA25	6	*Methanoculleus marisnigri* strain JR1	NR_074174	99	*Methanomicrobiales*	H	Marine sediment
SA4,SA7,SA14, SA16, SA23, SA28, SA30	7	*Methanofollis formosanus* strain ML15	NR_042767	98	*Methanomicrobiales*	H	Fish pond
SA6, SA29	2	*Methanoculleus submarinus* strain Nankai-1	NR_028856	99	*Methanomicrobiales*	H	Deep marine sediment
SA8, SA13, SA18, SA21, SA22, SA26	6	Uncultured *Methanobacterium* sp.	FR836474	98.5	*Methanobacteriales*	H	Lab scale anaerobic reactor
SA10, SA15, SA17	3	*Methanoculleus chikugoensis* strain MG62	NR_028152	98.5	*Methanomicrobiales*	H	Paddy field in Japan
SA20	1	Uncultured *Methanosaeta* sp.	AM998443	96	*Methanosarcinales*	A	Marmara Sea sediment
SA31	1	*Methanosphaerula palustris* strain E1-9c	EU156000	94	*Methanomicrobiales*	H	Peatland

[a] clone number based on S (sediment) and A (archaea). [b] based on routes by which methane production is achieved. H : Hydrogenotrophic, A: Acetoclastic

7.3.2 Composition and phylogenetic analysis of archaeal community in sludge inoculated reactors

A total of 44 randomly selected archaea clones from sludge-inoculated reactors were sequenced and phylogenetically analysed. All the clones were at least 92% similar to characterised archaea in the GenBank database (Table 7.2). Results obtained show the dominance of *Methanomicrobiales*-like archaea, which accounted for 80% (35 clones) of all archaea in sludge-inoculated reactors. Archaea related to *Methanobacteriales* and *Methanosarcinales* accounted for only 9% each, while 2% of the archaeal community were unclassified (Fig. 7.3).

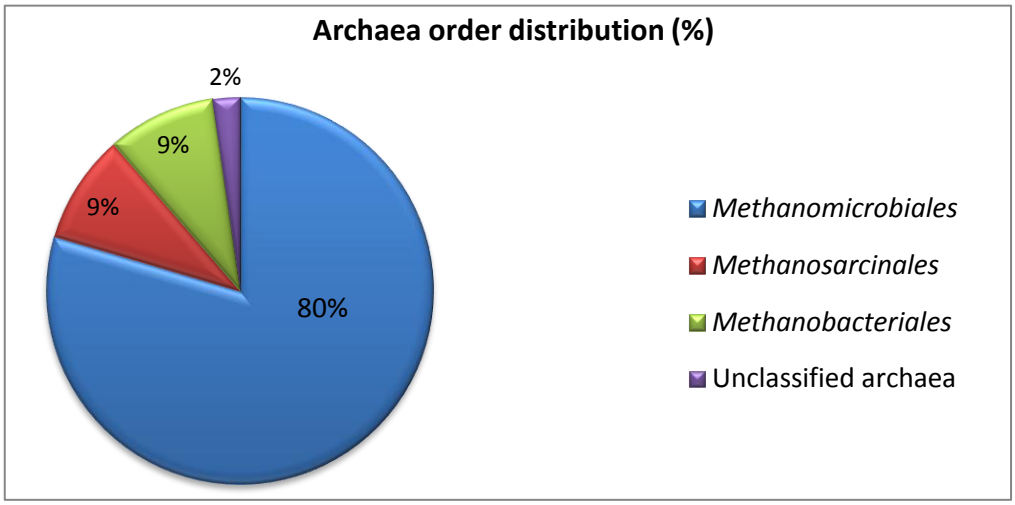

Fig. 7.3. Archaea (16S rRNA sequences) order distribution of clones from sludge inoculated reactors. n=44

Analyses of the various archaea orders retrieved from sludge-inoculated reactors revealed the presence of seven possible genera in the archaea community. Archaea related to the order *Methanomicrobiales*, apart from being the most dominant group, also represents the most diverse order of the archaea community with at least four possible genera (Fig.7.4).

Approximately a third (34%, 12 clones) of the order *Methanomicrobiales* are related to the genus *Methanospirillum*, while *Methanosphaerula*-like ones accounted for 26% (9 clones) of the order. Members of *Methanomicrobiales* related to the genera *Methanoculleus* and *Methanoregula* account for 17 and 14 % respectively, while 9% belongs to unknown genera (Fig. 7.4).

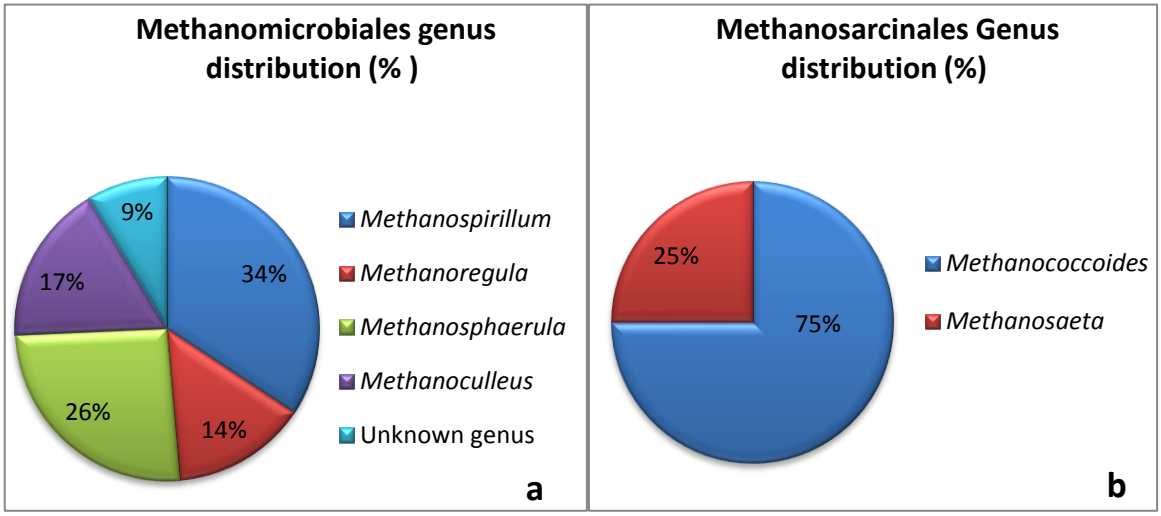

Fig.7.4. Genus distribution of archaea community belonging to the orders (a) *Methanomicrobiales* (n=35) and (b) *Methanosarcinales* (n=4) in sludge inoculated reactors

Archaea community related to the order *Methanosarcinales* were less diverse and only two genera were identified; *Methanococcoides* and *Methanosaeta* with the former being the dominant genus (Fig.7.4). Like in the sediment-inoculated reactors, archaea belonging to the order *Methanobacteriales* (n=4) was comprised entirely of the genus *Methanobacterium*.

Table 7.2. Relationship of archaeal nucleotide sequences from **sludge** inoculated methane reactor compared with GenBank database

Clone[a]	**Most closely related organisms**	**Accession number**	**Mean Sequence similarity (%)**	**Order**[b]	**Functional group**[c]	**Source of closest relative**
HA2, HA3, HA27, HA29, HA38	*Methanospirillum hungatei* strain JF-1	KM408634	99	Mm	H	Anaerobic sludge digester
HA4, HA31, HA42	*Uncultured Methanobacterium sp.*	FR836474	97.5	Mb	H	Anaerobic sludge
HA6	*Methanospirillum stamsii* strain PS	NR_117705	97	Mm	H	Anaerobic sludge
HA7	*Uncultured Methanococcoides sp*	AM980606	90	Ms	M	Sea sediment
HA8	Uncultured *Methanothermococcus* sp.	AM998446	92	Ms	A	Sea sediment
HA9, HA17, HA25, HA30, HA36	Uncultured *Methanoregulaceae* archaeon clone BNA156	KJ806528	96	Mm	H	Anaerobic sludge digester
HA10	*Methanospirillum hungatei*	M60880	98	Mm	H	Food waste digester
HA11, HA15, HA23 HA28, HA45, HA47, HA49	*Methanosphaerula palustris* strain E1-9c.	NR_074167	96.5	Mm	H	Organically rich wetland
HA12	Uncultured *Methanospirillum* sp. clone TS1A121	JF789588	98	Mm	H	Wetland soil
HA13, HA14, HA50	Uncultured *Mm* archaeon clone QECE1ZA081	KF198592	99	Mm	H	Anaerobic sludge

Clone[a]	Most closely related organisms	Accession number	Mean Sequence similarity (%)	Order[b]	Functional group[c]	Source of closest relative
HA16, HA19, HA20, HA43	*Methanospirillum hungatei* strain JF-1	NR_074177	98	Mm	H	Anaerobic sludge
HA21	Uncultured archaeon clone UAFB_TA_33_A29	KJ476548	98			anaerobic reactor
HA22	Uncultured *Methanobacterium sp.*	FR836474	99	Mb	H	Lab scale reactor
HA24, HA26, HA39, HA40, HA46, HA48	Uncultured *Methanoculleus sp.*	EU857631	97	Mm	H	Methane plant
HA33	Uncultured *Methanosarcinales* archaeon clone QEBH4ZF091	KF198803	99	Ms	A	Anaerobic sludge
HA34	Uncultured *Methanosphaerula sp.* clone 3_16_A9_a	JQ087676	97	Mm	H	Sediment core
HA37	*Methanosphaerula palustris* strain E1-9c	EU156000	95	Mm	H	Fen peatland
HA41	*Methanosaeta concilii* GP-6	CP002565	99	Ms	A	Lab scale reactor

[a] clone number based on H (sludge) and A (archaea). [b] Mm: *Methanomicrobiales*, Mb: *Methanobacteriales*, Ms: *Methanosarcinales* [c]based on routes by which methane production is achieved. H : Hydrogenotrophic, A: Acetoclastic, M: Methylotrophic.

Analysis of the prevalence or reported origin of the identified closest relatives of the archaea community in sludge-inoculated reactors indicates their prevalence in diverse anaerobic environments. Result obtained highlighted the similarity between sources of characterised archaea in the GenBank database and the source of inoculum in the current study. Most member of the archaea communities (78%, 34 sequences) have been previously isolated from anaerobic digester environment whether sludge and waste digestion or in lab scale reactors (Fig. 7.5) while about 20% have been isolated from soil or sediment environments.

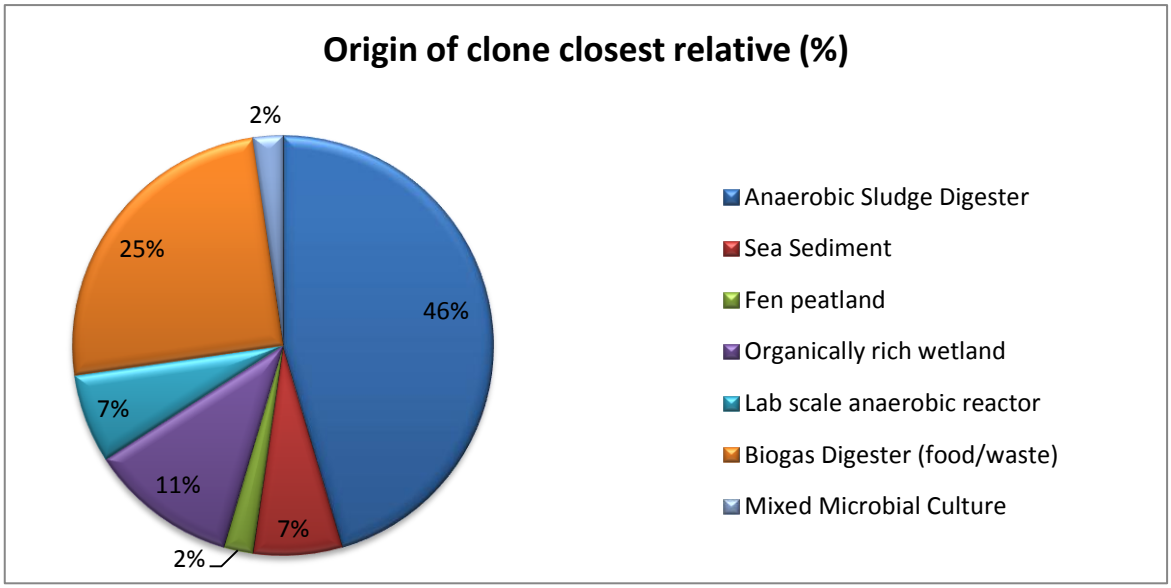

Fig. 7.5. Origin of closest relatives of archaea clones from sludge inoculated reactors. n=44

Comparison between archaea communities of sediment and sludge inoculated reactors reveals some distinct similarities and dissimilarities (Fig. 7.6). For instance, archaea community related to the order *Methanomicrobiales* dominated in both reactors. However, *Methanomicrobiales*-like population within the sludge-inoculated reactors was much more diverse. Within the *Methanomicrobiales*-like population, while there was no clearly dominant genus in the sludge-inoculated reactors, *Methanoculleus*-like archaea dominated

in sediment-inoculated reactors. Another very significant difference between sediment and sludge inoculated reactors is the occurrence of *Methanomicrobiales* related to the genus *Methanofolis* which constituted 37% (n=26) in sediment-inoculated reactors but were not detected in sludge inoculated reactors. In the same vein, archaea related to the genera *Methanospirillum* and *Methanoregula*, which accounted for 34 and 14% of the order *Methanomicrobiales* (n=35) in sludge-inoculated reactors respectively, were not detected in sediment-inoculated reactors (Fig. 7.6).

