**UNIVERSIDADE DO ALGARVE**

# Anaerobic Digestion of *Laminaria hyperborea* for biogas production

**Sara Alexandra Teixeira da Costa**

Dissertation
**MSc in Marine Biology**
**Scientific Area of Marine Biotechnology**

Supervisors:
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## Statement of authorship

## Declaração de autoria de trabalho

Anaerobic Digestion of *Laminaria hyperborea* for biogas production

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Sara Alexandra Teixeira da Costa

*The sea, the great unifier, is Man's only hope.*

*Now, as never before, the old phrase has a literal meaning: we are all in the same boat.*

Jacques Yves Cousteau

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## Resumo

Considerando o crescimento exponencial das necessidades humanas por energia e as altas emissões de gases de efeito de estufa atuais e esperadas, é crucial encontrar fontes de energia alternativas para um futuro sustentável, mas tecnologicamente avançado, com a consequente diminuição da dependência económica de combustíveis fósseis para geração de energia. A produção de bioenergia, a partir de biomassa e resíduos, será de grande importância para o futuro das energias renováveis. Em contraste com muitas outras fontes de energia de biomassa, as macroalgas não edíveis são uma fonte de energia que não compete com a produção de alimentos agrícolas, nem necessitam de água doce para irrigação. O presente estudo é dedicado à alga *Laminaria hyperborea* e ao seu potencial de ser um produtor bioenergético de metano aquando sujeito ao processo de digestão anaeróbia (DA). Usando um conhecido e eficiente inóculo para DA, este processo de fermentação foi otimizado quanto às seguintes variáveis: tipo de diluente, taxa de alimentação, temperatura ideal e tipo de agitação (contínua/ocasional). Processos de pré-armazenagem e pré-tratamento da alga também foram estudados. No fim, foi possível concluir que a utilização de água destilada como diluente é alternativa viável e menos dispendiosa que o uso de tampão fosfato-salino (PBS). Além disso, outros métodos de pré-armazenamento da alga para DA devem ser melhor investigados, tendo em consideração os resultados aqui apresentados. Foi igualmente observado que temperaturas mais elevadas de incubação (35 e 37 °C) produzem maiores quantidades de metano/g de sólidos voláteis/dia do que a 25 °C. Concentrações mais elevadas de alimento/massa algal (15 e 20% [pf/v]) levam mais tempo a ser digeridas pelo inóculo e não resultam em produtividades significativamente mais elevadas que a concentrações de 5 ou 10 % (pf/v). Nenhum pré-tratamento às algas pareceu melhorar significativamente a % de sólidos voláteis consumidos pelo inóculo, mas é aconselhado o pré-tratamento das algas com autoclave. Por último, uma vez que a agitação contínua em fermentadores de pequeno volume (200 mL) não aumenta a produtividade, recomenda-se uma agitação mínima ocasional.

**Termos-chave**: digestão anaeróbia, otimização, *L. hyperborea*, biogás, metano

# Abstract

Considering the exponential growth in human demand for energy and the current and expected high greenhouse gas emissions, it is crucial to find alternative sources of energy for a sustainable but technologically advanced future. This would also lessen the economic dependency on fossil fuels for energy generation. Bioenergy production from biomass and waste is expected to be of great importance in the future of renewable energy. Non-food macroalgae do not compete with agricultural food production nor require fresh water for irrigation, in contrast with other biomass energy sources. The present study is on the seaweed *Laminaria hyperborea* and its potential to be a bioenergetic producer of methane by anaerobic digestion (AD). Using a known efficient AD inoculum the fermentation process was optimised regarding variables such as diluent composition, feed rate, temperature and continuous or occasional stirred fermentation. Processes for the pre-treatment and pre-storage of seaweed were also studied. In the end it was possible to conclude that using distilled water as diluent was a viable, less expensive alternative to the use of PBS; storage methods should be more investigated, taking the herein results into consideration; higher temperatures of incubation (35 and 37 ºC) produce higher quantities of methane/g volatile solids (VS)/day than at 25 ºC; higher feed concentrations (15 and 20 % [ww/v]) results in longer digestion times and will not increase productivity significantly as compared to moderate concentrations, such as 5 or 10% (ww/v); no pre-treatment applied to the seaweed seemed to significantly improve the %VS used by the inoculum, but it is encouraged to pre-treat the seaweeds by autoclaving them; and lastly, as continuous stirring in small volume fermenters (200 mL) did not improve productivity, occasional mixing is recommended.

**Keywords**: anaerobic digestion, process optimisation, *L. hyperborea*, biogas, methane

# Índex

# Figure Index

# Table index

## Abbreviations and acronyms

| | |
|---|---|
| AD | Anaerobic Digestion |
| ANOVA | Analysis of variance |
| Btu | British thermal units |
| CCEG | Climate Change Expertise Group |
| $CH_4$ | Methane |
| $CO_2$ | Carbon Dioxide |
| d | Days |
| dw | Dry Weight |
| dw/v | Dry Weight per Volume |
| EIA | US Energy Information Administration |
| EU | European Union |
| FDA | Fluorescein DiAcetate |
| GC | Gas Chromatography |
| GHG | Greenhouse gases |
| HRT | Hydraulic Retention Time |
| IEA | International Energy Agency |
| IPCC | Intergovernmental Panel on Climate Change |
| mL $CH_4$/ g VS | Methane Yield |
| mL $CH_4$/ g VS/ d | Methane Productivity |
| NaOH | Sodium Hydroxide |
| NER | Nordic Energy Research |
| OECD | Organisation for Economic Co-operation and Development |
| OPEC | Organization of the Petroleum Exporting Countries |
| PBS | Phosphate Buffer Solution |
| PT | Phaeophycean tannins or Phycotannins or Phlorotannins |
| SRT | Solids Retention Time |
| TMC | Total Microbial Counts |
| TS | Total Solids |
| TWh | Tera Watt-hours |
| UNDCCC | United Nations Framework Convention on Climate Change |
| v/v | Volume per Volume |
| VFA | Volatile Fatty Acids |
| VS | Volatile Solids |

| | |
|---|---|
| ww | Wet Weight |
| ww/v | Wet Weight per Volume |

# 1. Introduction

## 1.1. Global environmental changes and world energy

Since solar variation, plate tectonics, volcanism and meteorite impacts are now less frequent, the major drivers of global changes are, nowadays, the exponential growth in human demand for energy, food, services and information and the disposal of associated waste products. These events have caused pollution, ocean acidification, fish-stock collapse, extinctions and a most obvious problem**,** climate change (temperature and precipitation rises). The chemical composition of the atmosphere has changed significantly in the last 150 years since the beginning of large scale industrialization, due to an increase of about 25% of greenhouse gases (GHG) (EIA, 2004). GHG (e.g. carbon dioxide, methane and nitrous oxide) contribute to a process referred to as the "greenhouse effect" wherein solar energy is captured by such gases and is re-emitted as infrared energy (heat) to the earth's surface, thus increasing the air temperature (IPCC, 2007).

GHG emissions are most of all caused by carbon dioxide release from energy-related processes, leading to a need for a worldwide energy policy. The United Nations Framework Convention on Climate Change (UNFCCC) set emission targets that even if achieved by the signatory countries would still leave the $CO_2$ levels about 60% above the required level to prevent a rise of 2°C in air temperature by 2035 (IEA, 2012a).

On a press release at 9th November 2011, the International Energy Agency (IEA) stated that the share of fossil fuels in global primary energy consumption will fall from about 81% today to 75% in 2035. Conversely, renewable energies accounted for 19.5% of global electricity generation and 3% of global energy consumption for road transportation in 2009. From 2000 to 2010, the global biofuel production grew from 16 billion litres to more than 100 billion litres, providing ~ 3% of the world's fuel for transport. Despite this age of fiscal austerity, renewable energies appear to be the world's fastest growing forms of energy, whose total energy use is projected to increase from 11% in 2010 to 15% by 2040 (EIA, 2013), taking into consideration the great efforts to subsidize them from $66 billion in 2010 to $250 billion in 2035. Delaying this investment action would be a false saving: for every $1 of investment avoided in cleaner technology before 2020, an additional $4.30 would be needed to compensate for the increased emissions (IEA, 2011).

On the 11th June 2012, IEA published new developments in energy technology – *Energy Technology Perspectives 2012* – where it demonstrated how by using electric vehicles and deploying smart grids global temperature rise can be limited to 2 ºC, enhancing a cleaner, securer and more competitive energy future (IEA, 2012a).

On the 20th November 2012, IEA together with Nordic Energy Research (NER) gave a preview of the project *Nordic Energy Technology Perspectives, considering the 11th June report, where it was shown a Carbon-Neutral Scenario for Denmark, Finland, Iceland, Norway and Sweden* in which energy-related $CO_2$ emissions are reduced by 85% (IEA, 2012b). The Nordic countries have this opportunity due to having a high level of renewable resources and fairly progressive policies. More initiatives like this are needed worldwide to counteract the dependence and unsustainable use of fossil fuels for energy generation, not just regarding environmental aspects, but also the economy. The rise of oil prices continues and it is much influenced by world events that in turn influence the major oil distributors/sellers, such as the Organization of the Petroleum Exporting Countries (OPEC). For example, at the end of 2010 and beginning of 2011, the oil price increased from $82 to more than $112 per barrel after a social and political crisis in the Middle East and Africa (EIA, 2013).

A long-term view of the energy market for all fuel sources in the world is given by EIA (2013) (Figure 1). Fossil fuel supplies are limited and although new reserves have been discovered, they do not satisfy the increasing needs of population growth and industrialization. The world energy consumption is projected to increase by 56% from 2010 to 2040 (EIA, 2013).

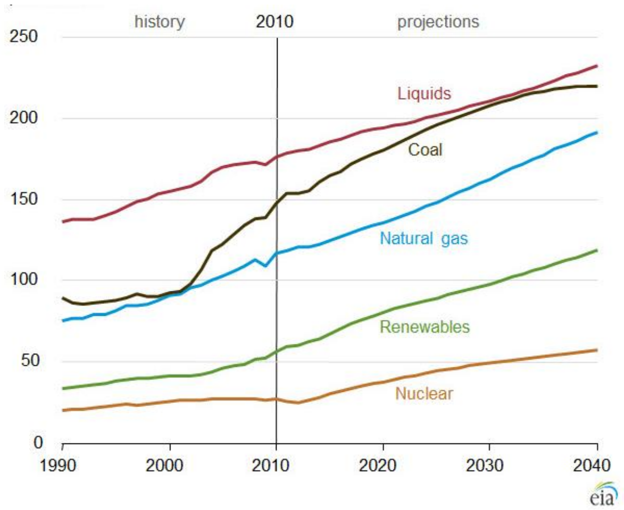

**Figure 1** World fuel energy consumption (quadrillion Btu) (EIA, 2013).

Liquid fuels (mostly petroleum) remain, within the fossil fuels, the most used energy source in terms of British thermal units (Btu) in spite of a small crash that took place in 2008 (Fig. 1). However, the liquids share of world market energy consumption is predicted to fall from 34% in 2010 to 28% by 2040, due to the switch from liquid fuels to cheaper sources such as fuel gases. Coal consumption will have a significant growth in non-OECD nations, especially in Asia (e.g. China and India), for electric power generation and industrial processes. Natural gas is gaining a strong competitive position due to its growth outlook for reserves and supplies, with the expansion of liquefied natural gas (LNG) production capacity and new drilling techniques, resulting in an increase in resource availability and low capital costs. Also natural gas is less polluting than coal or oil and has high efficiency yields, which makes it an attractive option to be used (EIA, 2013). Nuclear energy sources will have a rise from 5% in 2010 to 7% by 2040, despite the past events such as the Fukushima Daiichi plant disaster arising from a tsunami hitting the Japanese eastern coast (EIA, 2013). The development of new nuclear power plants raises concerns about safety, radioactive waste disposal and proliferation of nuclear material and, for some countries it, is still not attractive due to its high capital and maintenance costs.