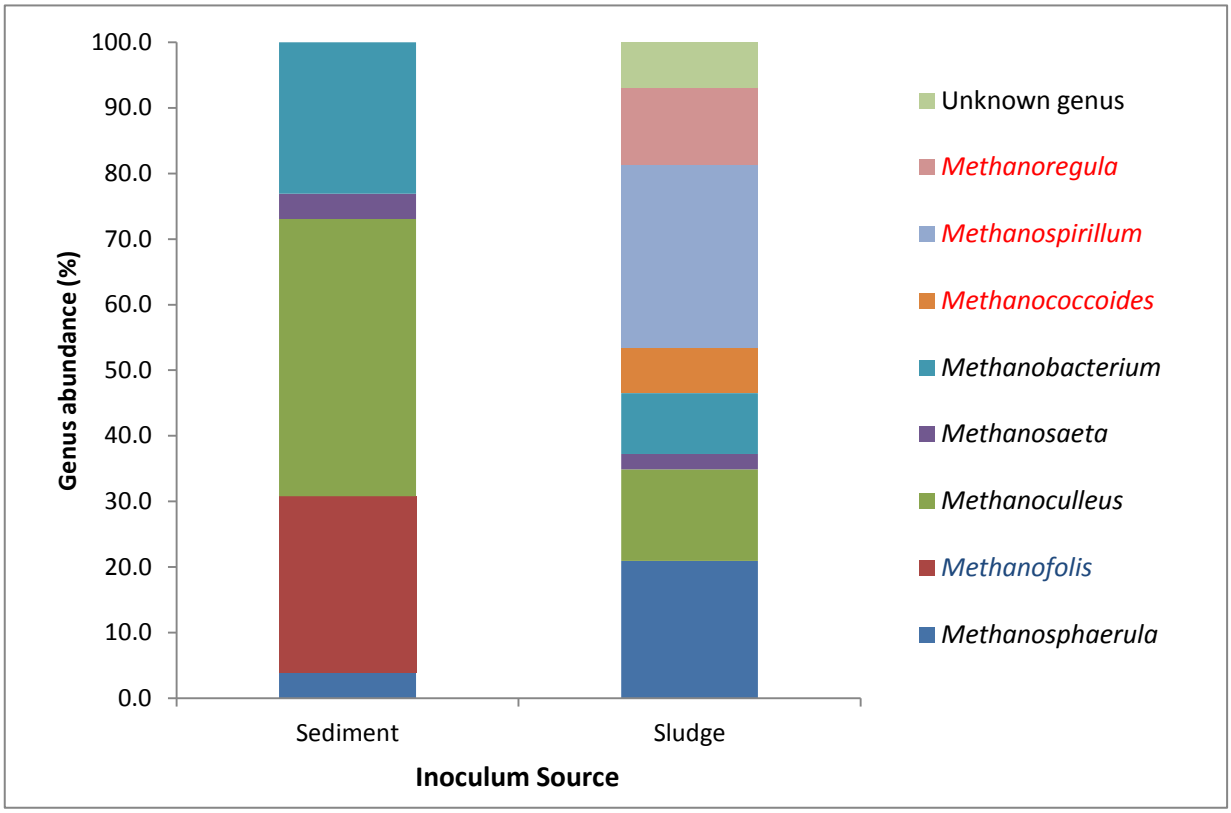

Fig 7.6. Genus distribution of the archaea population in sediment and sludge inoculated reactors. Genus in **blue** was detected only in sediment while genera in **red** were detected in sludge-inoculated reactors. Sediment n=26, Sludge n=44

The lower archaea diversity recorded in sediment-inoculated reactors also corroborates the results obtained from the DGGE analysis earlier reported (Chapter 6). This is an indication that the comparable amount of methane produced in sediment and sludge inoculated reactors was achieved by different groups of archaea.

Phylogenetic tree analysis of the 16S rRNA archaea sequence clones of both sediment and sludge inoculated reactors revealed a fairly diverse combined archaea community (Fig. 7.7). There were a few overlaps in the archaea community between the two sources of inoculum especially within those related to the order *Methanobacteriales*. However, clusters of different archaea community, based on the source of inoculum was observed within *Methanomicrobiales*-like archaea with most of the sludge-inoculated archaea closely related to the genus *Methanospirilium* while archaea from both sources of inoculum clustered closely to the genus *Methanoculleus*. Additionally, the order *Methanosarcinales* was populated almost entirely by archaea drawn from sediment-inoculated reactors (Fig. 7.7), an indication of the ability of environmental conditions to select for microbial composition of the ecosystem (Steinberg & Regan, 2008). However, results obtained revealed a more diverse archaea community within the sludge-inoculated compared to sediment-inoculated reactors. The reason for the limited archaea diversity in the anoxic sediment inoculum might be connected to the low temperature obtainable in the sediment which is not suitable for most organisms (Ciotola et al., 2013; Lianhua et al., 2010; Zhang et al., 2012). Nevertheless, the ability of archaea to thrive under extreme environmental conditions suggests that nutrient availability might also be the cause of limited archaea diversity in the sediment cores. Based on DGGE and clone library analyses, archaea diversity appears low in the sediment-inoculated reactors; this may be due a number of reasons, such as the limited number of sequences, especially from sediment-inoculated reactors. These results might therefore be different with a larger clone library in both cases.

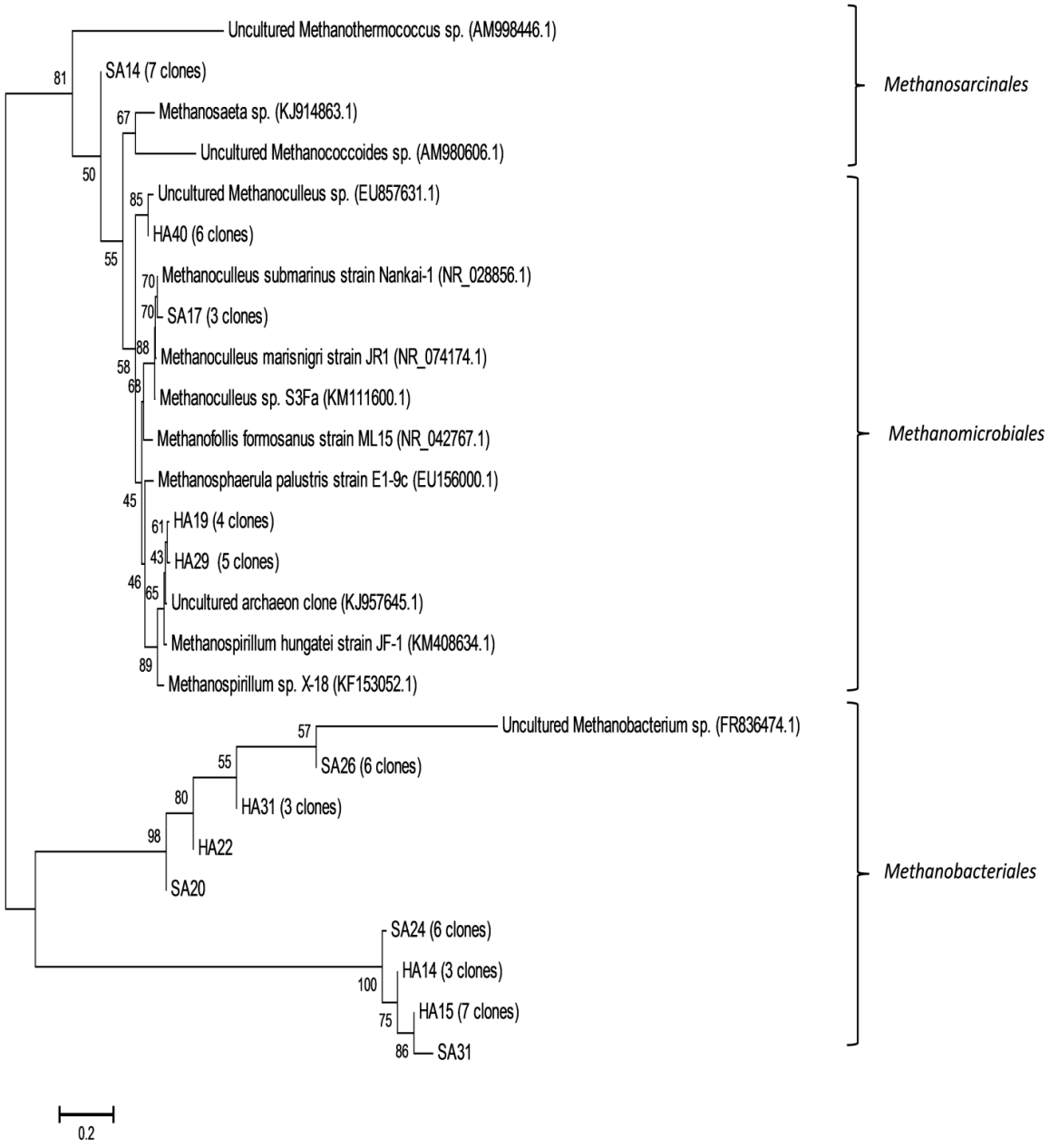

Fig. 7.7 . Phylogenetic tree showing the relationship between representative methanogen clones present in the sediment (SA) and sludge (HA) inoculated seaweeds reactors and reference organisms (sequences) retrieved from the GenBank database. Accession number of the reference sequences is listed in parenthesis. Phylogenetic tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 3.82933897 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 26 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1992 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

7.3.3 Phylogenetic analysis of methanogen's community in seaweed anaerobic reactors

7.3.3.1 Phylogenetic analysis of methanogen's community in sediment inoculated anaerobic reactors

Methanogenic archaea thrive in diverse anaerobic environments, and are responsible for methane production wherever they exist-whether in arctic sediment or hot springs. As different environments harbour various microbial communities, the current study attempts to exploit the degradative potential of microbial community of nutrient-rich, anoxic marine sediment for biomethane production from substrates of marine origin.

The *mcr*A gene fragment (~500bp) amplified and sequenced in this study impacted phylogenetic analysis resulting is as low as 72% methanogens similarity with entries in the NCBI database (Table 7.3). Similar approach by Steinberg & Regan, (2008), who conducted a phylogenetic comparison of methanogenic communities from acidic, oligotrophic fen and anaerobic digesters resulted in sequence similarity of as low as 69.3%. While the lowest similarity recorded for 16S rRNA gene study was 88%. The poor sequence similarity recorded in that study is likely due to the smaller (<500bp) fragment produced by available *mcr*A gene primers or the fact that there are currently fewer sequences in the *mcrA* databased compared to the 16S rRNA database.

Results of phylogenetic analysis of 28 randomly selected clones from sediment inoculated reactors reveals differences in order diversity and % distribution between methanogenic (*mcrA* gene sequence) and archaeal (16S rRNA gene sequence). Methanogen (*mcrA* gene) clone sequences were found to be 71-99% similar to known methanogens on the GenBank database (Table 7.3).

Although all the three orders detected within archaea community were also detected in the methanogens community, their distribution varied considerably (Fig. 7.8). Many studies

have demonstrated the significant variation between archaea detection with 16S rRNA gene and *mcrA* gene clone libraries depending on the environment under investigation (Cardinali-Rezende et al., 2009; Cho et al., 2013; Dhillon et al., 2005; Hwang, et al., 2010; Ma et al., 2013; Wirth et al., 2012). This is because not all the archaea community are potentially involved in the process of methane production. Differences in detected methanogenic archaea between 16S rRNA and *mcrA* clone libraries might also stem from lower available *mcrA* sequence database. As a result, less (*mcrA* gene) clone sequences might more appropriately represent the dominant methanogen groups within the reactors.

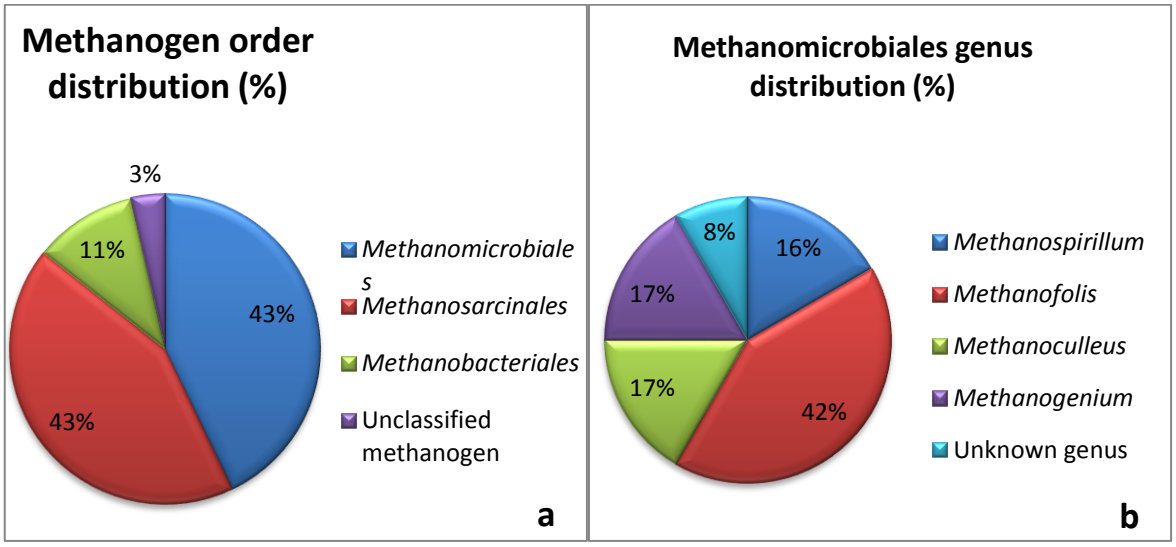

Fig. 7.8. Methanogen (*mcr*A gene sequences n=28) (a) community order and (b) *Methanomicrobiales* (n=12) genus distribution in the sediment inoculated seaweed reactors

For instance, while methanogens related to the order *Methanomicrobiales* (73%) dominated archaea (using 16S rRNA gene sequences) community in sediment-inoculated reactors with only 4% related to the order *Methanosarcinales*, both orders constitute similar proportion (43% apiece) of specific methanogen (using *mcr*A gene sequence) population in the sediment-inoculated reactors (Fig. 7.8). It appears these two orders of methanogens are very important to the process as demonstrated in literature. For example, a study of 21 different anaerobic sewage sludge digesters reported by highlighted the

dominance of methanogens related to *Methanomicrobiales* and *Methanosarcinales* detected by oligonucleotide probes (Garcia et al., 2006). This is an indication that some of the archaea (using 16S rRNA gene sequences) related to the order *Methanomicrobiales* were not likely involved in the actual methane production process. Although archaea related to the order *Methanobacteriales* accounted for 23% of the total archaea population when 16S rRNA gene was cloned, its prevalence dropped to 11% when the *mcr*A gene sequences were analysed (Fig. 7.8).

Interestingly, all the 43% *Methanosarcinales*-like methanogens are composed entirely of methanogens related to the genus *Methanosarcina*. This is very different from results of archaea 16S rRNA gene clone library, where archaea related to the order *Methanosarcinales* was made up entirely of the genus *Methanosaeta*. Research has shown that Methanogens belonging to the genus *Methanosarcina* are the most versatile of all methanogens as they are able to utilise a wide range of substrates including acetate and methyl compounds such as methanol and methylamines. They are also able to utilise H_2 to reduce methyl compounds whenever H_2 is available. In fact, they produce three CH_4 and one CO_2 for every methyl compound metabolised (Kendall & Boone, 2006). Apart from its ability to produce methane through both acetoclastic and hydrogenotrophic methanogenesis pathways, *Methanosarcina* show high growth rates, with a doubling time of 1-1.2 days (as opposed to 4-6 days in other methanogens) and are more tolerant to sudden changes in pH than other methanogens (S. K. Cho et al., 2013; Ma et al., 2013). With these qualities, anaerobic rectors dominated by this group of (*Methanosarcina*) methanogens would potentially by highly productive.

Table 7.3. Relationship of methanogen nucleotide sequences from **sediment** inoculated methane reactor compared with GenBank database

Clone(s)[a]		**Most closely related organisms**	**Accession number**	**Range of % similarity (mean)**	**Order**[b]	**Functional group**[c]	**Source of closest relative**
SMcr1, SMcr5, SMcr12, SMcr15, SMcr22, SMcr23, SMcr27, SMcr29	8	*Methanosarcina baltica* DSM 14042	LC015100	84-94 (93.5)	Ms	M, A	Deep sediment
SMcr2, SMcr17**,** SMcr24,	3	Uncultured *Methanobacterium sp*.	FR836474	93-99 (95)	Mb	H	Lab scale reactor
SMcr3		Uncultured archaeon clone R45_0d_D5	EU201180	92			Deep sea sediment
SMcr4		Uncultured *Methanoculleus sp*. clone PB11-0231-24	KJ487637	81	Mm	H	Methane plant
SMcr6		*Methanosarcina lacustris*	AY260443	84	Ms	A	Cold terrestrial habitat
SMcr7, SMcr10	2	*Methanosarcina semesiae* strain MD1	NR_028182	71-77 (74)	Ms	A	Mangrove sediment
SMcr8		Uncultured Methanospirillum sp.	JQ684567	91	Mm	H	Sewage sludge
SMcr9		*Methanogenium marinum* strain AK-1	NR_028225	95	Mm	H	Cold sediment
SMcr11, SMcr14, SMcr25, SMcr26	3	*Methanofollis formosanus* strain ML15	NR_042767	88-95 (90)	Mm	H	Fish pond

Clone(s)[a]		Most closely related organisms	Accession number	Range of % similarity (mean)	Order[b]	Functional group[c]	Source of closest relative
SMcr13		*Methanofollis ethanolicus* strain 104120	AB703643	90	Mm	H	Anaerobic sludge
SMcr18		*Methanosarcina barkeri* strain TR-Z13	HQ591417	90	Ms	A	Sediment
SMcr19, SMcr30	2	Uncultured *Methanomicrobiaceae* archaeon clone KM69	DQ085326	92-98 (95)	Mm	H	Landfill
SMcr20		*Methanogenium marinum*	DQ229159	72	Mm	H	Marine sediment
SMcr21		*Methanospirillum hungatei* strain JF-1	NR_112982	98	Mm	H	Sewage sludge

[a] clone number based on S (sediment) and Mcr (methanogen). [b] Mm: *Methanomicrobiales*, Mb: *Methanobacteriales*, Ms: Methanosarcinales

[c]based on routes by which methane production is achieved. H : Hydrogenotrophic, A: Acetoclastic, M: Methylotrophic.

Dominance of methanogens related to the genus *Methanosarcina* in anaerobic reactors studied using the functional (*mcrA*) gene analysis alongside methane production, is suggestive of active methanogenesis. For instance, in a study of dry anaerobic digestion of food waste under mesophilic conditions, Cho et al., (2013) reported the dominance of the methanogens belonging to the genus *Methanosarcina* (99%) resulting in high rate of methane production in those reactors. These qualities, no doubt contributed to the methane production in sediment-inoculated reactors despite low methanogen numbers and diversity.

Phylogenetic analysis of methanogens related to the order *Methanomicrobiales* from the sediment-inoculated reactors showed that unlike archaea 16S rRNA gene sequence analysis, where the order was made up of about three genera, methanogenic (*mcr*A gene sequences) order *Methanomicrobiales* was much more diverse with potentially four genera detected.

Studies of microbial ecology in bioreactors and other anaerobic environments have found significant variations in the contribution of the order *Methanomicrobiales* to the methanogenic community (Cho et al., 2013; Garcia et al., 2006; Juottonen, 2008; Ma et al., 2013; Milferstedt, et al., 2010; Narihiro & Sekiguchi, 2011; Narihiro et al., 2009; Yavitt, et al, 2012).

Two methanogen-like genera (*Methanospirillum* and *Methanogenum*) which, were not detected with 16S rRNA sequences were represented within the methanogen community of the sediment-inoculated reactors. This observation suggests their potential involvement in the methane production process (Garcia et al., 2006; Ma et al., 2013). However, one genus (*Methanosphaerula*-like), which was detected in the archaea *Methanomicrobiales* community (using 16S rRNA sequences), was not found in the methanogen community, an

indication of its lack of involvement in actual methane production in the sediment-inoculated reactors.

The disappearance or lack of involvement of the genus *Methanosphaerula* in active methanogenesis has been previously reported. For instance, during a microbial ecology study of anaerobic sequential batch reactor operated at short hydraulic retention time, Ma et al., (2013), showed the presence of *Methanosphaerula* in the general archaea community, but were not detected during active methanogenesis,. Cho et al., (2013) also reported the presence of *Methanosphaerula* at the start of a food waste dry AD process, which disappeared (was not detected) during active methane production stages.

The other two genera of the order *methanomicrobiales* (*Methanoculleus* and *Methanofolis*) were detected with both 16S rRNA and *mcr*A gene sequences, this illustrates that they are potentially active members of the community and may theoretically play a role in process function and stability.

Methanoculleus spp. have been reported to be involved in syntrophic oxidation of acetate in digesters containing high concentration of VFAs and as a result compete with acetoclastic methanogens for acetate (Cardinali-Rezende et al., 2009; Dhillon et al., 2005; Hwang, et el., 2010). They also have the ability to metabolise a wide range of substrates such as H_2+ CO_2 , formate, 2-propanol + CO_2 , or 2- butanol + CO_2 for methane production (S. K. Cho et al., 2013; Garcia et al., 2006). The contribution of *Methanoculleus* spp. to hydrogenotrophic methanogenesis in full-scale anaerobic reactor digesting activated sludge has also been reported (Guo et al., 2015; Kröber et al., 2009; Schlüter et al., 2008).

The abilities of members of *Methanogenium* and *Methanoculleus* genera of *methanomicrobiales* to thrive in marine and salty environment (Garcia et al., 2006) might

account for their widespread distribution in the current study at seawater salinity conditions. The genus *Methanofollis* is another halotolerant group of hydrogenotrophic methanogen able to metabolise a wide range of substrates such as H_2+ CO_2, formate, 2-propanol + CO_2, 2- butanol + CO_2, or cyclopentanol + CO_2 but cannot utilise acetate, methanol, ethanol or dimethylamine. It has been detected in a number of habitats such as oil fields (Kryachko, et al., 2012), fish ponds (Banning et al., 2005), rumen of buffaloes (Franzolin, et al., 2012) as well as in anaerobic sludge digesters (Nayak et al., 2009). However, it dominates the order *Methanomicrobiales* in sediment inoculated reactors and interestingly, it was only detected in sediment-inoculated reactors in the current study. The substantially high (>350 ml/gVS) methane produced from sediment-inoculated seaweeds' reactors in this study, suggests that most of the methanogens including the genus *Methanofollis* were actively involved in methane production.

Analyses of the reported origin of the methanogen clones closest relatives showed that majority of the methanogens (79%) were originally isolated from sediment or soil habitats (Fig. 7.9). This is an indication that inoculum (methanogens) capable of supporting efficient methane production from marine biomass could be obtained from marine sources.