Renewable energy comes from natural resources such as rain, wind, sun, tides and waves, geothermal and biomass, the first two (as hydroelectric and wind power) being responsible for 55% and 27% of the increase in renewable generation, respectively (IEA, 2011). The major problem of a few renewable forms of energy is their intermittency, which can be overcome by improving battery storage technology and generating facilities over wide geographic areas. Energy from biomass and organic waste (sewage sludge, municipal and industrial waste, manure and crop waste) is seen as one of the most promising future renewable energy sources, especially since a continuous electrical power generation from these sources can be guaranteed.

Renewable resources will play a crucial role in the $CO_2$-mitigation policies by reducing GHG emissions and their harmful effect on the world climate (IEA, 2011). Sustainable, cheap and non-polluting energy sources are thus required for the future. It is not likely, however, that a single form of renewable energy will solve all the problems, suggesting that diversification is the best solution. Efforts are being made so that renewables can successfully be included in long-term scenarios. Nevertheless this is difficult, as for instance electric or hybrid cars took time to penetrate the market, and are not yet available to everyone due to their elevated prices and/or range limitations (OECD/IEA, 2013). By 2020, the European Commission aims to have 20% of the EU’s energy produced from renewable sources. It also has a target of reducing 20% of GHG emissions and reducing the energy consumed by 20% (EC, 2011).

### 1.2. Bioenergy production

Bioenergy is energy that results from biological matter – biomass. Being a type of renewable energy it makes a strong contribution worldwide – 10% of world total primary energy supply in 2009 (IEA, 2013). In the European Union, however, some key factors were found to cause problems in granting permission for processes covering biofuel production, biogas, biomass combustion and co-firing installations, such as "too many process steps and permits issued by separate authorities", which "are subject to a wide range of legislative acts, lack of clear timetables, lack of local knowledge and capacity to analyse complex bio-energy permit applications, lack of clear procedures to obtain grid access, [and] local resistance to bio-energy projects" (EC, 2013).

Plants with high sugar content or oilseeds, and wastes from agriculture, urban or forestry activities are types of biomass that can be used for bioenergy production, independently from their physical state — solid, gaseous or liquid. Bioenergy can make a significant contribution worldwide, in part due to do the potential to be a net zero emissary of GHG, since the carbon dioxide released by the biomass, or resultant biofuel, when burned is compensated by the amount absorbed when the plant was originally grown (EC, 2013).

Bioenergy production is currently of great interest in Europe and the EU is greatly promoting the use of biofuels in transport, electricity generation and heating (EC, 2011). Modern technologies allow progress in biotechnology towards optimizing processes of biomass transformation into efficient biofuels that can be produced economically and in sufficient quantities to replace crude oils.

Biomass can become energy sources through three different conversion processes – physicochemical, thermochemical or biochemical (Fig. 2). Among physicochemical conversion processes biodiesel is probably the final product most recognized by the general public, since its inclusion into transport networks has been greatly promoted. Nevertheless, the most known ancient use of biomass is by combustion (a type of thermochemical conversion process) to generate heat. Currently, several industries also use combustion to produce high pressure steam that in turn generates electricity. Other thermochemical conversion processes do not burn the biomass; instead, they simply use heat and low $O_2$ levels to form gases (*e.g.* syngas, $CH_4$, and ethane), liquids (*e.g.* oils, chemicals, and methanol) and solids (char). Finally, if organisms such as bacteria, archaea or yeasts are added to biomass, a biochemical conversion process may take place.

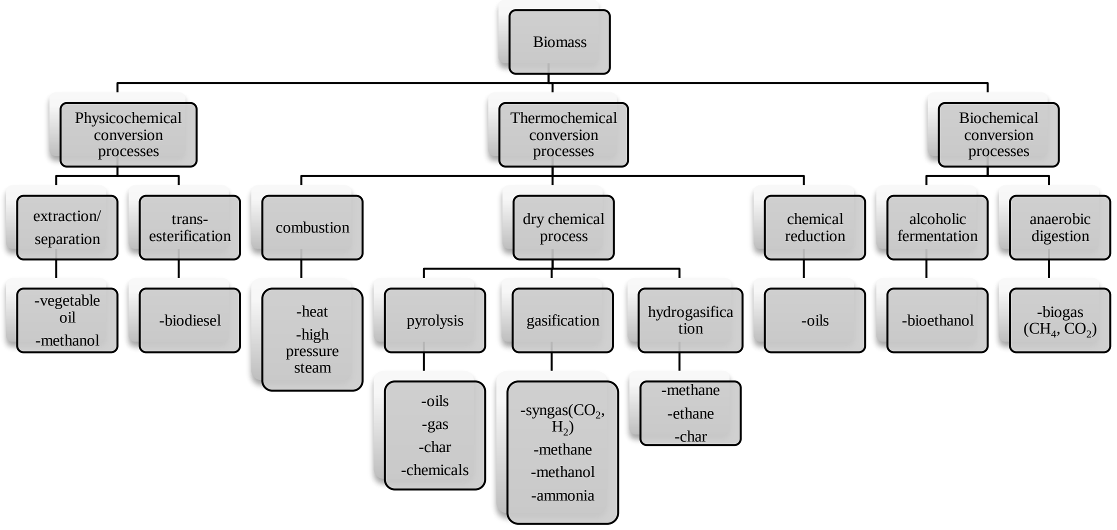

**Figure 2** Schematic synthesis of the different conversion processes to which biomass can be subjected (after Slesser & Lewis [1979] and Apeels *et al.* [2011]). Special emphasis goes to anaerobic digestion, a biochemical process employed in this dissertation. For further details please see the accompanying text.

Wet biomass, such as algae, and other biomass sources with high water content can only efficiently produce energy by aqueous processes (represented in Fig. 2 by chemical reduction, alcoholic fermentation and AD) due to the high energy requirement for drying the material artificially (Slesser & Lewis, 1979). Biologic degradation can occur in aerobic conditions too, by the known composting process, when biomass is converted into microbial biomass, $CO_2$ and $H_2O$, releasing heat and having a biomass yield of about 50% (Henze *et al.*, 1997), but little energy is produced. In contrast, AD results in microbial biomass, biogas (50:50% $CH_4$:$CO_2$) and residual undigested biomass as its main products, thanks to a complex mixed microbial population that ferments the biomass.

Because it involves several groups of anaerobes AD is a challenging process, specially as it involves several different steps (Figure 3): step 1 - hydrolysis of organic polymers, such as polysaccharides, lipids and proteins forms monomers like sugars, fatty acids, short peptides and amino acids using extracellular enzymes, which is followed by step 2 - acidogenesis due to the release of volatile fatty acids [VFAs]: propionate, butyrate and alcohols; step 3 - acetogenesis comprising three possible ways of forming acetate from VFAs, direct fermentation of hexoses and hydrogen and step 4 - methanogenesis: formation of $CH_4$ and $CO_2$, also through three different ways: acetate, hydrogen or formate and methylated substrates (Deppenmeier *et al.*, 1996). The main groups of microbes operating on the described process are: hydrolytic and fermentative organisms (step 1), acidogenic bacteria (step 2), acetogenic (use VFAs) and homoacetogenic (reduce $CO_2$) bacteria (step 3), and hydrogenoclastic (use $H_2$) and acetoclastic (use acetate) methanogenic archaea (step 4) (Leschine, 1995). Methanogenic archaea are strictly anaerobic, but the other microorganisms involved are facultative aerobes. Other anaerobes, such as sulphate- and sulphur-reducing bacteria can out-compete methanogens (when sulphate is in abundance) and prevent methane formation, both because of their higher affinity for hydrogen and acetate, and their higher growth rate (Brock *et al.*, 1994).

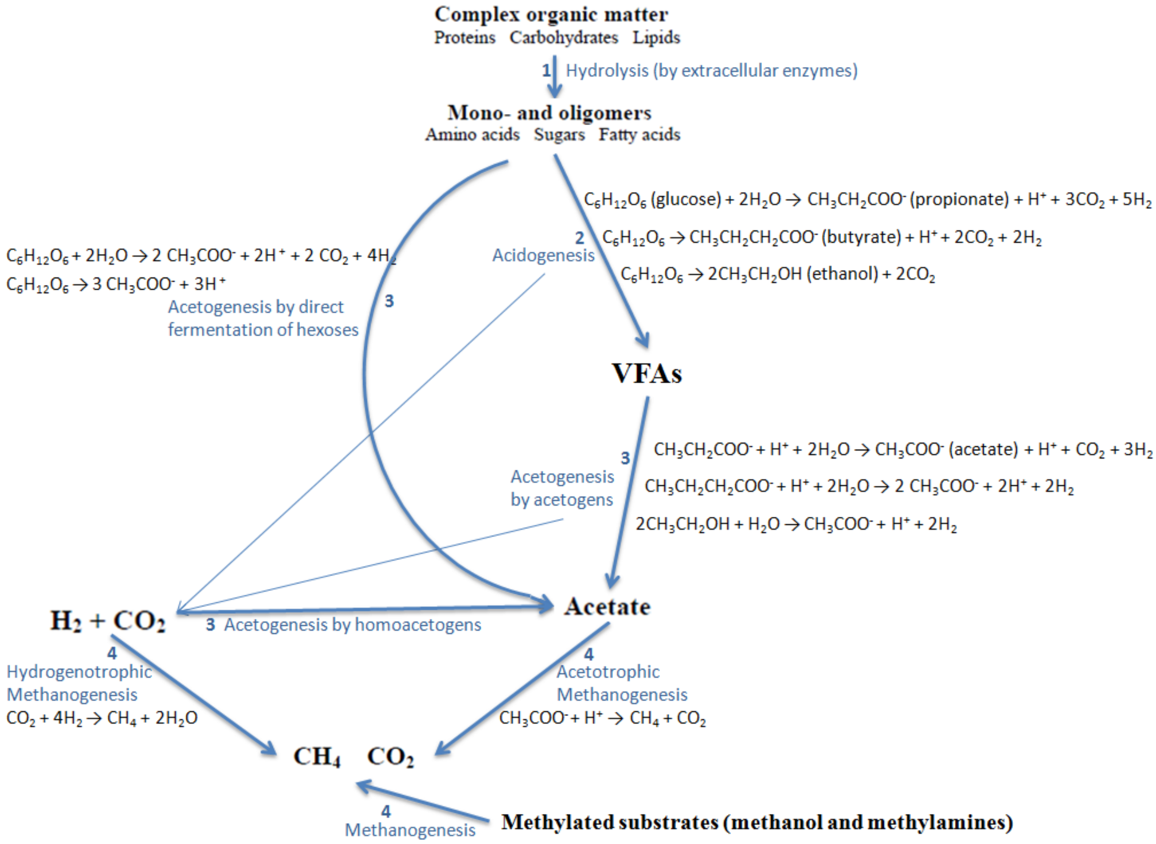

**Figure 3** Schematic summary of AD of organic matter (Brock *et al.*, 1994), the chemical reactions and microorganisms involved (Klass, 1998).

The methane present in biogas is chemically the same as natural gas (Bhatia, 1990) and is an excellent vehicle fuel, which production is regarded as one of the most energy efficient and environmentally friendly way to produce biofuels (Borjesson & Mattiasson, 2008). Biotechnology nowadays allows recreating this natural process (AD) for the ultimate goal of energy production. AD experiments began in the first half of the twentieth century, and its use for organic waste treatment increase 25% annually (Buffiere *et al.*, 2008). AD can also be applied on a small scale, which provides opportunities for developing countries and rural areas, where it is difficult to have or benefit from centralised energy production (Appels *et al.*, 2011). The future use of biomass requires additional measures to ensure its sustainability, since it can indirectly promote deforestation or forest degradation and excessive soil use. For the Renewable Energy Directive, the European Commission will investigate the use of bioenergy after 2020, according to the EU energy and climate ambition for 2030, where it will be taking into account environmental, social and economic aspects of production (EC, 2012).