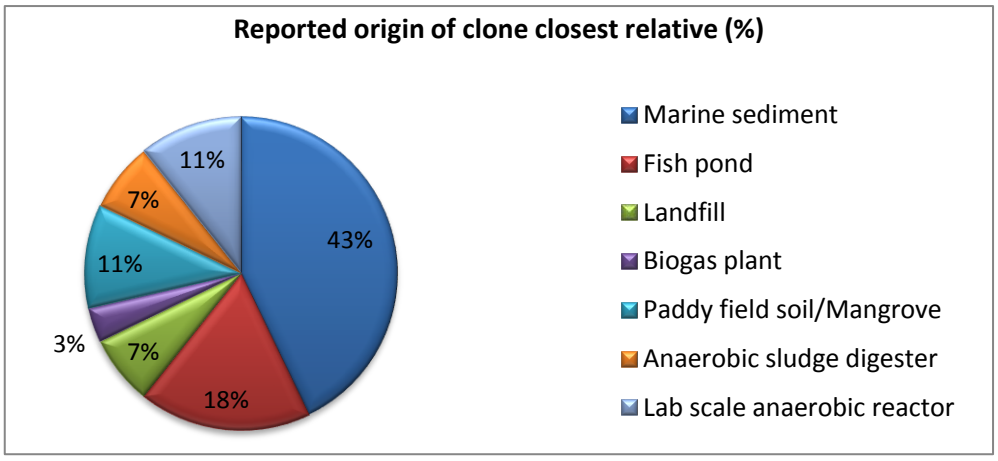

Fig. 7.9. Origin of closest relatives of methanogen clones from sediment inoculated reactors. n=28

Generally, results obtained suggest that although general diversity and numbers were low at earlier stages during the digestion process, highly efficient methanogens were selected and were active in the sediment-inoculated reactors. These efficient methanogens brought about the substantially high methane production recorded in the sediment inoculated seaweed reactors.

7.3.3.2 Phylogenetic analysis of methanogen's community in sludge-inoculated anaerobic reactors

Phylogenetic analysis of 28 randomly selected cloned *mcr*A gene sequences from sludge inoculated seaweeds reactors revealed the presence of at least three methanogen-like orders namely: *Methanomicrobiales* (39%), *Methanosarcinales* (36%) and *Methanobacteriales* (21%) with about 4% unclassified methanogens (Fig. 7.10). Cloned sequences were found to be 75-99% similar to methanogens in the GenBank database (Table 7.4). The result corroborates reports of the dominance of genera *Methanomicrobiales* and *Methanosarcinales* in various anaerobic digesters (Narihiro et al., 2009; Nayak et al., 2009; Sundberg et al., 2013; Tabatabaei et al., 2010). It is also an indication that acetoclastic, hydrogenotrophic and methylotrophic methanogenesis were all potentially active during methane production in the sludge-inoculated reactors.

Further phylogenetic analyses to the genus level showed that methanogens related the order *Methanomicrobiales* were less diverse in sludge-inoculated reactors than in sediment inoculated ones. Three genera were recorded namely *Methanospirillum* (37%), *Methanoculleus* (36%) and *Methanogenium* (27%). Interestingly, methanogens related to the genus *Methanofollis*, which made up the bulk (42%) of the order *Methanomicrobiales* in sediment-inoculated reactors were not detected in the sludge-inoculated reactors. Previous research has shown that the genus *Methanofollis* is present mainly in sediments

and fishponds (Banning et al., 2005; Garcia et al., 2006; Parkes et al., 2012; Kryachko et al., 2012); this might be the reason it was not detected in the sludge inoculated reactors.

Results of phylogenetic analysis of methanogens related to the order *Methanosarcinales* to the genus level revealed a more diverse order (compared to sediment-inoculated reactors) with three possible genera represented. Incidentally, *Methanosaeta*-like methanogens, which were not detected in sediment inoculated reactors accounted for half (50%) of the order *Methanosarcinales* in sludge inoculated reactors (Fig.7.10). *Methanosaeta* is the only genus of methanogen that thrives exclusively on acetate. It is one of the most reported genera of methanogens, occurring in a wide range of habitats (Calli et al., 2006; Demirel & Scherer, 2008; Dhillon et al., 2005; Ellis, et al., 2012; Galand, et al., 2005; McHugh, et al., 2003; Narihiro et al., 2009; Rincón, et al., 2008; Salvador, et al., 2013; Smith & Ingram-Smith, 2007; Tabatabaei et al., 2009; Yu et al., 2014). Although it thrives under low acetate concentrations (Garcia et al., 2006), its dominance in anaerobic reactors and other anaerobic environment has been widely reported (Fernandez et al., 2000; Glissmann, et al., 2004; Parkes et al., 2012; Keyser et al., 2006; McHugh, et al, 2003; Salvador, et al, 2013; Smith & Ingram-Smith, 2007; Song, Shin, & Hwang, 2010; Williams et al., 2013; Yu et al., 2014; Zhang et al., 2012). This observation forms part of the unique distinctions between the two sources of inoculums.

The second genus of methanogens related to the order *Methanosarcinales*; *Methanosarcina* accounting for 40%, is the most versatile of all methanogens, in many respect. It is the fastest growing methanogen group, with a doubling time as low as 24 hours and can metabolise a wide range of substrates including acetate, for which it outcompetes *Methanosaeta*. It also has the ability to tolerate and cope with drastic changes in environmental conditions such as pH better than any other methanogens (Kendall &

Boone, 2006; Von Klein, et al, 2002). These qualities likely make members of the *Methanosarcina* dominate anaerobic environments especially under special conditions; such as in anaerobic reactors operated at short retention time (Ma et al., 2013), abandoned coal mines (Beckmann et al., 2011), in salt-mesh creek sediment (Parkes et al., 2012) and during anaerobic digestion of brown algae under high salinity (Miura et al., 2014). The presence or dominance of *Methanosarcina,* is therefore seen as an indication of high acetate concentration as well as increased methanogenesis.

Table 7.4. Relationship of methanogen nucleotide sequences from **sludge** inoculated methane reactor compared with GenBank database

Clone(s)[a]	**Most closely related organisms**	**Accession number**	**Range of % similarity (mean)**	**Order**[b]	**Functional group**[c]	**Source of closest relative**
HMcr1, HMcr2	*Methanospirillum stamsii strain PS*	NR_117705	94 (94)	Mm	H	Anaerobic batch digester
HMcr4	*Methanolobus taylorii*	MTU22243	78	Ms	A	sludge anaerobic digester
HMcr6	*Methanobrevibacter smithii isolate ACE6.*	LK054626	86	Mb	H	Rumen methanogens
HMcr9, HMcr10	*Uncultured Methanosaeta sp. clone JM-ASBR*	JQ684541	88 (88)	Ms	A	Anaerobic batch reactor
HMcr29	*Methanosarcina baltica* DSM 14042	AB973356	95	Ms	M, A	Deep sediment Baltic Sea
HMcr11	Uncultured Methanosaeta sp. clone HALEY_A16	AM998443	98	Ms	A	Anoxic marine sediment
HMcr12	Uncultured archaeon clone mcrA_dig_E17	EU980410	99			Sludge anaerobic digester
HMcr24	Uncultured *Methanobacterium sp*.	KM259858	94	Mb	H	hypersaline soda lakes
HMcr14	Uncultured *Methanobacterium* sp. clone G2-13_090821	KJ487582	77	Mb	H	mesophilic grass silage fermenter
HMcr25	Uncultured *Methanosaeta* sp. clone	KC618385	82	Ms	A	anaerobic digester fed with swine manure
HMcr15	*Methanosarcina barkeri* str. Fusaro	NR_074253	99	Ms	M, A	mud at a freshwater lake

Clone(s)[a]	**Most closely related organisms**	**Accession number**	**Range of % similarity (mean)**	**Order**[b]	**Functional group**[c]	**Source of closest relative**
HMcr3, **HMcr7**, HMcr16	Uncultured *Methanoculleus sp*.	EU857631	75-91 (83)	Mm	H	Methane plant
HMcr17	*Methanosarcina barkeri* str. Wiesmoor	CP009526	78	Ms	M, A	Iron corrossion-inducing microbial community
HMcr22, HMcr23	*Uncultured Methanobacterium sp.*	FR836474	89	Mb	H	Lab scale anaerobic reactor
HMcr26	Uncultured *Methanobacterium* sp. clone SB1	KJ442934	99	Mb	H	maize silage fed anaerobic digester
HMcr21	*Methanoculleus marisnigri* strain JR1	NR_074174	90	Mm	H	Cold sediment
HMcr27	*Methanogenium cariaci strain JR1*	NR_104730	96	Mm	H	marine sediments
HMcr18	Methanogenium organophilum	AB353222	92	Mm	H	Methanotrophic communities
HMcr30	*Methanosarcina barkeri*	HQ591417	95	Ms	M,A	Sediment
HMcr19	*Methanothermobacter thermautotrophicus*.	X07794	78	Mb	H	Paddy soil
HMcr28	*Methanogenium marinum strain AK-3*	DQ177345	94	Mm	H	Marine sediment
HMcr8, HMcr20	*Methanospirillum hungatei* strain JF-1	NR_074177	80-97 (87)	Mm	H	anaerobic digester sludge

[a] clone number based on H (sludge) and Mcr (methanogen). [b] Mm: *Methanomicrobiales*, Mb: *Methanobacteriales*, Ms: Methanosarcinales [c]based on routes by which methane production is achieved. H : Hydrogenotrophic, A: Acetoclastic, M: Methylotrophic.

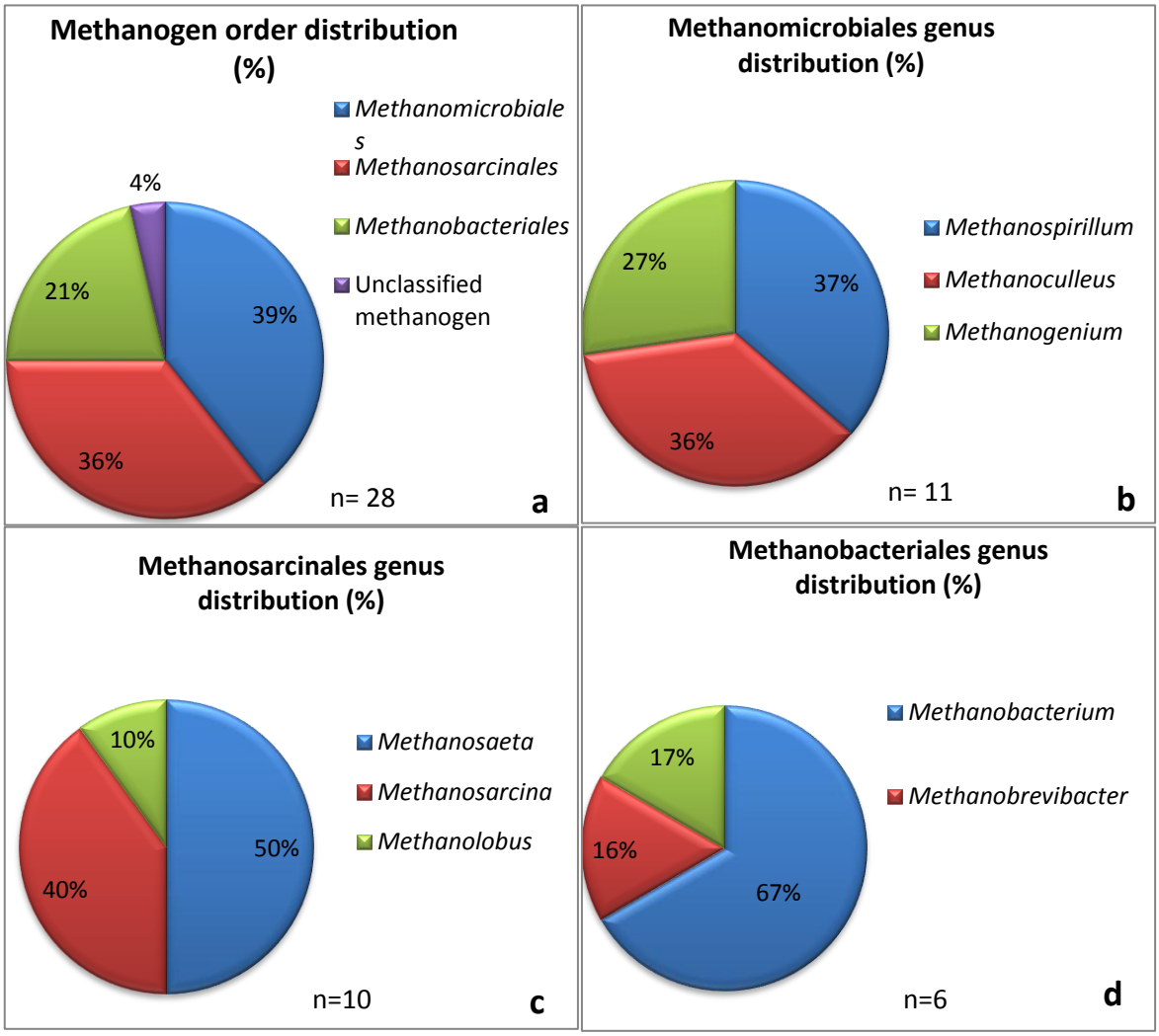

Fig. 7.10. Methanogens distribution by (a) Order and genus distribution of the Orders (b) *Methanomicrobiales* (c) *Methanosarcinales* and (d) *Methanobacteriales*.

The third *Methanosarcinales*-like genus, *Methanolobus* which constitute 10% of the order (Fig.7.10), is both halophilic and exclusively methylotrophic; metabolising methanol, methylamines and sometimes methyl sulphides (Kendall & Boone, 2006). Although not detected in the archaea community using 16S rRNA sequences, identification of methanogens related to *Methanolobus* within the *mcrA* (functional) gene clone library is an indication of the likelihood of their involvement in actual methane production during the process. This genus of methanogens was also not detected in the methanogen community of sediment-inoculated reactors (Fig. 7.11). Its ability to utilize substrates other than

hydrogen and acetate for which other methanogens compete has been suggested as a way to carve a niche for itself and avoid competition for substrates, especially in harsh conditions (Parkes et al., 2012). In fact, pyrosequencing of methanogen sequences from coal bed reservoir which showed methylotrophic methanogenesis as the main route towards methane production showed that the genus *Methanolobus* was the dominant methanogen responsible methane production in that environment (Guo et al., 2012). *Methanolobus* has also been detected in large numbers in mesothermic oil fields (Kryachko et al., 2012).

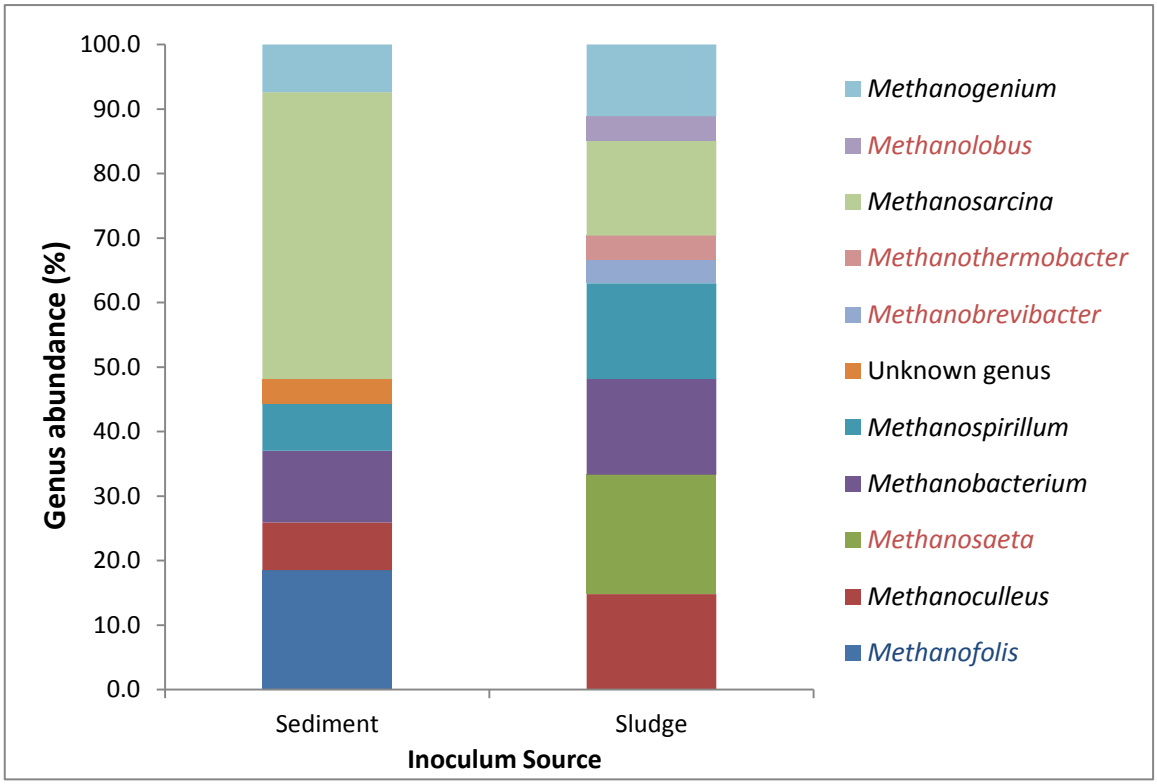

Fig 7.11. Genus distribution of the methanogen population in sediment and sludge inoculated reactors. Genus in **blue** occurs only in sediment while those in **red** were detected only in sludge-inoculated reactors.

Methanogens related to *Methanobacteriales* (third methanogen order) retrieved from the sludge-inoculated reactors in this study are likely to be more diverse than those from sediment-inoculated reactors; with potentially three genera represented. Those related to the genus *Methanobacterium* constitute the bulk of the order accounting for 67%, while

those related to the genera *Methanothermobacter* and *Methanobrevibacter* accounted for 17 and 16% respectively. All three hydrogenotrophic genera have been detected in various AD processes. For instance, Franke-whittle, et al., (2014), while investigating the effects of high concentrations of volatile fatty acids on methanogenic communities during anaerobic digestion, reported the detection and stability of the *Methanobacterium* during stages of increased VFA levels. The authors posited that the numbers or activities of *Methanobacterium* are not affected by the VFA concentrations. *Methanobacterium* has also been detected in landfills (Luton, et al., 2002), as well as in gut of animals such as buffalo (Chaudhary, et al., 2012). *Methanobacterium* cannot survive the absence of utilisable hydrogen, which affect their important role in granulation involving production of extracellular polymer for granules formation (Keyser et al., 2006). *Methanothermobacter* on the other hand has been reported to dominate thermophilic anaerobic reactors because of its ability to withstand high temperatures (Franke-whittle et al., 2014). *Methanobrevibacter* is another genus of *Methanobacteriales* detected only in the sludge-inoculated reactors. Like the other members of the order, it is hydrogenotrophic and use formate as a carbon source (Tabatabaei et al., 2010). As in the current study, it was detected in low quantity (9%) in methane plants treating maize silage (Nettmann et al., 2008), but dominate in rumen of sheep (Chaudhary et al., 2012; Yu, et al., 2008). It has been described as acid tolerant due to its ability to thrive at below pH 6 (Hao, et al., 2012).

Previously reported cloning and sequencing as well as pyrosequencing techniques have shown significant variation in the archaea and methanogen community of various anaerobic bioreactors. Many factors are responsible for these variations, which include operational and environmental conditions. This was attested to by Franke-whittle et al., (2014), who investigated the effects of high concentration of volatile fatty acids during anaerobic digestion on methanogenic communities and reported that although the

community composition of methanogen may be similar, the structure in highly dependent on operational temperatures. The authors concluded that mesophilic conditions favours higher archaea (and methanogen) diversity. Other factors such as substrate composition and the stage of the anaerobic digestion process also play important roles in determining microbial ecology of AD facilities. For instance, microbial population studies of laboratory scale solid waste reactors investigated by Nayak et al., (2009), showed the detection of representatives from three different archaea orders; *Methanomicrobiales*, *Methanosarcinales* and *Methanobacteriales* at the start of the process, however in the latter stages of the process, only archaea related to the order *Methanomicrobiales* were detected. Similarly, Sundberg et al., (2013), utilized 454 Pyrosequencing technique to analyse microbial richness of 21 full scale methane reactors and found the dominance of archaea belonging to the order *Methanosarcinales* and the genus *Methanosaeta* in particular, during active methanogenesis, suggesting that acetoclastic methanogenesis was the favoured route towards methane production in those reactors. Another study of the diversity of archaea in wastewater treatment plant carried out by Fredriksson, et al., (2012), also found that 63% of the total archaea population belong to the genus *Methanosaeta*. Similarly, a study by (Narihiro et al., 2009) found that *Methanosaeta* constitute 51-70% of the total archaea population of anaerobic digestion treatment plants studied using sequence-specific rRNA cleavage method.

However, Song, et al., (2010), while investigating methanogenic population dynamics in upflow anaerobic digester sludge blanket treating swine wastewater reported the dominance of methanogens related to the order *Methanobacteriales*, suggesting that hydrogenotrophic methanogenesis was the main route during methane production. Zhu et al., (2011), also found the dominance of *Methanobacteriales* (58%) in anaerobic methane reactor fed with swine faeces using *mcr*A gene-sequence analysis.

Conversely, other reports on archaea and methanogen population studies have shown the dominance of archaea and methanogens belonging to the order *Methanomicrobiales*. For instance, the methanogenic community of methane reactor treating cattle dung investigated by Rastogi et al., (2008), showed that *Methanomicrobiales* constituted 41% of the total methanogen population in the summer months. Their proportion increased considerably to 98% by winter months. Similarly, Munk et al., (2010) while studying the population dynamics of methanogens during acidification of methane fermenters treating maize silage, found that *Methanomicrobiales* was the dominant group of methanogens in the fermenters. Using pyrosequencing approach to phylogenetically characterize the microbial community of anaerobic digesters fed with maize silage, Kröber et al., (2009) reported that archaea belonging to the order *Methanomicrobiales* was the dominant methanogens in the reactors. Again, microbial community dynamics studies of low temperature (15^{o}C) anaerobic wastewater treatment bioreactor showed that even at low temperatures, *Methanomicrobiales* was the dominant archaea (O'Reilly et al., 2010).

The current study found representatives of *Methanomicrobiales*, *Methanosarcinales* and *Methanobacteriales* within the archaea and methanogen populations. This was also the case at the start of a microbial population studies of lab-scale solid waste reactors in the presence or absence of biosolids carried out by Nayak et al., (2009). It demonstrated the dominance of *Methanomicrobiales* within the archaea (16S rRNA gene sequence) and methanogen (*mcr*A gene sequences). Results in this study suggest that hydrogenotrophic methanogenesis was the main route towards biomethane production. However, the contribution of other groups (orders) of archaea and methanogens likely proved important to process functions, stability and productivity.

Among the few reports of anaerobic digestion of seaweeds (whether inoculated with sediment or sludge), only a couple of reports contains information about the activities of

the microbial consortiums (Hinks et al., 2013; Miura et al., 2014) during the process. In one of the studies, Miura et al., (2014), evaluated the use of anoxic sediment as the source of inoculum for seaweeds anaerobic digestion under high salinity. The results of the study showed that considerable amount of methane could be produced using this method. However, methane production was inhibited after day 27. Analysis of the microbial community in that study revealed the dominance of archaea belonging to the order *Methanococcus* (hydrogenotrophic) and low *Methanosarcinales* (mostly acetoclastic) numbers resulting in the inability to utilise VFAs produced-especially acetate, which resulted in poor methane production. The report of Miura et al., (2014) on the dominance of *Methanococcus* in the AD sub-culture was based on partial sequence of 16S rRNA gene, which could be different, should *mcrA* gene analysis have been carried out.

Phylogenetic trees analysis based on *mcrA* gene sequences of selected clones reveals the diversity obtained from the methanogen clone library (Fig.7.12). Results obtained highlighted the dominance of methanogens belonging to the order *Methanomicrobiales* as well as its distribution across the type and source of inoculums. As earlier observed, the results showed that the methanogen community obtained from mcrA gene sequences were more diverse than archaea community using 16S rRNA gene clone libraries. A similar scenario was reported by Steinberg & Regan, (2008) who conducted a phylogenetic comparison of methanogens from oligotrophic fen and anaerobic digesters. In that study, although lower sequence similarity was reported for *mcr*A gene sequences compared to 16S rRNA gene sequences; higher diversity was recorded within the methanogen community than within the archaea community.