### 1.3. Seaweeds as bioenergy sources

Some biomass types used for bioenergy production have competitive issues with the already hard-pressed food-crop production. The first generation biofuels (*e.g.* bioethanol and biodiesel produced with food crops) have socio-economic inconveniences due to their large-scale use of arable land, pesticides and potable water and influence on food prices (*e.g.* corn and soybean). As a result, it was necessary to consider non-food crop biomass (*e.g.* cellulosic biomass such as wood or agriculture wastes – second generation biofuels) (Kraan, 2013). Likewise the use of seaweeds as biomass source (third generation biofuels) revealed to be a good alternative too. Unlike land plants, seaweeds have generally no lignin, a clear advantage as the latter polymer is inhibitory and refractory to biodegradation. Other advantages of using seaweeds as feedstock are connected to the fact that these photosynthetic organisms do not need arable land or freshwater to grow (Vivekanand *et al.*, 2012).

Seaweeds can be harvested from natural environments (about 1 million tonnes are harvested annually) or they can be cultivated in farms worldwide (over 15 million tonnes) (FAO, 2006). Marine environments have a great potential to be exploited for bioenergy production since they are responsible for approximately 50% of the global biomass (Carlsson *et al.*, 2007). In Europe, seaweed cast-up on beaches is frequent due to tides and storms, and it is known as ‘total drift’ (Guiry & Blunden, 1991). In addition, the high amount of ‘seaweed proliferation’ (Briand, 1989) – rapid growth due to eutrophication – leads them to be considered as pollutants on leisure beaches, having a waste disposal cost to landfill for local authorities. Seaweeds can also be harvested for exploitation when they grow near- or offshore. In Europe the main countries harvesting seaweeds for commercial purposes are Norway and France (Bruton *et al.*, 2009). Norway accounts for about 120,000 tonnes of *Laminaria* spp. harvested annually from a standing stock of 10 million tonnes (Jensen, 1998) and have an estimated standing stock of 1.8 million tonnes of *Ascophyllum nodosum* (Moen *et al.*, 1997b). France harvests about 50,000 – 70,000 tonnes of seaweeds annually, especially

*Laminaria* spp. for hydrocolloid production (Bruton *et al.*, 2009). According to FAO numbers (2006), aquaculture of seaweeds is of great importance in Asia, where China alone can produce 10,800,000 tonnes of seaweeds. Other places in the world are suitable for industrial seaweed farms; however, as they are not explored yet, the potential of this industry is much larger than the current production (Vivekanand *et al.*, 2012).

Using seaweed as a biofuel ($CH_4$) source was first investigated after the crisis of the 1970s and continued into the 1980s (reviewed by Forro, 1987). Currently bioethanol, biogas and syngas produced by seaweeds generate heat and electricity, and residual biomass can be used as fertilizer (Braun, 2007; Dahiya & Vasudevan, 1986). A new proposed way of using seaweeds relies on combined processes, *e.g.* production of energy parallel with alginates extraction. However, it is known that any extraction step (of alginates, laminarin and fucoidan) would reduce the potential energy yield, by lowering about 50% of the fermentable compounds (Bruton *et al*., 2009). In Portugal and Norway, a commercial company interested in using seaweeds for energy production has stated that, in relation to the potential of seaweeds aquaculture, there is a vast opportunity for Europe, considering its offshore area – 7 million $km^2$. For example, the implementation of five seaweed farming clusters representing 2,500 $km^2$ of an area between Norway and Portugal would yield 50 million tonnes seaweed annually. This biomass could then in turn be transformed in 2.1 billion litres of bioethanol or 1 billion $m^3$ biomethane (12.6 TWh) (SES, 2009-2011).

In this project, using the brown seaweed *L. hyperborea* as feedstock, a biochemical conversion process widely applied (Fannin *et al*., 1983) – anaerobic digestion (AD) – was employed to produce biomethane.

### 1.3. Anaerobic digestion of seaweeds

AD, sometimes called fermentation, is a process where a substrate is metabolized without an exogenous electron acceptor (Singleton & Sainsbury, 1993). It is performed by microorganisms in the absence of $O_2$ to digest biomass, producing biogas – mainly $CH_4$ and $CO_2$ (Horn, 2000). The referred microorganisms are a mixed population of strict and facultative anaerobes interacting through competitive and synergetic relations. They have an important role in the global carbon cycle, especially concerning the re-mineralization of organic matter (Horn, 2000). AD occurs in aqueous environments, and so any highly hydrated biomass source is suitable to be digested without any pre-treatment such as drying (Ward *et al.*, 2008). Biogas with methane content higher than 45% is flammable (Deublein &

Steinhauser, 2008). The fermentation intermediates produced (principally VFAs) are also the starting point for the long-term production of fossil fuels.

It has been previously shown that brown seaweed polysaccharides are very suitable for methanogenesis exhibiting high conversion rates and efficiency (Fannin *et al.*, 1983; Chynoweth *et al.*, 1987; Vergara-Fernández *et al.*, 2008). Most developmental studies in AD, however, focused on reactor design and substrate preparation. Little was done to examine or develop the actual composition of the microbial inoculum involved in seaweed AD – probably because of the difficulties of dissecting such a complex consortium of organisms at that time. It was recognised that the methane-producing methanogens represent the largest and most diverse group within the Archaea domain. Woese *et al*. (1990) and Deppenmeier *et al.* (1996) uncovered three pathways that methanogens use to form their products. It was previously considered by some (Chynoweth *et al.*, 1981) that a microbial consortium from waste-water or municipal-waste treatment plants may adapt to hydrolyse seaweed polysaccharides. The most efficient hydrolytic bacterial strains with the relevant enzymatic profiles were, however, considered highly unlikely to exist in these waste treatment consortia, considering that seaweeds contain unusual polysaccharides such as alginate, laminarin, fucoidan, agar and carrageenan, which are rare or absent in terrestrial plants. Indeed Rao *et al*. (1980) and Hanisak (1981) both noted the benefits of seaweed AD when using specific inocula derived from marine sediments and active in hydrolysis of seaweed polysaccharides (reviewed by Morand *et al*., 1991).

It was recently considered by A. D. Sutherland’s group at Glasgow Caledonian University (Williams *et al.*, 2012) that ruminants eating seaweed contain anaerobic microbiota that have evolved over thousands of years towards a very efficient degradation of seaweed polysaccharides. This evolutionary process may explain how they can rapidly and efficiently obtain the VFAs required for energy utilisation. This research group has therefore recently isolated and shown that rumen bacteria from seaweed-eating North Ronaldsay sheep includes bacterial species that are highly hydrolytic for seaweed polysaccharides and the entire consortium is very effective in both the acidogenic and acetogenic phases, as well as in methanogenesis (Williams *et al.*, 2012). When comparing this consortium with other potential inocula such as sewage sludge, marine sediments and consortia from naturally degrading seaweeds in lab scale fermenters, it was found that a mixture of all of these inocula was the best anaerobic digester for the brown seaweed *L. hyperborea* (A. D. Sutherland, personal communication).

### 1.4. *Laminaria hyperborea* (Gunnerus) Foslie 1884

The brown algae (Ochrophyta) have the Laminariales and Fucales as the two most economically important orders, as a result of their food potential (Madlener, 1977; Boisvert, 1987; Boivert, 1988) and polysaccharide contents. They are useful for animal and human nutrition, agriculture use, cosmetics, bioconversion, waste water treatment, pharmaceutical use and biotechnology (Guiry & Blunden, 1991).

Laminariales are distinctive by their diploid parenchymatous thallus resulting from an intercalary meristem between the stipe and the blade and its oogamic reproduction (Lee, 1989). *L. hyperborea* has, in comparison to the other species of the same genus, its biology and ecology exhaustively studied due to its economic importance (Guiry & Blunden, 1991). In the cold temperate region of the European Atlantic, the geographic distribution of this species reaches the northern coast of Iceland and the Russian coast (70ºN), having as the southern limit Cape Mondego, Portugal (40ºN) (Kain, 1967; Lüning, 1990;

Figure **4**). This distribution may have to do with the need of a temperature below 10-15ºC, so the gametophytes can produce gametes (Lee, 1989).

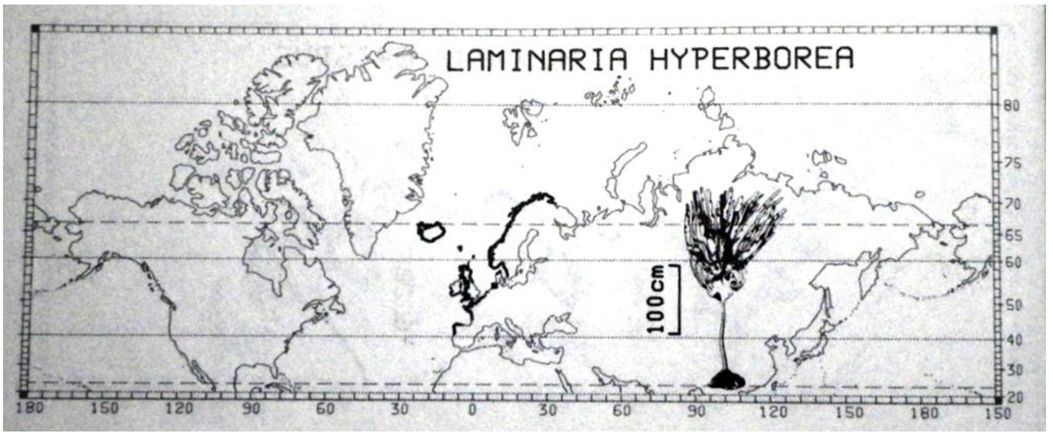

**Figure 4** *Laminaria hyperborea* distribution on the north cold temperate region (Lüning, 1990).

This species is able to occupy the mid-sublittoral zone (below the mean low water of spring tides), at about 1.5 – 4 m depth, forming the known 'laminarian forests' (Lüning, 1990). Individuals may live up to 15 years attached to the rocky substratum thanks to its rigid stipe (Lüning, 1990), which is able to support a dense blade canopy that absorbs the downwelling light (Kitching, 1941; Norton *et al.*, 1977). *L. hyperborea* stipes and leaves have been collected in the British Isles, northern France and Norway for alginate extraction (Guiry & Blunden, 1991). *Laminaria*, along with *Macrocystis*, have the highest primary production rates in the world, reaching a net production of about 1000 to 2000 g/m$^2$ of carbon annually (Mann & Chapman, 1975).

The chemical composition is one of the most important things when considering the brown seaweeds for bioenergy production or commercial products. Their cell structure has large amounts of extracellular polysaccharides surrounding the protoplast and a double cell wall of cellulose microfibrils (Lee, 1989). This cell wall has an amorphous component made up of alginic acid and fucoidan, whereas the mucilage and cuticle are composed primarily of alginic acid (Evans & Holligan, 1972; Vreeland, 1972). Alginic acids are also known as phycocolloids and are extracted and used as thickeners in the food and cosmetic industries (Guiry & Blunden, 1991). Chemically they are made up of linear copolymers consisting of β-1,4-linked-D-mannuronic acid with variable amounts of 5-epimer α-1,4-L-guluronic acid (Lee, 1989). Fucoidan is, in turn, a polymer of α-1,2-, α-1,3-, and α-1,4-linked residues of L-fucose sulphated at C-4 (Lee, 1989). Algal fucoidans have been extensively characterised, and a wide range of activities such as anti-inflammatory (Cumashi *et al.*, 2007), anticoagulant (Boisson-Vidal *et al.*, 2000; Thorlacius *et al.*, 2000; Chandía & Matsuhiro, 2008), antiviral (McClure *et al.*, 1992; Hayashi *et al.*, 2008), antitumoural (Coombe *et al.*, 1987; Alekseyenko *et al.*, 2007), gastric ulcer-protective (Hwang *et al.*, 2008) and renal failure-protective (Zhang *et al.*, 2003) capacities have been established. However, because of their potential antibacterial activity they may possibly reduce the AD potential of algal biomass, possibly due to the release of sulphide-containing compounds (Morand *et al.*, 1991). Laminarin (a β-1-3-linked glucan chain) is the main product that serves as a long-term storage product for the algae, although the sugar alcohol D-mannitol is the accumulation product of photosynthesis (Lee, 1989), having importance in osmoregulation and in sugar transportation to the thallus (Sze, 1998). Laminarin concentration greatly varies during the year being notably higher in summer/autumn than in winter/spring seasons (Sze, 1998). In general, seaweeds do not contain lignin, which may ease their use via AD as compared to land plants, making them a suitable source for bioenergy (reviewed by Kraan, 2013).

Other compounds, such as water soluble polyphenolic compounds called phaeophycean tannins, phycotannins or phlorotannins (PT) are present in the thallus of brown algae, located in intracellular physodes (major cytoplasmic vesicle-like bodies formed by dictyosomes and endoplasmic reticulum), and can inhibit the AD process, as they usually have antimicrobial activity (Schoenwaelder, 2002). They are structurally less complex than terrestrial tannins, and are polymers of phloroglucinol, *i.e.*, 1,3,5-trihydroxybenzene (Regan & Glombitza, 1986). Besides its antimicrobial properties, multiple functions of PT have been reported, including antioxidant activities and avoidance of herbivory. Significantly, the polyphenols of *Laminaria* spp. are found in the outer sheath of the stipe being a possible predation deterrent of the organ that is responsible for bringing forth new fronds, transport nutrients and growth (Moen *et al.*, 1997a). Barwell *et al*. (1989) found that a soluble, non-dialysable polyphenol fraction was inhibitory for enzyme activities (amylase, lipase and trypsin) and was readily isolated from *A. nodosum* and *Fucus serratus* and *F. vesiculosus*, but not *Laminaria* spp. Considering the above published studies and a personal communication of A.D. Sutherland, unpublished findings, it seems that the polyphenol levels in *Laminaria* spp. are unlikely to affect AD. However, it should be noted that extraction of polyphenols from *Laminaria* spp. may offer a valuable resource to enhance the economic viability of *Laminaria* AD.

## 2. Main study aims

As part of a European Research Project supported by a Marie Curie Fellowship – Seaweed AD – the present study focuses on the potential of the seaweed *L. hyperborea* to be a feedstock for the bioenergetic production of methane by AD. To fulfil this goal, using a known efficient AD inoculum (inoculum 8 supplied by Dr A.D. Sutherland), the following specific objectives concerning the fermentation process were pursued: optimisation of (i) diluent composition (seawater, distilled water and PBS); (ii) temperature (37, 35, 30 and 25 $^{o}$C); (iii) feed rate (concentrations of 2, 5, 10, 15 and 20 % [ww/v]); and (iv) continuous or occasional stirred fermentation. Processes of pre-treatment (alkali, acid, autoclaved, heated and untreated) and anaerobic pre-storage of seaweed were also investigated for this algal species for the first time.

## 3. Material and Methods

### 3.1. Seaweeds

*L. hyperborea* (15 Kg), collected as fresh, beach cast seaweed on the Firth of Forth, near Edinburgh, Scotland was washed under tap water to remove sand and debris. The fronds and stipes were roughly grounded with a Braun kitchen mince. The resulting biomass was stored at -20$^{o}$C upon receipt from Scotland via courier delivery in frozen condition.

### 3.2. Inoculum

For the mixed inoculum (inoculum 8) used in the fermenters, the samples were either previously collected in Scotland or collected in Portugal, and frozen at -70°C on collection. The Scottish inoculum samples were sent by courier airmail as ‘Biological substance, category B, UN 3373’ in January 2012. The inoculum used was a mixture of seven separate inocula: North Ronaldsay sheep rumen contents, obtained at slaughter in Kirkwell, Scotland; North Ronaldsay sheep faeces, obtained at slaughter in Kirkwell, Scotland; Normal sheep (grass eating) rumen contents, obtained at slaughter in Paisley, Scotland; Normal sheep (grass eating) faeces, obtained at slaughter in Paisley, Scotland; A mixture of municipal AD fermenter leachates from Biogen Greenfinch, UK sites: Branston, MWPD, Biocycle A and Biocycle B; Human sewage AD leachate from ETAR, Lagos, Portugal; and Marine sediments (anaerobic, black mud collected from the Ria de Formosa, Faro, Portugal mixed with naturally rotting seaweed which was anaerobically fermented for 48 hours). All inocula were stored at -20°C in 100 mL aliquots (completely filled bottles) until required.

### 3.3. Fermentation

In the start up of fermenters all materials and reagents used were pre-reduced by gassing in a sealed plastic bag flushed with a mixture of 80 % nitrogen, 10 % carbon dioxide, and 10 % hydrogen in the presence of a palladium catalyst (Don Whiteley, Scientific) to convert oxygen to water. Strict anaerobic conditions were observed by the reduction of a resazurin-based indicator strip (Oxoid, UK) from pink to colourless.

Reactor systems consisted of 500 mL flasks filled until 200 mL. These mini-fermenters were fitted with a gas collector (volumetric buoyant inverted cup) on side-arm from where biogas could be collected, the volume measured and sampled for gas analysis (Figure 5).

**Figure 5** Bioreactor system: conical flask fermenter with the gas collector on side-arm.

## 3.4. Determination of %$CH_4/CO_2$, cellular viability and total solids/volatile solids

In every experiment three main tests were performed as data collectors: determination of %$CH_4/CO_2$, cellular viability and total solids/volatile solids.

### 3.4.1. Determination of %$CH_4/CO_2$

The %$CH_4/CO_2$ in the biogas was determined by injection of an approximately 0.5 mL sample of biogas onto a Perkin Elmer 8500 Gas Chromatograph equipped with a methaniser and a flame ionisation detector. Helium was used as a carrier gas under a flow rate of 20mL/min from a 6 feet x 1/8in column packed with 80/100 carbosphere (Speck and Burke, UK). The partition was run isothermally with an oven and injector temperature of 150°C, and 300°C for the detector. Peak area was transformed into compound percentage using a Hewlett Packard HP3396 series II in-line integrator.

### 3.4.2. Determination of cellular viability

The fluorescein diacetate (FDA) assay was performed to measure metabolic viability of the inoculum, which can be used to estimate the number of viable bacteria, when a correlation and a regression are determined for fluorescence versus total microbial count (TMC). The method relies on the fact that, when microorganisms are viable, they actively convert the FDA non-fluorescent compound into a green fluorescent compound – the fluorescein – by an esterase activity. This fluorescence can then be quantified, using a fluorimetric spectrophotometer, and correlated to the total microbial counts of a fixed and stained sample.

The FDA assay was carried out essentially as published by Peeters *et al.* (2008). A 1:50 dilution of FDA stock solution (10 mg/mL in acetone) was made in 100 mM MOPS buffer and stored at -20 ºC covered with foil. Volumes (100 µL) of this were added to duplicate 100 µL volumes of each inoculum sample ($10^{-1}$ and $10^{-2}$ dilution) in black multilabel microtitre plates. Samples were incubated for 1h and fluorescence read (Biotech, Synergy4 microplate reader) using 518 and 494 nm as emission and excitation wavelengths, respectively, after subtraction of the blank reading of 100 µL sterile distilled water plus FDA working solution. For this method dilutions of $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$ were used. TMC were determined from direct microscopy using the New Portman grid (May, 1965) after the samples were fixed with glutaraldehyde 0.2 % (v/v) and stained with acridine orange 0.1 % (w/v). Results were given in cells/mL, following the formula below:

$$\mathrm{TMC}(\mathrm{cells.L}^{-1}) = \frac{\mathrm{x.A.d}}{\mathrm{a.n.v}}$$

Where:

$x$ = number of cell counted

$A$ = area of the filtration chimney ($mm^2$)

$d$ = dilution factor due to the addition of glutaraldehyde (final vol. /sample vol.)

$a$ = area of each field viewed ($mm^2$)

$n$ = number of fields counted

$v$ = volume of the filtered sample (in L)

The samples analysed had a dilution of $10^{-6}$, $10^{-7}$ and $10^{-8}$.

In further direct readings of FDA values, the dilution factor of the analyzed sample was eliminated by adding the inverse $\log_{10}$ factor of dilution to the $\log_{10}$ fluorescent reading.

### 3.4.3. Determination of total solids/volatile solids

Residual seaweed total solids (TS), ash and volatile solids (VS) were measured after each Hydraulic Retention Time (HRT, time needed to completely change the leachate from a fermenter) or feed, by first drying the samples at 100 $^{o}$C overnight in an oven (Binder) in small ceramic crucibles. After the samples were cooled down in desiccators, they were weighed to determine TS or dry weight. Finally, the dry residues were further heated at 530$^{o}$C overnight in a furnace (Cassel) and weighed again to obtain the ash content, and by subtraction from TS, the VS content (Larsen, 1978). From the result obtained, the proportion of seaweed VS remaining after AD could be calculated by comparison with the VS in the original seaweed feed (to get the %VS used during the AD).

Methane yield was calculated as mL of methane/g of seaweed VS and methane productivity as mL of methane/g of seaweed VS/day. Yield depends upon seaweed concentration added and solids retention time. Productivity depends upon concentration, feed rate and dilution rate.

## 3.5. Diluents composition study

A culture of inoculum 8 already adapted to *L. hyperborea* was established by defrosting 100 mL of the frozen leachate recovered from week 4 of inoculum replicate 8c, and recovering the culture by adding 300 mL of PBS, supplementing this with 0.025% glucose/yeast extract and growing for 24 hours anaerobically. Two 100 mL volumes of this culture were refrozen and the remaining 200 mL were added to a mini-fermenter, 20 mL of 20% (ww/v) *L. hyperborea* was also added and the pH adjusted to 7.5. This amount of seaweed was then added every second day (as a fed-batch culture) with pH adjusted to 7.5 until the fermenter culture reached 300 mL in volume. The culture was then split into three mini-fermenters (100 mL each) and 20 mL of 20% (ww/v) seaweed was added to each fermenter every second day until 200 mL volume was reached in each fermenter.

Each fermenter was fed every second day with 50 mL of 8% (ww/v) seaweed (equivalent to 1% [ww/v] seaweed/day) and pH adjusted to between 7.3 and 7.5, after removing 50 mL of leachate. Seaweed residue was removed weekly for TS/VS analyses. The fermenter cultures were tested weekly for FDA fluorescence, biogas volume and %$CH_4$/$CO_2$ produced until it was considered they were in adequate conditions for the experiment to start. After this, one culture was slowly adapted to distilled water diluent and other to seawater diluent (both sterilised by autoclaving); the seawater was collected from the Atlantic Ocean on Faro Island, Portugal. These were compared with a third fermenter culture which was continually treated with PBS. Adaption process was done by adding the new diluent in place of PBS to each culture at 50 mL every second day. Once adapted, if survived, each replicate culture was then grown successively for three HRT in its respective diluent. The cultures were monitored weekly for FDA fluorescence, biogas volume and %$CH_4$/$CO_2$ produced and TS/VS used.

The fermenter run with sterile distilled water as diluent was continued as a long term fermentation and supply of inoculum for further optimisation experiments.

### 3.6. Pre-storage of seaweed

To six sandwich bags 100 g ww of *L. hyperborea* seaweed were added. To half of the bags were added 10 mL of distilled sterile water and to the other half 10 mL of inoculum 8 (the inoculum used in this thesis AD experiments). The bags were degassed by filling and emptying three times, with a mixture of 80% N, 10% $CO_2$ and 10% $H_2$, creating an anaerobic environment. Storage bags needed to be empty of any gas at the start to know if any was produced during the storage period, and if it was the case, volume and methane % were measured. They all were left at ambient temperature outside for either 4, 8 or 12 weeks and minimum and maximum temperatures of storage were recorded using a digital thermometer. The condition of the seaweed in each storage bag was assessed by visual examination for obvious microbial growth. The moisture content, TS and VS of the stored seaweeds were also measured and compared with the original seaweed.