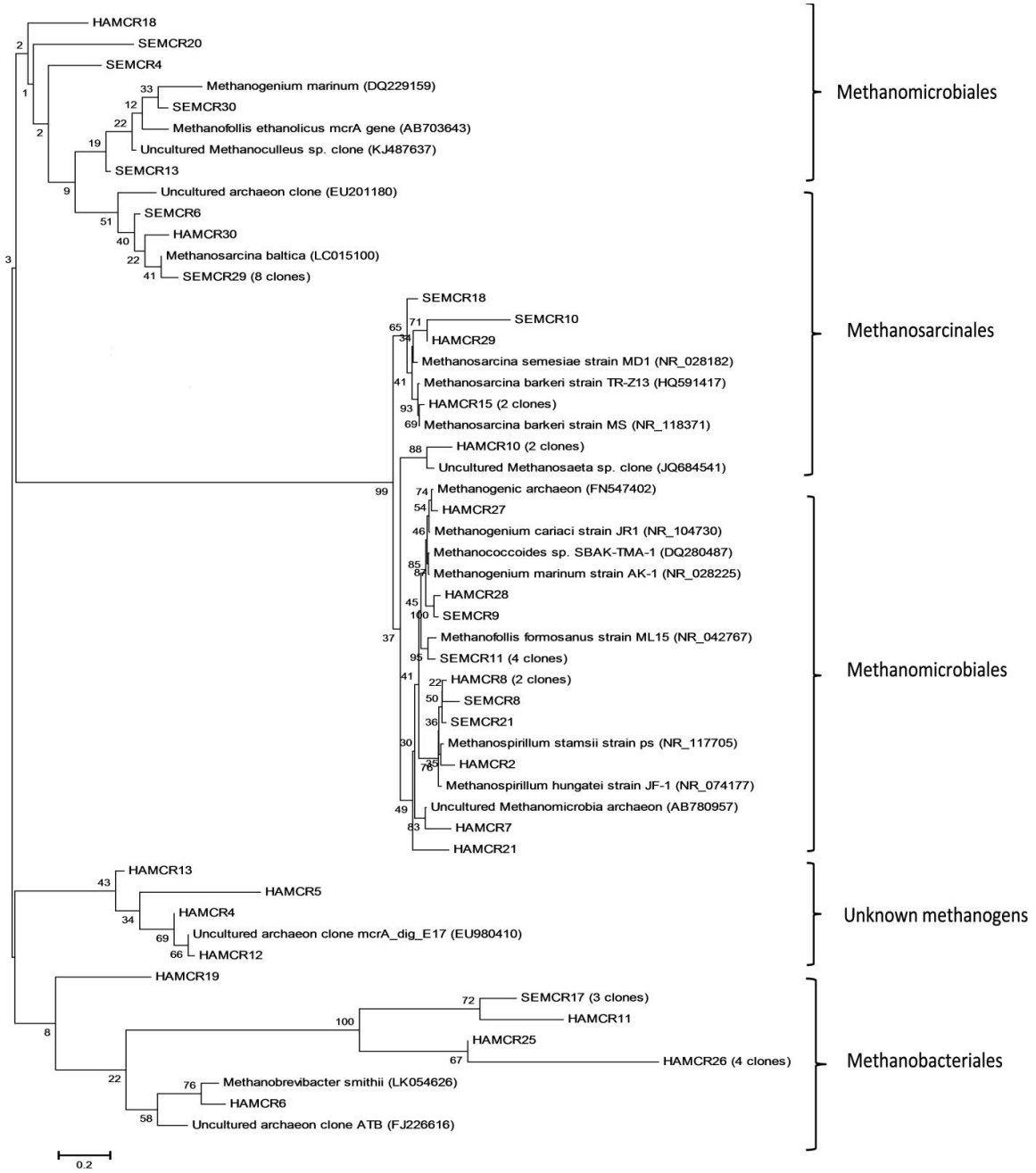

Fig. 7.12. Phylogenetic tree showing the relationship between representative methanogen clones present in the sediment (SEMCR) and sludge (HAMCR) inoculated seaweeds reactors and reference organisms (sequences) retrieved from the GenBank database. Accession number of the reference sequences is listed in parenthesis. Phylogenetic tree was inferred using the Neighbour-Joining method (Saitou N. and Nei M. **1987**). The optimal tree with the sum of branch length = 11.01316005 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein J. **1985**). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method. (Tamura K., Nei M., and Kumar S. **2004**) and are in the units of the number of base substitutions per site. The analysis involved 53 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1845 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Tamura K., et al. **2013**).

Unlike the archaea clones phylogenetic analysis, members of the methanogen community were widespread throughout the three orders detected. There are however differences in genera affiliations across the different orders (Fig.7.12). For instance, a number of methanogens clones from the sediment-inoculated reactors closely related to the genus *Methanofollis* were not detected in sludge-inoculated reactors while the reverse was the case with the genera *Methanosaeta*, *Methanobrevibacter*, *Methanothermobacter*.

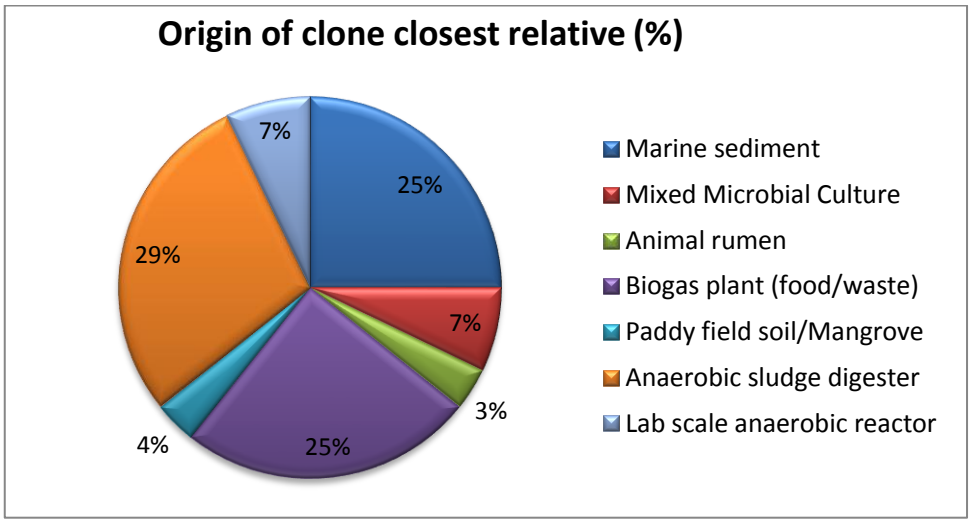

Fig. 7.13. Origin of closest relatives of methanogen (*mcr*A gene) clones from sludge inoculated reactors. n=28

Analysis of the origin of the methanogens closest relatives of sludge-inoculated reactors showed that the methanogens present in the seaweeds digesters can be found in various anaerobic environments including animal rumen (Fig 7.13). However, the results obtained showed that the bulk of the methanogens (>60%) were originally isolated from anaerobic sludge digester or other methane plants, which is consistent with the source of the inoculum in the current study.

7.4 Conclusion

There is currently a lack of consensus on the main cause of variation in microbial ecology or bioreactors during anaerobic digestion processes. However, it appears there are functional drivers in AD systems as a result of some thermodynamic and functional requirements of the process, resulting in microbial ecology determination. In other words, the stage of the process (which is a function of the process at play/required) essentially determines the nature and types of substrate availability and in essence dictates the types of prevalent group of microbes present at that stage.

Variations in microbial ecology of anaerobic digestion occur even when environmental and operational conditions are similar. However, factors such as the source of inoculum, type of substrates, nature and size of digesters are determinants of microbial community structure of AD reactors. The lack of consensus observed in the studies of microbial ecology of AD systems, stems from most importantly a lack of clarity on the specific stage (day) of digestion process when digestates are collected for microbial ecology studies. It is also as a result of differences in other parameters such as substrates/inoculum ratio, pH, nature of substrates and so on, which are not always well defined.

To achieve sufficient knowledge of microbial interactions within AD systems, there would be a need for clearer reporting of every details that may contribute to changes in microbial community structure. Additionally, comparison of microbial ecology of various AD systems would not produce the desired results until there are some forms of standardization in process and operational conditions.

Going forward, it is important to continue to consistently identify and monitor major trends in microbial interactions of specific AD systems (such as batch systems, seaweeds

digestions etc.) and for more specifics in terms of timing of sampling. This consistency will enable researchers draw valuable conclusions from consistently observed trends in such a complex microbial community interactions and functions in specific AD systems.

Although the dataset available for this study was limited, this study has shown for the first time, the dominant archaea and methanogen community during active methanogenesis in seaweeds anaerobic digestion. It shows that hydrogenotrophic methanogenesis appears to be the favoured route to methane production during anaerobic digestion of seaweeds at seawater salinity conditions -irrespective of the source of inoculum. Nevertheless, it also suggests that substantial methane production depends on efficient interaction and cooperation between various groups of the microbial community involved in the process. One pertinent conclusion to be drawn from the current study is that, despite the similarity in the actual methane production, archaea and methanogen community of sediment and sludge inoculated reactors responsible for the methane production were significantly different-both in numbers, distribution and diversity.

In summary, the microbial community structure in the current study appears to be driven first by the type and source of inoculums and then by the functional requirements of the reactors which is dependent on the stage of the process. Nevertheless, substrates composition also plays significant role in determining microbial composition and variation during the process.

As the results of this chapter are based on a limited number of clones, results obtained are presented as representatives of the likely archaea and methanogen community. Further research would be needed to establish some of the findings of this chapter.

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8 Summary and conclusion

"Correlation …does not indicate causation. In fact, it often does not. The rooster might believe it causes the sun to rise, but the reality is much more complex"

- The Atlantic

The potential for sustainable methane production from marine biomass is enormous, and if fully harnessed could contribute significantly to climate change mitigation through carbon sequestration, bioremediation (seaweeds farming) and renewable methane for heat or electricity (Adams, et al., 2011; Hughes, et al., 2012). This is important, particularly for coastal communities with comparative advantage for marine bioenergy. Apart from the possibility of seaweeds farming for bioenergy production, the west coast of Scotland harbours large seaweeds deposits which sometimes constitute health hazards and source of greenhouse methane (Hermannsson & Swales, 2013). Reasonable harvest of these wild beach-cast seaweeds as proposed by Hermannsson & Swales, (2013) could be a form of readily available bioenergy source, especially in the short term.

A number of studies have looked at various means of improving the process of methane production from seaweeds, but they are mainly focused on the optimization of the physical and chemical parameters. Very few reports on the microbial ecology studies of seaweeds reactors are available in literature and as such, little is known about the intricate interactions between microbial community and process functions, as well as microbial responses to changing conditions during anaerobic digestion of seaweeds.

The overall aim of the current study therefore, was to better understand anaerobic digestion of seaweeds from microbial viewpoint and evaluate how the microbial community (especially methanogens) respond to, and cope with changing reactor conditions during the process. This study also attempts to demonstrate the feasibility of sustainable biomethane production from marine materials (seaweeds) by exploiting the intrinsic potential of the marine environment by using marine sediment as a source of inoculum. To achieve the aim of this study, a range of molecular approach was utilised to evaluate the interactions between process functions and microbial community dynamics during anaerobic digestion

of seaweeds. A good knowledge of the microbial ecology of seaweeds reactors could provide bases for the process monitoring and the prevention of possible system failure.

A number of objectives, which are addressed in different chapters of this study, were proposed to enable the actualization of the main aim of this project. Considering the salty nature of the substrates (seaweeds), one of the objectives was set out to test the hypothesis that:

- *Washing of seaweeds prior to anaerobic digestion does not enhance methane production*

The high salt content of seaweeds has been shown to inhibit microbial activities during anaerobic digestion (Miura et al., 2014). It is therefore important to ensure that this inhibition does not negatively affect the process, resulting in system failure. So to demonstrate the feasibility and sustainability of using seaweeds as the sole substrates during the anaerobic digestion process anaerobic batch tests was carried out using washed and unwashed seaweeds inoculated with digested sludge. The impact of washing was assessed on methane production and microbial community structure (using DGGE) over time.

Results of the preliminary investigations carried out (chapter 3) on washed an unwashed *Laminaria digitata* suggested that washing seaweeds prior to anaerobic digestion has little or no impact on actual methane production. This is because there was no significant difference in methane production between the two set ups (washed and unwashed). The results of the microbial ecology studies (using DGGE) of the digestates collected at interval show similarities in the bacterial (up to 56%) and archaeal (up to 81%) community between washed and unwashed seaweeds reactors during the anaerobic digestion process. This further supports the results obtained from methane analyses that washing of seaweeds

prior to AD has little impact on the productivity of the process. Besides, since the overall profitability of the digestion process depends on the cost effectiveness of the inputs, the fact that washing of seaweeds prior to anaerobic digestion might be unnecessary will further boost its sustainability and economic viability.