In a further study 100 g ww of *L. hyperborea* were added to four other sandwich bags. Two bags were exposed to two different treatments – autoclaving for 15 min or heating (80 ºC) for 1 h – and these were then stored at 2-4 ºC. The other two bags suffered no pre-treatment and were respectively stored outside at ambient temperature (with maximum and minimum temperature recorded) and in the refrigerator (2-4 ºC). They all were left for 12 weeks and then the condition of the seaweed in each storage bag was assessed by visual examination for obvious microbial growth. Also, the TS and VS of the stored seaweeds were measured and compared with the original seaweed. The two first bags stored in the refrigerator (autoclaved and heated) and the untreated bag stored outside were tested for total sugars (Dubois *et al.*, 1956) and tannins (Ayaz *et al.*, 2008) to try to find if the amount of sugars available for AD were preserved or consumed during the storage period, and also if there was any alteration in tannins concentration. No other bags were tested for this, because of their higher degree of contamination.

### 3.6.1. Sugar quantification

The sugars quantification method (Dubois *et al.*, 1956) consisted in adding 5 µL of 80% phenol to a 10-µL sample, followed by the addition of 200 µL of concentrated $H_2SO_4$ and an incubation period of 10 min at room temperature. The absorbance was later read at 490 nm and a standard curve was determined using glucose as standard with the concentration of sugars given in mg/mL.

### 3.6.2. Total tannins quantification

The total tannins quantification method (Ayaz *et al.*, 2008) consisted in adding 100 µL of 10-fold diluted Folin-Ciocalteu reagent to a 10-µL sample, followed by an incubation period of 5 min at room temperature in the dark. After that, 100 µL of $Na_2CO_3$ (75 g/L) were added and the microplate was incubated for 90 min at room temperature in the dark. The absorbance was read at 725 nm and a standard curve was drawn using Gallic Acid Equivalents. The concentration of tannins was given in mg/mL.

## 3.7. Temperature optimization

From a 200 mL long term grown inoculum 8 supplied by Dr. A. D. Sutherland, 50 mL were distributed into three mini-fermenters, adding 50 mL of sterile distilled water to each (considering the results from the diluent experiment). Then 20 mL of 20 % (ww/v) *L. hyperborea* seaweed in sterile distilled water were given every second day as a fed-batch culture, until the fermenters volume reached 200 mL. All fermenters were incubated at 37°C and metabolic viability, biogas and $CH_4$ productions were confirmed (see section 3.4). When ready, individual fermenters were incubated separately at 35, 30 and 25°C by dropping 5°C every day, until each reached the desired temperature. The fermentation process was then continued until one of the fermenters reached four HRT. Each HRT happened when 4 feeds were given, because the feeding procedure consisted of removing 50 mL of leachate followed by the addition of 2 % (ww/v) *L. hyperborea* chopped and mixed in a meat grinder in distilled autoclaved water (4 g ww seaweed in 50 mL). The used parts of *L. hyperborea* were the leaves and stipes. Feeds were given only when biogas, in the inverted cup gas collector, no longer increased. The pH was adjusted to between 7.3 and 7.6 with 2M NaOH or HCl. If the pH dropped below 6.5, several microorganisms within the inoculum consortia were at risk. Only the acetate-using methanogenic archaeons belonging to the *Methanosarcina* genus are known to support this value of pH (Deublein & Steinhauser, 2008).

Upon every HRT, residual seaweed was removed and total biogas produced, % of $CH_4/CO_2$ in the biogas, bacterial count equivalent and %VS used were calculated. To see if the inoculum survived after lower temperatures incubation (25 ºC), a recovery process was performed by moving all fermenters to the initial temperature of 37 ºC.

### 3.8. Influence of feed rate

Feed concentrations of 5, 10, 15 and 20% (ww/v) were tested in four fermenters. Higher concentrations could not be used, as the suspension would be too viscous to pump and would probably be toxic due to excessive phlorotannins. The time necessary to fully ferment the various concentrations of seaweed was determined by measuring biogas volume produced. Previous experiments have shown that a feed concentration of 2% (ww/v) / 2 days, and a dilution rate of 25% of the fermenter volume (50 mL from a 200 mL total fermenter volume) every second day in a semi-continuous fermenter system when adding feed was of potential benefit in reducing product feedback inhibition (*e.g.* from VFA; A.D. Sutherland, personal communication). However, in this experiment, considering the amount of seaweed given per feed, the dilution rate was of 50% of the fermenter volume at each given feed to avoid feedback inhibition. The pH was checked every day and adjusted to between 7.3 and 7.6 and biogas volume registered. Residual seaweed was removed after every feed for TS/VS analysis, and the % $CH_4/CO_2$ in biogas and FDA fluorescence were measured weekly. The inoculum used for the four mini-fermenters of this experiment came from a combination of mini-fermenters from the temperature experiment, after a maintenance period to ensure continued good biogas production.

On the results analysis, these different feed concentrations were also compared with the 2% (ww/v) distilled water fed fermenter from the diluent experiment (see section 3.5) which was subjected to the same conditions of incubation.

### 3.9. Pre-treatment of seaweed

Inside an anaerobic bag, four empty autoclaved mini-fermenters, one Duran bottle with 300 mL autoclaved PBS and a bottle of long term grown inoculum 8 frozen on October 10th, 2012 (defrosted at 30ºC) were all left for 2 h to equilibrate with bottle tops loose. Then 100 mL of the inoculum, 0.5 mL 20% (w/v) glucose and 0.5 mL 20% (w/v) yeast extract were added to PBS. The pH was adjusted to 7.4 and the culture was incubated overnight at 37 ºC. After this, 100 mL volumes were distributed through the four mini-fermenters and a 2% (ww/v) feed were given to each. They were all left for 48h at 37ºC, the pH being checked and adjusted every day. Upon 6 feeds of 2% (ww/v) every second day the flasks were all combined into one, adding the leachate of two other flasks (from the feed rate experiment, section 3.8,) known to have good biogas productivity. The feeding process was the same for more 4 feeds. After this, and considering the good $\log_{10}$ FDA and methane production results, it was chosen to start the experiment of the different feed pre-treatments. Five mini-fermenters were fed with feeds of 2% (ww/v) every second day which were either untreated, autoclaved, or heated at 80°C while immersed in 0.5M HCl or 0.5M NaOH.

The first 3 pre-treatment feeds were done by grinding 4 g ww seaweed in a 100 mL pot with little sterile distilled water, after which it was added more to make up to 50 mL. Then, the pots were either autoclaved, stirred for 1 hour at 80ºC or left as an untreated control. The other pre-treatments consisted on leaving 4 g ww ground seaweed in 10 mL 0.5M HCl or 0.5M NaOH stirring for 6 hours at 80°C (low molarity, moderate temperature), followed by dilution until 50 mL with sterile distilled water. The HCl or NaOH was neutralised to pH 7.5, with 2M NaOH or 2M HCl before addition of the seaweed to fermenters.

When giving a feed to each fermenter, 50 mL of leachate were replaced from the 200 mL mini-fermenter, so that after 4 feeds an HRT occurred. Residual seaweed was removed at every HRT for analyses of the %TS/VS used. Measures of total biogas produced, % $CH_4/CO_2$

and $\log_{10}$ FDA fluorescence were also recorded weekly. All fermenters were left for 4 HRT with the biogas volume produced checked every day.

### 3.10. Continuous or occasional stirred fermentation

To test how stirring influenced fermentation, a new fermenter was made using 100 mL inoculum from a known productive fermenter. It was fed with 2% (ww/v) autoclaved seaweed (every second day) until it reached 200 mL. The next feeds were given by replacing 50 mL of leachate by a new feed, and the mini-fermenter was left for one week to be monitored for suitable biogas productivity. When ready, the experiment was started with the fermenter being continuously stirred for 4 HRT with feeds given every second day as previously explained (see section 3.7). The stirring effect was performed using a magnetic stirrer with enough intensity to apply some movement in the seaweed inside the mini-fermenter.

For comparison to the previous fermenter, data from one occasional stirred fermenter of a previous study (pre-treatment experiment section 3.9) was used, since that fermenter was fed with similar 2% (ww/v) autoclaved seaweed.

### 3.11. Data analyses

All comparisons of data means and variances were done on the software programme STATISTICA 7 (StatSoft ®) for Windows, with an alpha ($\alpha$) of 0.05.

Whenever the pre-requisites of normality and homoscedasticity were fulfilled for the data studied, ANOVA parametric test was performed under the null hypothesis of no differentiation between the means of different variables in study (three diluents, four temperatures, three feed concentrations and five pre-treatments). If the null hypothesis was rejected, Post-hoc test (Tukey HSD) was performed to discover which variables were significantly different. Data subjected to this analysis were from the diluent experiment (yield, productivity, $\log_{10}$ FDA, %VS used), feed rate experiment (yield and $\log_{10}$ FDA), and pre-treatment experiment ($\log_{10}$ FDA).

If normality and homoscedasticity were not found, Kruskal-Wallis non-parametric test was applied. If significant differences were found, then a Post-hoc test (Dunn) was done. Dunn's Post-hoc test critical value used was: $Qc_{0.05,\,4} = 2.639$, for $\alpha = 0.05$ and 4 variables. Data subjected to this analysis were from the temperature experiment (yield and productivity), feed rate experiment (productivity and %VS used) and pre-treatment experiment (yield, productivity and %VS used).

When the data had just two variables as the stirring experiment (occasional and continuous) all data (yield, productivity, %VS used and $\log_{10}$ FDA) were statistically investigated using the T-student parametric test.

# 4. Results

## 4.1. Cellular viability

To evaluate the cellular viability of the inoculum along the different experiments, a correlation was performed between the fluorescence of the fluorescein diacetate (FDA) assay and the total microbial counts (TMC). This determination allowed the replacement of the latter method by the former, being faster and more intuitive for the operator.

To obtain the TMC data, several dilutions of a inoculum sample were made. However, only one ($10^{-7}$) was selected for further calculations, considering the field microscope effort needed (*n* = 30) to have at least 200 microbial counts, making it statistically acceptable (see formula in section 3.4.2). TMC of the remaining dilutions were extrapolated from the $10^{-7}$ dilution.

The FDA assay data obtained from technical triplicates of the dilutions $10^{-1}$, $10^{-2}$ $10^{-3}$ and $10^{-4}$ of the leachate sample (table 1, Figure 6) showed that the equipment used (Biotech, Synergy4 microplate reader) was unable to quantify the high fluorescence produced by the sample $10^{-1}$ dilution. Taking that into consideration, $10^{-1}$ dilution was not included in the correlation graph. Dilutions higher than $10^{-4}$ were not done because, after subtracting the blank value (430 fluorescence units [FU]), the fluorescence values were zero. So the detectable fluorescent range was within $10^{-2}$ and $10^{-4}$ dilutions.

**Table 1** Logarithmic FU means of the Fluorescein Diacetate (FDA) assay (*n* = 3) with standard deviation in brackets, and the Total Microbial Counts (TMC) used to construct the correlation graph.

| **Dilution sample** | **$\text{Log}_{10}$ (FDA)** (FU) | **$\text{Log}_{10}$ (TMC)** (microbial cells/mL) |
|---|---|---|
| **$10^{-2}$** | 4.63 (± 0.04) | 10.67 |
| **$10^{-3}$** | 3.43 (± 0.04) | 9.67 |
| **$10^{-4}$** | 2.45 (± 0.04) | 8.67 |

When a $\log_{10}$ conversion was applied to the FDA and TMC values for the same sample dilution, a good positive correlation was obtained ($R^2 = 0.99$) (Figure 6). Using the regression equation obtained ($y = 0.91x + 6.46$), the logarithmic number of cells per mL (y) for any microbial sample can be easily quantified using just the logarithmic fluorescence values of a sample ($x$). In conclusion, it can be said that whenever a $\log_{10}$ FDA value is close or higher than 3.4 FU, the inoculum is in a good state of cellular viability ($\geq \log_{10}$ 9.67 microbial cells/mL).

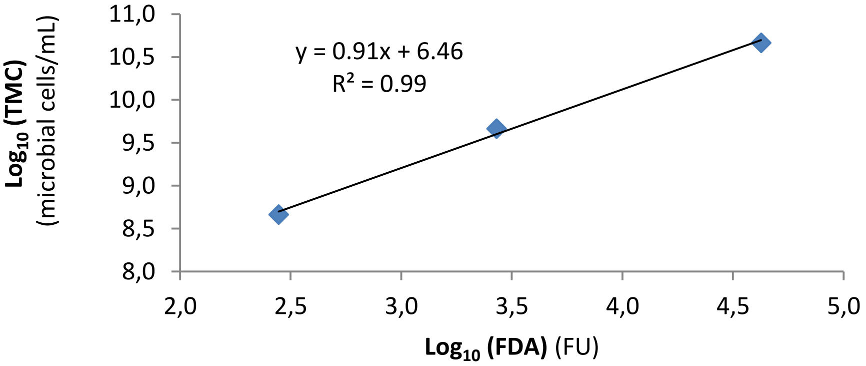

**Figure 6** Correlation between the log $_{10}$ Total Microbial Counts (TMC) and the log $_{10}$ Fluorescent Units (FU) measured by the Fluorescein Diacetate (FDA) assay.

### 4.2. Diluents composition study

Three different diluents (PBS, $H_2O$d and seawater) were compared about their ability to act as a buffer solution in AD process. Several parameters were registered throughout the experiment and are shown below (Table 2). Each HRT represents four feeds of 4 g ww seaweed in 50 mL diluent, each taking 8 days to completely change the diluent volume. Total biogas produced *per* HRT was not significantly different ($p > 0.05$) between the three fermenters in study neither the percentage of methane in the biogas ($p > 0.05$). The mean percentages of volatile solids used by the fermenters after each HRT were between 58.8 and 67.4% and the cellular viability *per* HRT reflected the inoculum good conditions, since $\log_{10}$ FDA was between 5.7 and 6.5 FU.

**Table 2** Results for the diluents composition experiment (*n* = 36). Total biogas produced, % of $CH_4$ in biogas, %VS used, cellular viability (FDA), yield and productivity with their respective means and standard deviation for the different diluents (PBS, $H_2$Od and Seawater) are shown over three HRT.

| | **PBS** | **Distilled water** | **Seawater** |
|---|---|---|---|
| **Total Biogas Produced (mL)/HRT** | | | |
| **1st HRT** | 455 | 360 | 285 |
| **2nd HRT** | 445 | 480 | 290 |
| **3rd HRT** | 450 | 435 | 280 |
| **Mean** | 450.0 | 425.0 | 285.0 |
| **SD** | 5.0 | 60.6 | 5.0 |
| **% Methane in biogas/HRT** | | | |
| **1st HRT** | 51.8 | 52.2 | 51.2 |
| **2nd HRT** | 51.6 | 53.7 | 47.2 |
| **3rd HRT** | 58.3 | 57.5 | 58.4 |
| **Mean** | 53.9 | 54.5 | 52.4 |
| **SD** | 3.8 | 2.7 | 5.6 |
| **% Volatile Solids Used/HRT** | | | |
| **1st HRT** | 65.7 | 67.2 | 60.1 |
| **2nd HRT** | 67.4 | 62.3 | 57.5 |
| **3rd HRT** | 69.2 | 57.8 | 58.8 |
| **Mean** | 67.4 | 62.4 | 58.8 |
| **SD** | 1.8 | 4.7 | 1.3 |
| **$\log_{10}$ FDA/HRT** | | | |
| **Mean** | 6.5 | 6.3 | 5.7 |
| **SD** | 0.3 | 0.3 | 0.2 |
| **Yield (mL methane/g of VS)/HRT** | | | |
| **Mean** | 74.7 | 71.4 | 45.9 |
| **SD** | 5.4 | 11.8 | 4.1 |
| **Productivity (mL methane/g of VS/day)/HRT** | | | |
| **Mean** | 9.3 | 8.9 | 5.7 |
| **SD** | 0.7 | 1.5 | 0.5 |

PBS = Phosphate Buffer Solution; HRT = Hydraulic Retention Time; FDA = Florescein Diacetate.

The mean yields obtained from the mini-fermenters having PBS and $H_2$Od as diluents were significantly higher ($p < 0.05$) from the mean yield obtained by the mini-fermenter with seawater diluent. There were no significant differences in the yield between the fermenter fed with PBS or $H_2$Od ($p > 0.05$). Figure 7 illustrates the experiment development of the three fermenters along their HRTs, where it is possible to see that the $H_2$Od fed fermenter recovered after the 1st HRT (the inoculum was originally grown in PBS, see methods section 3.5) showing a yield improvement on the subsequent HRT.

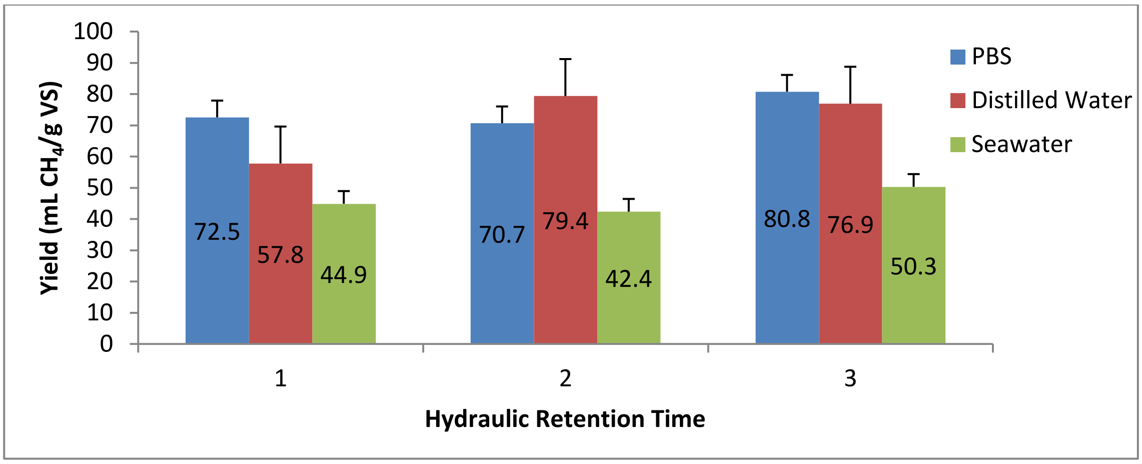

**Figure 7** Yield of mini-fermenters fed with different diluents during three HRT. For each diluent column *n*=4; PBS = Phosphate Buffer Solution. Error bars show standard error.

The low biogas production of the seawater-fed fermenter was mirrored by a drop in its bacterial viability measured by the FDA assay (Figure 8). The mean $\log_{10}$ (FDA) FU of PBS was significantly higher ($p < 0.05$) from the one obtained from the seawater fed fermenter, but no different from the $H_2O$d one ($p > 0.05$).

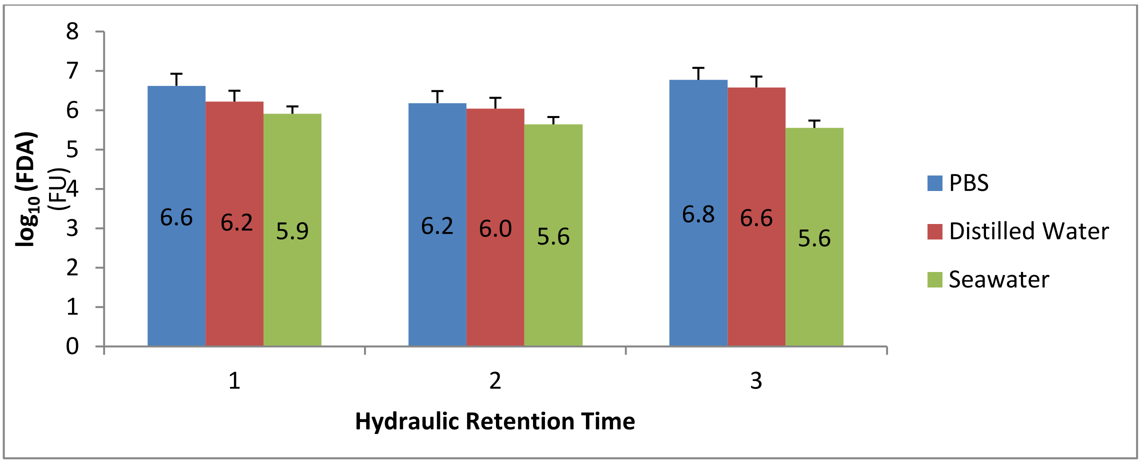

**Figure 8** Cellular viability of mini-fermenters fed with different diluents during three HRT. For each diluent column *n*=4; PBS = Phosphate Buffer Solution; FDA = Fluorescein Diacetate. Error bars show standard error.

When comparing the means of the %VS used (Table 2, Figure 9) it was found that only the fermenter with seawater diluent was significantly lower ($p < 0.05$) than the fermenter with PBS diluent. The %VS used by the $H_2O$d fed fermenter was not significantly different from the other means (seawater or PBS) ($p > 0.05$).

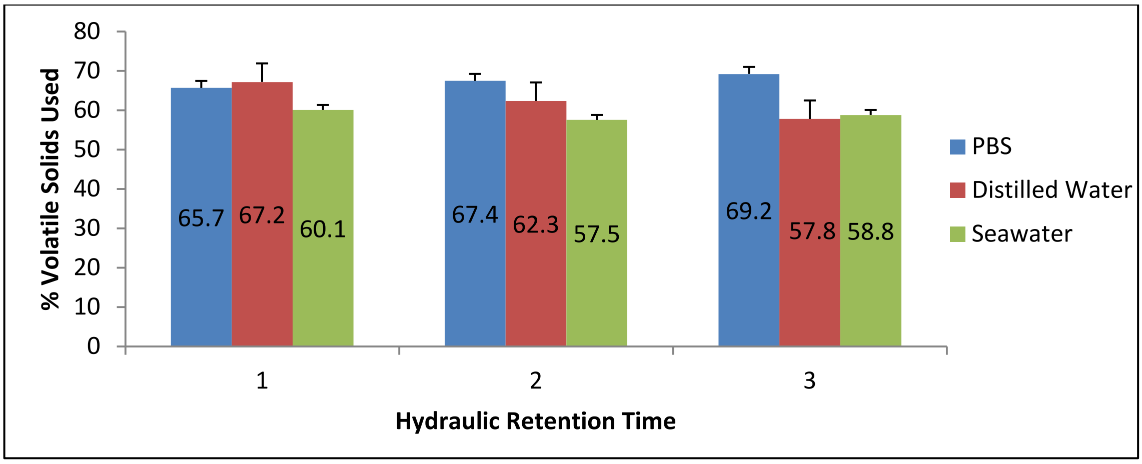

**Figure 9** Percentage of Volatile Solids used by mini-fermenters fed with different diluents during three HRT. For each diluent column *n*=4; PBS = Phosphate Buffer Solution. Error bars show standard error.

When analysing the different means of productivity obtained (Table 2, Figure 10), there are again no differences in whether using PBS or $H_2Od$ as diluent ($p > 0.05$). Distilled water can therefore clearly replace PBS without adversely affecting methane productivity, culture viability or usage of VS.

Distilled water and PBS productivities were significantly higher ($p < 0.05$) than the seawater diluent productivity. Although the seawater diluent quickly reduced its methane production down to 47.2 % $CH_4$ by the 2nd HRT (see table 2) its mean methane production was no different as the other diluents ($p > 0.05$).