Based on the results above, subsequent anaerobic digestion tests were carried out using unwashed seaweeds.

Comparative biomethane production between sediment and sludge inoculated reactors of various substrates.

In Chapter 4 of this study, specific biomethanation potential of three seaweeds (*Laminaria digitata, Saccharina lattissima* and *Fucus serratus*) was tested using a unique source of inoculum (anoxic sediment) from the same marine environment as the seaweeds substrates. Marine sediment (containing microbial community adapted to salty conditions) was considered as a source of inoculum in order to exploit the intrinsic potential of the marine environment. For the purpose of comparison, digested sludge and cellulose are utilised as inoculum source and alternative substrate, respectively.

The hypothesis for this chapter is to demonstrate that: *There is no significant difference in methane production when either anoxic sediment or digested sludge is used as the source of inoculum*. The anaerobic digestion test was conducted on the three seaweeds (dried), which are widely distributed across the UK and especially on the west coast of Scotland during a 50-day process study. To demonstrate the sustainability of the process and its potential application for rural coastal communities, experiments were designed to minimise preparation stages and additional energy requirements such as washing seaweed or use of freshwater. Comparison was made between process performances and microbial ecology of reactors inoculated with anoxic sediment and digested sludge.

Volatile fatty acids measure at intervals during the process indicated that extensive hydrolysis was achieved in all sediment and sludge inoculated reactors. Results show the commencement of exponential increase in VFAs concentrations in both sediment and sludge inoculated reactors at about the same time (after day 8). This was the first indication of similarity in the performances of the two sources of inoculum. Nevertheless, accumulation of VFAs produced appeared more likely in sediment-inoculated reactors where higher VFAs concentration were recorded at specific time points compared in sludge inoculated reactors (Fig. 4.7). This accumulation might be as a result of lower VFAs uptake by the archaea and methanogens community present in the sediment inoculum rather than higher actual VFAs production. In addition, this accumulation of VFAs, which resulted in pH drops below 6.5 in seaweeds reactors, might also have inhibited methanogens (Chynoweth, et al., 2000; Khalid, et al., 2011; Raposo, et al., 2012) and affected any increase in methane production at the early stages in those sediment inoculated reactors.

Results of methane production between blank and substrate (seaweeds and cellulose) reactors under both inoculations suggest that the methane produced results from the biodegradation and metabolism of the added substrates. It shows that the substrates provided sufficient carbon and energy source to support the growth of the various groups of microorganism present in the reactors (Fig. 4.12 and 4.14).

Generally, the results obtained indicated that the use of anoxic sediment as the source of inoculum during anaerobic digestion of seaweeds can bring about methane production (Fig. 4.16) similar to, or more than the amount produced when sludge is the source of inoculum (Fig. 4.17). This is particularly important for remote coastal communities, as all inputs for the process could be locally sourced. Statistical analysis of the results showed that there were no significant differences between the use of either source of inoculum on

overall methane productions from anaerobic digestion of all three seaweeds. This suggests that the two sources of inoculum harbour sufficient microbial population for effective biomethanation of seaweeds. Nonetheless, when non-seaweed substrate (cellulose) was digested, different outcomes were observed. The results obtained revealed that cumulative methane produced by sludge inoculated cellulose reactors was significantly (8 times) higher than cellulose reactors inoculated with anoxic sediment, suggesting the inability of some of the microbial community in the sediment inoculum to metabolise cellulose or some of its derivatives through to methane.

It is evident from the results of the biomethane produced that *L. digitata* and *S. latissima* were the most hydrolysed of the three seaweeds. Methane production in these two seaweeds was similar under either inoculations and in most cases concentrations of methane are double that produced by *F. serratus*. As such, it appears that some components of *F. serratus* hampered the activities of the microbial community resulting in reduced productivity. This was reflected in the quantity of ash present in the seaweeds (Fig. 4.3), which was significantly higher in *F. serratus* compared to the other two seaweeds. The ash content represents the solid fraction of the seaweed biomass that is not amenable to biodegradation, and can have an inhibitory effect on methanogenesis. Research has shown that polyphenol, which is a major inhibitor of microbial activities, is more prevalent in *F. serratus* compared to the other seaweeds in this study (M. S. Kelly & Dworjanyn, 2008).

Generally, results from volatile fatty acids and methane analyses highlight the suitability of two (*L. digitata* and *S. latissima*) of the three seaweeds for large-scale bioenergy production. These two seaweeds consistently showed positive results for biomethane production and possess huge potential for cultivation (Hughes, et al., 2012; Schiener, et al., 2014; Wei, et al., 2013), which would prove valuable when commercial/large-scale

methane production becomes feasible. This is consistent with previous research which has demonstrated the suitability of these two seaweeds for biomethane production (Adams, Toop, et al., 2011; Adams, Ross, et al., 2011; Bruhn et al., 2011; Hanssen et al., 1987; Nielsen & Heiske, 2011) using various approaches.

The use of anoxic sediment inoculum in this study seemed efficient for the biomethanation of all experimental seaweeds especially *L. digitata*, and *S. lattissima*, an indication that the intrinsic potential of the marine environment could be exploited for sustainable bioenergy production in coastal communities. Similar observation has been reported by Miura et al., (2014), on the use of inoculum from similar sources as the substrates during anaerobic digestion of biomass.

Overall, the results of this study suggest that the microbial community in the two sources of inoculum (especially sludge) are not deterred or inhibited by the salt and metal content of the seaweeds or that they are able to remain viable and active (adapted) in spite of the peculiar composition of the seaweeds.

In summary, the results obtained from this chapter suggested that anoxic sediment used in this study has the potential to bring about similar methane production with digested sludge inoculum during anaerobic digestion of seaweeds. Results of this study support the hypothesis that *there is no significant difference in methane production between both sources of inoculum when seaweeds are digested*. Further research would be required to demonstrate and test these findings in large-scale reactors.

Microbial ecology studies of various AD reactors under different inoculations

To evaluate the similarities and distinctions in the productivity (activities), distribution and composition of the microbial community in the two sources of inoculum, the ecology of the various microbial groups were studied during the process using different molecular techniques.

Determination of organism (gene copy) numbers in relation to process performance during anaerobic digestion of seaweeds (using Q-PCR).

First, the quantity (numbers) of the different groups of the microbial community present at specific time points (day 2, 13, 20, 27) were analysed. These are important points where substantial microbial activities were anticipated based results from process study experiments (chapter 4). Microbial community within both sources of inoculums (day 0) were also quantified.

The hypothesis for this chapter was that *the number/amount of microorganism in the anaerobic reactors is a determinant of system functions and productivity during anaerobic digestion of seaweeds*. To address the hypothesis, a number of questions were proposed.

- *Are there differences in microorganism numbers between all reactors within the same inoculation treatment?*
- *Are there differences in microbe's numbers between specific reactors across different inoculation?*
- *Are there evidences to suggest microbial growth/increase is as a result of substrates availability (between blank and others)?*
- *How does microbes number change over time between the two inoculations?*
- *How does organism numbers relate to other process functions and performances?*

The results of q-PCR quantification of bacteria, archaea and methanogen communities in the (sediment and sludge) inoculums indicated that the two sources of inoculum are rich in microbial communities needed for anaerobic digestion. Although the q-PCR technique

employed does not differentiate between viable and dead cells, the results showed similar numbers of bacteria and archaea (within same order of magnitude) in the two inoculums (Fig. 5.3 and 5.5); however, methanogen numbers were significantly higher in the digested sludge (Fig 5.7). The higher methanogen numbers recorded in the sludge inoculum is likely due to the mesophilic conditions at which the wastewater treatment plant is operated.

Based on results presented in this thesis it can be hypothesised that the substrates (seaweed and cellulose) provided sufficient carbon and energy to support microbial (bacteria, archaea and methanogen) growth and development, which led to the increase in number, recorded between the inoculum (day 0) and day 2 and throughout the anaerobic digestion process.

Since the q-PCR techniques employed in this study does not distinguish between living and dead cells, the possibility of matching organisms' numbers with process functions might be difficult. Again, research has shown that large number of specific organism does not always determine activity, as some of the organisms might contribute little or nothing to the process functions (C. J. Smith & Osborn, 2009). However, results obtained from the quantitative PCR of bacterial, archaea and methanogen amplification (vis-à-vis other process functions) provide evidences to suggest that the organisms quantified were largely viable and active, and were responsible for the process functions recorded.

Although there were similarities in the overall, trend in microbial numbers observed between sediment and sludge inoculated reactors there were observable differences with a decline in number of microorganisms (bacteria, archaea and methanogen) between day 2 and 13 within sediment-inoculated reactors, whilst the sludge inoculated reactors showed the opposite trend. In addition, while organisms' numbers peaked between day 13 and 20 in sludge-inoculated reactors, peak organism numbers were recorded between day 20 and

27 of the digestion process. These results suggest that there was a delay in the attainment of optimum microbial growth in sediment-inoculated reactors. This is likely due to the time required by microbes from the marine environment to become adapted to the mesophilic conditions in the reactors. The transfer of sediment inoculum from marine environment (~8^{o}C and pH 7.5-8.4) to mesophilic conditions (37^{o}C, pH <7.5) might have also led to the initial short lag recorded in both biomethane production and microbial growth as the microbial community attempted to become adapted to the new conditions. Another possibility is the competition for substrates from other organisms such as sulphur reducing bacteria (SRBs) which are prevalent in marine sediment (Ali Shah, et al., 2014; Head, et al., 1997; Marquez, et al., 2013; Minderlein & Blodau, 2010; Mitterer, 2010; Nayak, et al., 2009; Nercessian, et al., 2005) but unlikely in the digested sludge.

In summary, the results of this section indicate a general correlation between microorganism numbers and other physical and chemical functions (VFAs formation, pH variation, methane production) in all reactors except in cellulose reactors (see section 5.3.4.1). It suggested that the organisms were largely viable and active and that they were mainly responsible for the recorded process functions and productivity.

Determination of microbial composition/richness and distribution during anaerobic digestion of seaweeds (using DGGE)

So far, previous results (from volatile fatty acids formation, methane production (except in cellulose; chapter 4) and organism (gene) numbers amplification; chapter 5) have shown little differences between sediment and sludge inoculated reactors. It could therefore, be assumed that the two sources of inoculum and their activities within seaweeds AD reactors were similar. The hypothesis for this section of the study is to show, based on the results obtained in chapter 4 that *there is no difference in microbial community composition and diversity between sediment and sludge inoculated reactors*. This hypothesis was tested with a fingerprint technique (DGGE) to provide a snapshot of microbial composition, richness and distribution during the process using the following questions:

- *Are there differences in microbial composition and community structure between the inoculums (prior to AD process) and digestates during the process?*
- *Are there differences in microbial community composition and diversity between sediment and sludge inoculated reactors?*
- *What are the main drivers/determinants of microbial variation during anaerobic digestion of seaweeds?*
- *Are there differences between seaweeds and cellulose reactors in terms microbial community over time?*

Although there was no significant difference in bacterial numbers (using q-PCR in chapter 5) between sediment and sludge inoculums, results of DGGE analysis indicated significant differences in bacteria community composition between the two sources of inoculum. Multi-dimentional scaling (MDS) plot and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analyses performed on the DGGE gels clearly showed that different groups of bacteria were present in the two sources of inoculum prior to anaerobic digestion (chapter 6). This result is an indication of the ability of different

ecosystem to select for microbes that thrive in various environments based on environmental conditions and substrate availability (Guo et al., 2012; Guo et al., 2015; Liu, et al., 2012).

Despite the differences observed in the bacteria community composition (DGGE), results obtained suggest that both inoculums harbour sufficient hydrolytic, acidogenic and acetogenic bacteria to support efficient bioconversion of the algal biomass into volatile fatty acids (VFAs in chapter 4). Nevertheless, a succession process was observed over time after the introduction of seaweeds and cellulose substrates, resulting in the replacement of the some of the original bacteria communities present in the inoculums. Changes in reactor conditions, type of substrates and process requirements appeared to be the main drivers of the observed shifts in microbial composition (especially bacteria) as well as their richness. This observation is similar to previous reports of shifts in microbial community structure during anaerobic digestion processes over time (Cho, et al., 2013; Ciotola, et al., 2013).