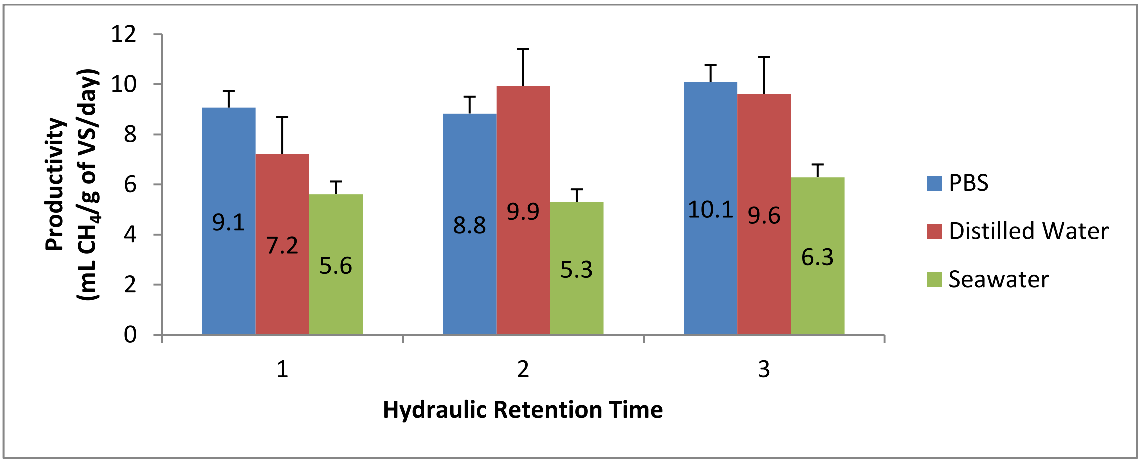

**Figure 10** Productivity of mini-fermenters fed with different diluents during three HRT. For each diluent column *n*=4; PBS = Phosphate Buffer Solution. Error bars show standard error.

### 4.3. Pre-storage of seaweed

It was considered that to store seaweed aerobically would likely result in a net loss of energy due to growth of aerobic bacteria without methane production. Grown anaerobically precursors for methanogenesis may accumulate and anaerobic bacteria may be digested too or contribute to AD in the fermenter. Possibly then, seaweed pre-fermented anaerobically in sealed bags by exogenous flora was taken to be more suitable for future AD than others stored with no AD precursors.

A subjective scale system to evaluate the degree of contamination was elaborated having six levels, from the most contaminated to the non-contaminated status: very high, high, medium, low, very low, and zero (Table 3). None of the methods tested in this experiment allowed a good conservation of the seaweeds, so the ‘zero degree contamination’ was never achieved. The % of TS and VS obtained from the seaweeds after the storage period was compared with its original state, and so in the analysis the original ratio VS/TS was compared with the post-storage ratio. When significant differences were found, it meant VS were lost or consumed, and the storage method was not suitable. The total sugar content of the untreated bag left outside (0.318 mg/mL) and the autoclaved bag (0.340 mg/mL) decreased approximately by the same degree when compared to the original seaweed sugar concentration (0.474 mg/mL), whereas the heated bag had just 35% of the original seaweed sugar concentration (0.166 mg/mL). In relation to tannins (PT), it seems that storing seaweed independently from the pre-treatment given increase their concentration (original: 0.074 mg/mL; autoclaved: 1.808 mg/mL; untreated left outside: 1.562 mg/mL; and heated: 1.173 mg/mL).

In the first experiment, all bags (first six storage bags on Table 3) were gassed with a mixture of an anaerobic gas; the bag of seaweed inoculated with 10 mL of inoculum 8 used as a microbial preservative (bag 1) was compared with the bag with 10 mL of water used as control (bag 2). These bags were analysed 4 weeks after the start of the experiment (21st December 2012). The temperature ranged between 4.2 – 28.2ºC. Before the opening of the latter bags both released a bad smell, suggesting that the sealing method was not perfect and they were most probably contaminated with a fungus, which was not identified. The origin, internal or external, of the contamination also remained undetermined (results not shown).

**Table 3** Results for the storage experiment. The inoculum/water (control) to which each bag was exposed is given in the first column, followed by the storage period and condition, the final degree of contamination, the % of TS and VS before and after the experiment and the concentration of sugars and tannins (PT) before and after the experiment.

| **Method** | **Storage period** (weeks) | **Storage condition** | **Degree of contamination** | **TS** (%) | **VS** (%) | **Original TS** (%) | **Original VS** (%) | **Sugars** (mg/mL) | **Original Sugars** (mg/mL) | **PT** (mg/mL) | **Original PT** (mg/mL) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Bag 1 (Inoc 8) | 4 | O | High | - | - | - | - | - | - | - | - |
| Bag 2 (water) | 4 | O | Medium | - | - | - | - | - | - | - | - |
| Bag 3 (Inoc 8) | 8 | O | Very high | - | - | - | - | - | - | - | - |
| Bag 4 (water) | 8 | O | Medium | 14 | 10 | 26 | 20 | - | - | - | - |
| Bag 5 (Inoc 8) | 8 | O | Very high | - | - | - | - | - | - | - | - |
| Bag 6 (water) | 8 | O | Very high | - | - | - | - | - | - | - | - |
| Autoclaved | 12 | R | Low | 75 | 55 | 20 | 15 | 0.340 | 0.474 | 1.808 | 0.074 |
| Heated | 12 | R | Very low | 34 | 25 | 20 | 15 | 0.166 | 0.474 | 1.173 | 0.074 |
| Untreated | 12 | R | Low | - | - | - | - | - | - | - | - |
| Untreated | 12 | O | High | 78 | 54 | 20 | 15 | 0.318 | 0.474 | 1.563 | 0.074 |

TS = Total Solids; VS = Volatile Solids; PT = Phlorotannins; Inoc 8 = inoculum 8; O = Outside; R = Refrigerator; (-) = not done.

The bag with inoculum 8 (bag 1) had some gas inside, contrasting with the control bag (bag 2), which was in a complete vacuum condition (so the smell of this bag could possibly be coming from the outer side of the bag caused by some spillage during preparation). At the top of both bags seaweed leaves were lighter coloured, probably due to photoxidation from sunlight. Considering the fungal contamination, the bags were not kept for future fermentation. It was clear then that gassing with a mixture of anaerobic gas was not sufficient to stop fungal growth and spoilage of the bagged seaweed.

The second sampling (bags 3 and 4) happened after 8 weeks of storage (18th January 2013). The thermometer recorded approximately the same temperature range as above (4.2 – 28.4ºC). The bags were again both contaminated with fungus (Figure 11 and Figure 12) as also were bags 5 and 6 (Figure 13 and Figure 14), which were supposed to continue in storage for an additional 4-week time period. However, since spoilage was apparent in all bags the experiment was not continued any further. When analysing the bags (3 to 6) more closely, it was possible to see that all of them contained some air inside; the ones which were inoculated also showed zones with water condensation on the wall of the bags. The contaminations seemed to appear more in the upper part of the seaweed material and there was some photo-oxidation on the fronds distributed in the upper part of the bags. The less affected bag (bag 4) was not inoculated and although it showed signs of contamination (small white dots) it had small bubbles of gas inside (Figure 12). Being the less affected, the seaweed of bag 4 was the only one analysed for TS/VS, having 14% TS and 10% VS after storage. When compared to the original seaweed state (26% TS and 20% VS) the ratio VS/TS did not change considerably (before storage = 0.769; after storage = 0.714). The decrease in the %TS and VS was most likely due to the wetter state of the seaweed, caused by the addition of 10 mL of water.

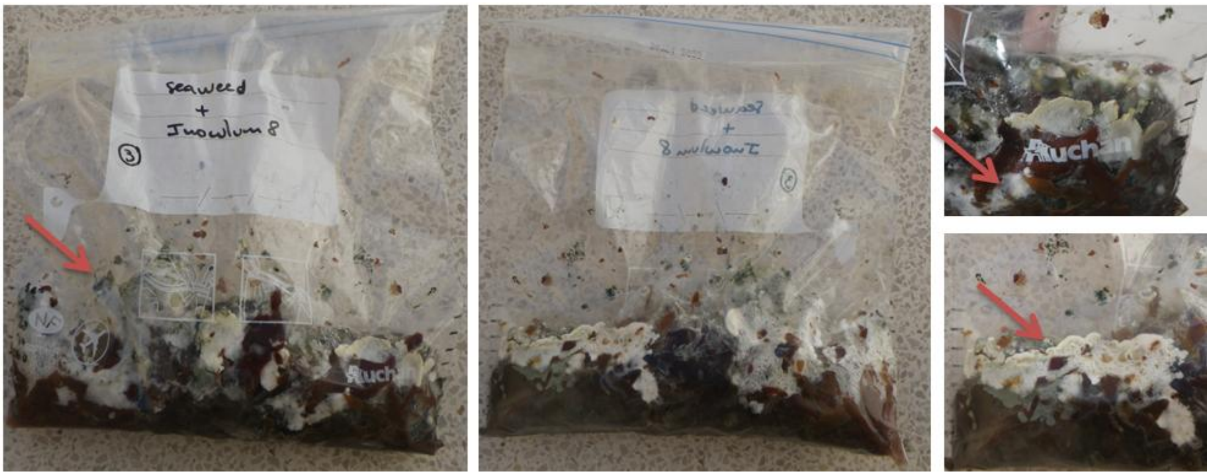

**Figure 11** Storage bag no.3 of *L.hyperborea* with 10 mL inoculum 8. Arrows indicate different types of contaminations.

**Figure 12** Storage bag no.4 of *L. hyperborea* with 10 mL distilled sterile water. Arrows indicate lighter seaweed fronds and air bubbles among moisture inside the storage bag.

**Figure 13** Storage bag no.5 of *L. hyperborea* with 10 mL inoculum 8. Arrows indicate lighter seaweed fronds and different types of contaminations.

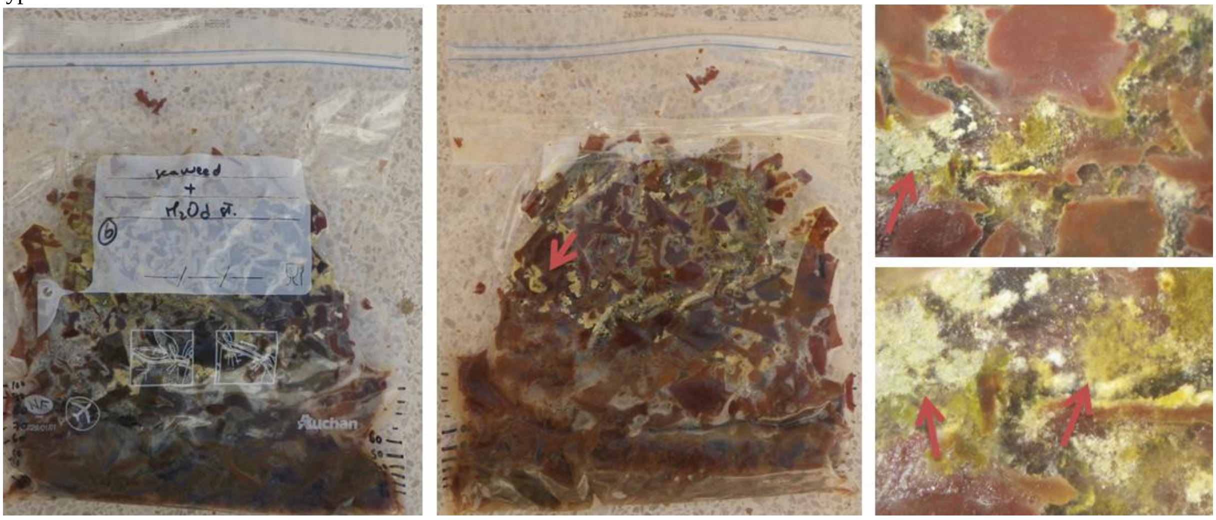

**Figure 14** Storage bag no.6 of *L. hyperborea* with 10 mL distilled sterile water. Arrows indicate different types of contaminations.

The second pre-storage experiment went on to compare different pre-treatments (autoclaved, heated at 80ºC and untreated) and storage conditions (outside ambient temperature and refrigeration temperature) with each bag examined after various storage periods.

The first result was from the untreated bag in the refrigerator (2-4ºC) (19th April 2013) (Figure 15). Although the bag seemed not to have air inside, the conservation of the seaweed was compromised since microbial contamination could be seen in the lower part of the bag distributed in patches (green and white). On the outside of the bag, small white round colonies of microorganisms were also found, but their origin (in or outside) was not certain. When the bag was opened, the seaweeds were very dry. No TS/VS, sugars or tannins tests were performed. At this time the seaweed in all the other bags was in good condition.