At the initial stages of the process, inoculum-like populations dominate within the bacteria community, but are later replaced by other bacterial groups as a result of microbial succession and changes in process functions. Studies have shown that only a minor proportion of bacterial community of inoculum is represented at the end of the digestion process. For instance a study of the microbial community response to seasonal temperature in small scale anaerobic digesters by Ciotola et al., (2013), found the bacterial community at the end of AD process no longer has the same structure present in the inoculum prior to the shift. In that study, the authors reported that the retention of inoculum-like bacteria community structure during anaerobic digestion lasts only a few days and at the end of the process only a fraction of the original bacterial community is retained. This observation however, does not indicate that the successive microbial groups were not initially present

in the inoculums; it is likely that their numbers were low due to initial low activity and lack of suitable substrates.

While bacterial diversity appeared to decline over time in sediment-inoculated reactors, the opposite was observed in sludge-inoculated reactors where bacterial community diversity increased after a brief decline. This report is opposite of what was observed in the archaea and methanogen community in sediment and sludge inoculated reactors, as diversity increased over time peaking around day 20 and 27 in most cases. Most of the variations observed in microbial community structure occurred after day 2 and before day 27 in all reactors (Fig. 6.1, 6.3). This period corresponds with considerable microbial activities (relation to volatile fatty acids formation and methane production) and suggests microbial responses to changing substrates conditions.

Most of the variations in bacteria community occurred during stages of suspected intensive microbial activities (between day 2 and day 20) as different substrates selected bacteria groups needed to perform the process. After the extensive fluctuation within bacterial community, a form of stability and similarity was observed at day 27 in all reactors, which suggest that similar bacterial community were present at this stage and are probably carrying out similar activities (Fig 6.1).

However, as a result of limited diversity, variations within the archaeal and methanogenic communities did not appear to be as pronounced (Fig. 6.5, 6.7, 6.11 and 6.13) as that of bacteria. More so, as there are far more bacteria types than archaea and methanogens.

Generally, there are observable differences in the microbial community structure between sediment and sludge inoculated reactors during the digestion. Nonetheless, different reactors harboured different microbial communities at different time points. Results obtained highlight the impact of time or the stage of the process on the microbial

community structure in all reactors and disprove the hypothesis that *there was no difference in microbial community structure and composition between sediment and sludge inoculated reactors*.

What are the dominant archaea and methanogens involved in methane production during anaerobic digestion of seaweeds?

To further investigate differences between the sediment and sludge-inoculated seaweeds' reactors, dominant archaea and methanogen component of *L. digitata* reactors were identified using cloning and sequencing techniques.

Source of inoculum as a determinant of the dominant archaea and methanogen population

Previous reports (chapter 7) highlighted the closest relatives of dominant archaea and methanogen populations within the sediment and sludge inoculated seaweed reactors. While a form of similarity in the archaea community was observed, particularly with the dominance of archaea related to *Methanomicrobiales*. Further identification to the genus level revealed a few distinctions between the two communities. For instance, within the sediment-inoculated reactors, the order *Methanomicrobiales* was made up of five identified genera while at least seven genera were identified in the sludge-inoculated reactors (Fig. 7.6), suggesting a more diverse archaea community within the sludge inoculated reactors. Furthermore, one of the dominant genus within the sediment-inoculated; *Methanofolis* was not detected from the sludge-inoculated reactors. In the same vein, *Methanospirillum*-like archaea, which was the dominant genus in the sludge-inoculated reactors, was also not detected in the sediment-inoculated reactors. Although, there are a few overlaps (similarities) in genera composition between the two experimental set ups, their proportional representation also varies. For example, while both sediment and sludge inoculated reactors harboured archaea related to the genus *Methanoculleus,* it formed the bulk (42%) of the archaea community in sediment-inoculated reactors while accounting for only 14% in sludge inoculated ones (Fig. 7.6).

Although there were similarities in the number of archaea orders detected between sediment and sludge inoculated seaweed reactors, there were noticeable differences in the archaea communities when identified to the genus level. There were also differences in the proportional order representation within the archaea community between the two sets of reactors. The results suggest that the source of inoculum potentially introduced different groups of microbes into the reactors.

Influence of source of inoculum on actual methanogen populations

Results of methanogens sequence identification and analysis gave a different picture from that of archaea in both set of reactors (sediment and sludge). Three orders, like in the archaea community, were identified for the methanogen community, with methanogen related to the orders *Methanomicrobiales* and *Methanosarcinales* accounting for similar proportions in both sediment (43% apiece) and sludge-inoculated reactors (39 and 36% respectively). The dominance of the two orders appears to be a major similarity observed between the two set of reactors. However, identification to the level of genus highlighted some unique distinctions. For instance, the methanogen community within the sludge-inoculated reactors appeared more diverse with at least nine identified genera compared to six in sediment-inoculated reactors (Fig. 7.11). Furthermore, *Methanofolis* related methanogen, which formed 19% of the methanogen in the sediment-inoculated reactor, was not detected in the sludge reactors. In contrast, *Methanosaeta*, *Methanothermobacter* and *Methanolobus* related methanogen, which formed 19, 4, and 4% respectively in sludge-inoculated reactors were not detected in sediment-inoculated ones (Fig. 7.11).

Even within the common genera (overlaps), proportional variations were observed. For instance, while both set of reactors harbour methanogens closely related to the genus *Methanosarcina*, it formed 44% of methanogens in sediment-inoculated reactors with only

about 14 % presence in the sludge-inoculated ones (Fig. 7.11). This observation is particularly important due to the differences in the activities of these methanogens in relation to substrates utilization and methane production.

Although the result of DGGE profile of the methanogen in sediment-inoculated reactors revealed very little band presence especially at the earlier stages of the process, the dominance of methanogens closely related to *Methanosarcina* might help explain the productivity of the process in relation to methane production in the reactors. Previous reports supported the versatility of *Methanosarcina*. For instance, apart from ability to withstand sudden changes in process conditions, it is the fastest growing methanogens and is able to utilise the widest range of substrates (K. Cho et al., 2013; Ma et al., 2013). Its prevalence in the sediment reactors (even in small numbers), might account for the substantial methane production recorded in those reactors.

Overlaps in genera composition within archaea and methanogen community of sediment and sludge inoculated reactors

Methanogen component of the archaea community represents those potentially involved in methane production during anaerobic digestion processes. The use of functional gene marker (*mcrA*) which is peculiar to methanogens enables the separation of methanogens from the general archaea community. Apart from being used for the determination of methanogen diversity, the *mcrA* gene is also a potential biomarker of methane yield during anaerobic reactions (Ma et al., 2013; Steinberg & Regan, 2009).

Although, similar methanogens were identified with both genes at the order level, the use of 16S rRNA and *mcrA* clone library gave strikingly different profile of the digesters' taxonomic genus compositions and suggest that the majority of the archaea population (dominated by *Methanomicrobiales*) were not potentially involved in methane production.

These results indicate high level of redundancy within the archaea community, an observation consistent with previous reports by Wilkins, et al., (2015). It also highlighted that the reactors harboured distinct methanogen and archaeal communities, which can be hypothesized to result from differences in the methanogen components of the inoculums or discrepancies introduced from PCR amplification process.

Earlier reports suggested that the use of either 16S rRNA or *mcrA* clone library alone cannot provide a complete community structure (Ma et al., 2013), results of this study further confirms that a combination of the two genes is essential to obtain a full spectrum of methanogen diversity in anaerobic digesters. This study attests to the usefulness of the *mcr*A gene as a suitable marker for both methanogen taxonomy and metabolic activity, used in conjunction with the 16S rRNA gene marker, it reinforces the values of using multiple gene studies for microbial diversity (Ma et al., 2013).

Although, clones analysed were limited expecially for archaea 16S rRNA gene clone library, the general result obtained suggested that the methanogen population identified with 16S rRNA gene clone library were fairly different from those identified using the functional (*mcrA*) gene clone library. One likely reason for this might be due to the differences and biases introduced by the techniques employed in the PCR amplification process. Any biase or errors introduced into the process at the amplification stage could affect other downstream applications sucha s cloning and sequencing (Nayak et al., 2009). It might also be as a result of lower entries in the *mcrA* gene database compared to the 16S rRNA database. As a result, *mcrA* gene clone library would more closely represent the identified methanogen communities than the 16S rRNA.

In summary, this study highlights some important quantitative and qualitative information of seaweeds AD reactors between the microbial community and system performance. It provides insights into the microbial ecology of the seaweeds anaerobic reactors and highlights some of the intricate linkages between the microbial community structure and other system functions.

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General Conclusion

"None of this research may make us behave better, not right away at least. But, all of it can help us understand ourselves – a small step up perhaps, but an important one"

-The Situationist

The aim of the current study was to better understand anaerobic digestion of seaweeds from microbial viewpoint and evaluate how the microbial communities respond and cope with changing reactor conditions during the process. Attempts were made to demonstrate the feasibility and sustainability of obtaining efficient seaweeds biomethane fermentation using various inoculum sources. This is the first time the microbial ecology study of seaweeds anaerobic reactors is carried out using a wide range of molecular techniques to unravel microbial community interactions and functions over time.

Some of the key findings obtained in this study are as follows:

- Washing of seaweeds prior to anaerobic digestion might not be necessary.
- The inherent potential of the marine ecosystem could be exploited by using anoxic sediment sourced from the same ecosystem as the seaweeds as a rich source of inoculum for sustainable methane production.
- Biomethane production from *Laminaria digitata, Saccharina lattissima* compare very favourably with most of the currently employed feedstock for methane production, and coupled with its added advantages in bioremediation, CO_2 sequestration and job creation, the potential could be enormous.
- Anoxic sediment employed in the current study harbour a rich community of microorganisms for efficient methane production from seaweeds.
- Anoxic sediment as a source of inoculum does not appear suitable for anaerobic digestion of cellulosic materials.
- The source of inoculum appears to be a significant determinant of the composition of the community structure. Substrate type is also an important factor that contributes to the microbial community composition and structure.

- Both acetotrophic and hydrogenotrophic methanogenesis appear to be important routes towards methane production during anaerobic digestion of seaweeds.
- Despite the similarity in the actual concentration of methane produced, the archaea and methanogen community of sediment and sludge inoculated reactors responsible for the methane production were significantly different-both in numbers, distribution and diversity.
- Microbial community structure in the current study appears to be driven first by the type and source of inoculums and then by the functional requirements of the reactors which is dependent on the stage of the process. Nevertheless, substrates composition also plays significant role in determining microbial composition and variation during the process.
- This project has improved our understandings of the interactions and interplay between process functions, and microbial community dynamics/structure during anaerobic digestion of marine biomass. It has also shown how different reactors select for various groups of microbial components based on process requirement and periodic substrates compositions.

Overall, this study provides insights on microbial ecology of seaweed anaerobic reactors and of the microbial responses to changing conditions within the reactors. It has also provided some microbiological dimensions to the understanding and possible optimization of the anaerobic digestion process. This knowledge would potentially be useful for process monitoring and prevention of system failure during large-scale seaweeds anaerobic digestion.

Recomendations

- Future research on seaweeds anaerobic digestion should consider *Laminaria digitata* and *Saccharina latissima* as suitable substrates for methane production, considering their digestibility and cultivation potential.
- There is a need for more clarity on the reporting of microbial ecology studies to allow for useful comparison among reactors.

Further Research

- Anoxic sediment used in this study was sourced from below a fish farm. It appears fish farming activities contributed in the microbial richness of the inoculum source. A fresh study is underway to determine the contribution of fish farming activities to the sediment's microbial richness, by using sediment away from fish farms and also studying the microbial ecology within the gut of the fish (from the same location).
- A study involoving the analysis of specific VFAs should be carried out to obtain more insight on their utilization by the methanogens. This could help explain the differences in methane production between sediment and sludge inoculated cellulose reactors.
- Further studies involving cloning and sequencing of digstates from sediment inoculated cellulose reactors would be useful to unravel the reason for the failure of methane production from those reactors.
- Further research based on a larger data set and clone libraries or the use of pyrosequencing would be needed to obtain a more comprehensive microbial community composition and process functions.
- Further studies are needed to determine and establish the various factors responsible for the selection and variation of microbial community within seaweeds anaerobic reactors over time.

- Further studies involving RNA and primers for specific groups of methanogens in quantitative PCR, would be needed to measure activities of the methanogens and their contribution to methane production