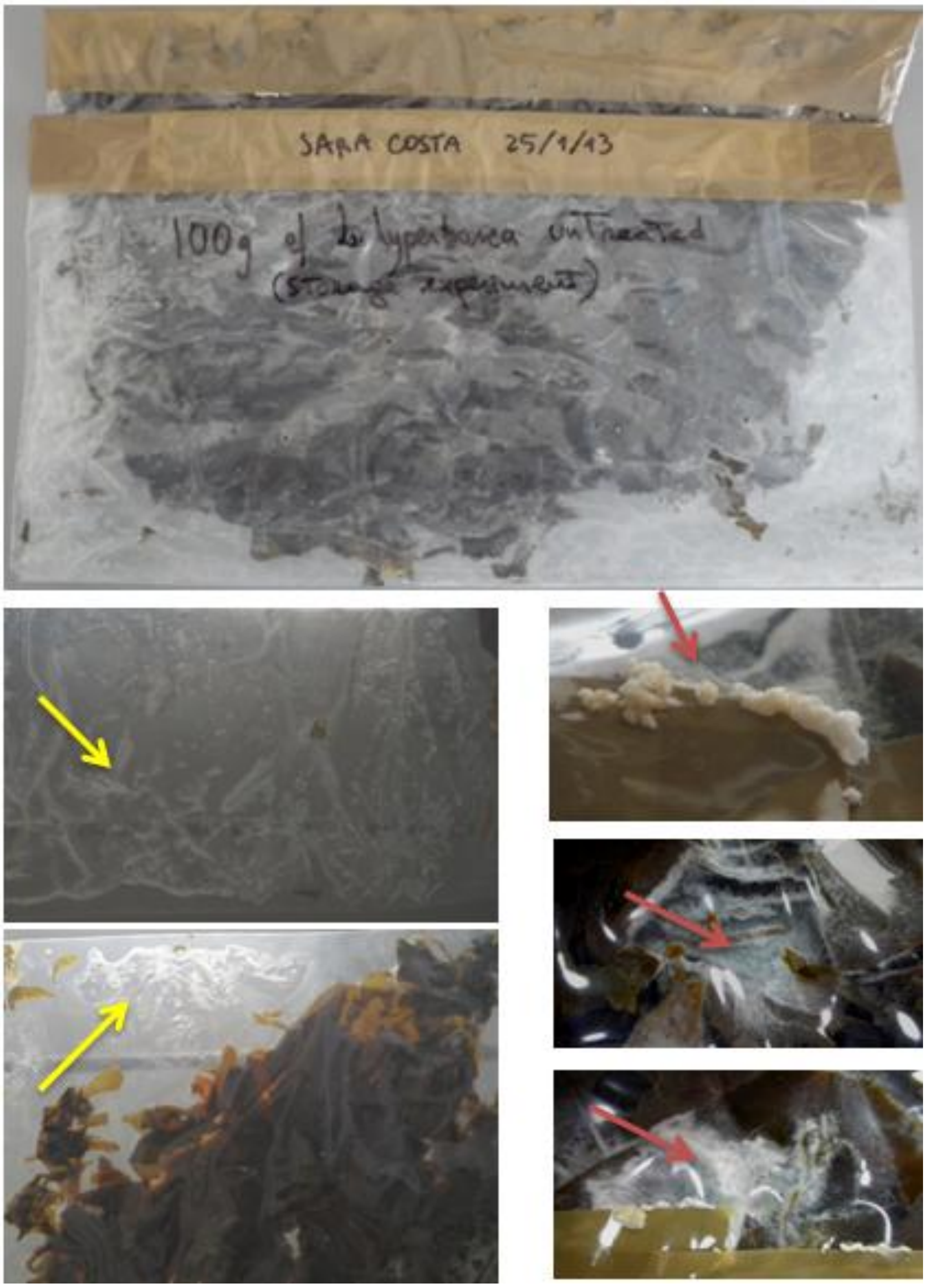

**Figure 15** Storage bag of *L. hyperborea* untreated and stored in the refrigerator. Red arrows indicate contaminations (small round colonies outside the bag, green patch inside and white patch inside close to the colonies). Yellow arrows indicate a different type of contamination (branched white formations along the bag).

The second examination made was of the untreated bag stored outside and the heated and autoclaved bags stored in the refrigerator (2-4ºC) (6th May 2013). None of the bags seemed to have gas inside.

The untreated bag stored outside experienced a range of temperatures between 7.1 – 32.7ºC. Some salt crystals were seen and microbial contaminations were found and photographed (Figure 16). The TS/VS results gave 78% TS and 54% VS, contrasting with the original state of 20% TS and 15% VS, so that, there was a small loss on the VS contents due to the storage process (ratio of VS/TS before storage = 0.750, after storage = 0.692). The seaweeds inside the bag were considerably desiccated after the storage for 12 weeks. After storage, analysis showed that the seaweed had a small decrease in its sugar contents (0.318 mg/mL of sugars) and a great increase in its tannins contents (1.563 mg/mL of tannins), compared to their original state of 0.474 mg/mL of sugars and 0.074 mg/mL of tannins.

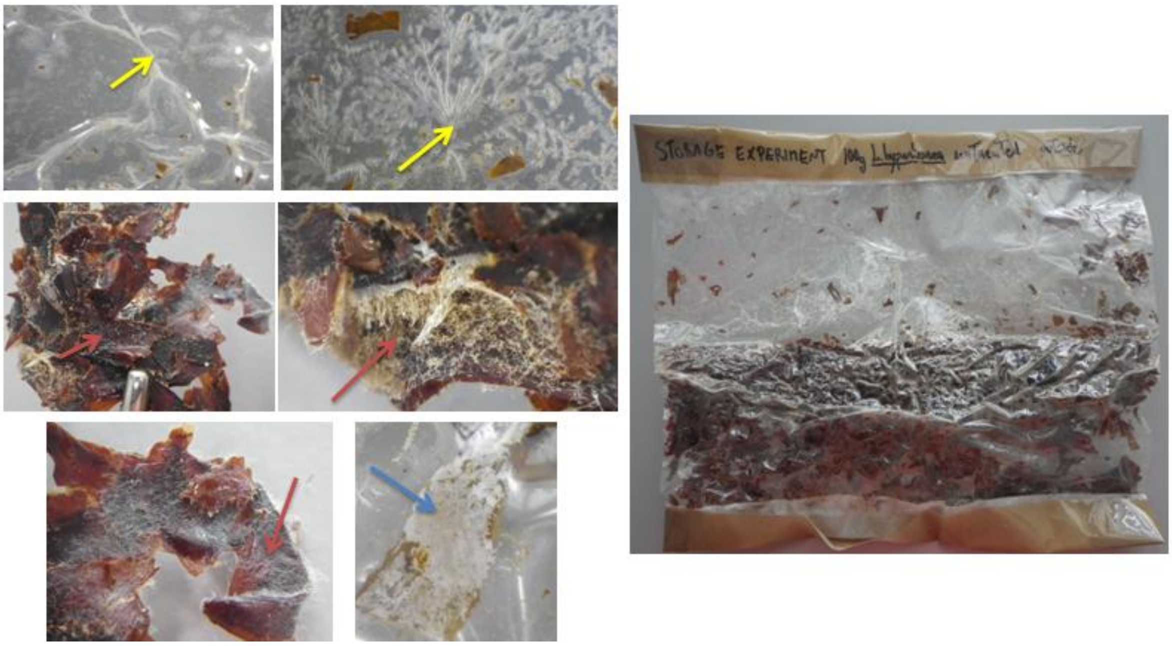

**Figure 16** Storage bag of *L. hyperborea* untreated and stored outside. Yellow arrows indicate an unidentified white branched formation; red indicate contaminations (small white dots and a fungal contaminant), and blue indicates dried salt.

In relation to the autoclaved storage bag, some contaminations and dried salt were seen (Figure 17). The seaweeds were again very dry with 75%TS and 55%VS contrasting with the original figures of 20% TS and 15% VS. The VS/TS ratio showed no significant loss of contents (before storage = 0.750; after storage = 0.733). After storage, chemical analysis revealed a small loss in the seaweed sugar contents (from 0.474 mg/mL to 0.340 mg/mL of sugars) and a significant increase in its tannins contents (from 0.074 mg/mL to 1.808 mg/mL of tannins), as observed in some previous bags.

**Figure 17** Storage bag of *L. hyperborea* autoclaved and stored in the refrigerator. Red arrows indicate contaminations; blue indicates dried salt and yellow indicates an unidentified white branched formation.

The heated bag had no contaminations on the fronds; only some branched white formations along the bag (Figure 18). The seaweeds were not as dry as in the other bags and the results for the TS/VS residues were 34%TS and 25%VS, with no significant decrease on the available VS contents for AD (VS/TS ratio after storage = 0.738). The chemical analysis showed a great decrease in sugar contents (from 0.474 mg/mL to 0.166 mg/mL of sugars) and a great increase in tannins contents (from 0.074 mg/mL to 1.173 mg/mL of tannins).

**Figure 18** Storage bag of *L. hyperborea* heated and stored in the refrigerator. Red arrow indicates an unidentified white branched formation.

In summary, it can be said that none of the storage conditions examined offered a good method for preservation of seaweed biomass prior to AD. However, it seemed that preserving the seaweeds in the refrigerator was, perhaps unsurprisingly, a better method than exposing it to ambient temperatures.

### 4.3. Temperature optimization

To optimize the AD process, four different temperature levels (25, 30, 35 and 37 ºC) were compared in relation to their ability to produce methane. Feeds were given only when biogas no longer increased and so the Hydraulic Retention Time (HRT) varied between fermenters at different temperatures. The first mini-fermenter to complete the 4th HRT was the one at 35 °C taking 37 days (~6 weeks). After that, all mini-fermenters were moved from their test temperature to 37 °C showing a good recovery process (data not shown).

It took nine days to reach each HRT for the mini-fermenter incubated at 35 °C, while incubation at 30 °C required fourteen days. The mini-fermenter at 25 °C took fourteen days to reach the 1st HRT, but even after a further 28 days it had not reached a 2nd HRT. The mini-fermenter at 37 ºC had 3 HRTs, each feed lasting for 2 days, *i.e.* after the second day there was no more biogas production. By the end of the experiment yield and productivity values were possible to compare over 3, 4, 2 and only 1 HRT from the fermenters at 37 ºC, 35 °C, 30 °C and 25 °C, respectively. This period of study covered 61 days of AD and additional time was not available to extend the study further.

Several parameters were registered throughout the experiment; some are given *per* feed, others *per* HRT (table 4). When comparing the means of the total biogas produced *per* feed, it was found that the 25 ºC fermenter had a significantly lower mean than all the others ($p < 0.05$). Also the productivity means determined for 37 and 35 ºC fermenters were significantly different ($p < 0.05$), being the fermenter at 35 ºC more productive. The cellular viability of the inoculum during the experiment showed good status condition since the means $\log_{10}$ (FDA) FU were between 4.9 and 6.3 units. Yield and productivity were statistically investigated for a better comparison of using different temperatures to AD *L. hyperborea*.

**Table 4** Results for the temperature experiment (*n* = 40). Total biogas produced, % $CH_4$ present in biogas, %VS used, cellular viability measured by FDA assay and yield and productivity for four different incubation temperatures are shown over 4 HRTs or 16 feeds with respective means and standard deviation.

| | **37 ºC** | **35 ºC** | **30 ºC** | **25 ºC** |
|---|---|---|---|---|
| | **Total Biogas Produced (mL)/feed** | | | |
| **Feed 1** | 55 | 120 | 160 | 80 |
| **Feed 2** | 100 | 120 | 105 | 65 |
| **Feed 3** | 120 | 120 | 75 | 110 |
| **Feed 4** | 85 | 120 | 120 | 70 |
| **Feed 5** | 120 | 130 | 80 | |
| **Feed 6** | 120 | 120 | 110 | |
| **Feed 7** | 120 | 140 | 125 | |
| **Feed 8** | 120 | 120 | 120 | |
| **Feed 9** | 120 | 120 | | |
| **Feed 10** | 85 | 130 | | |
| **Feed 11** | 110 | 150 | | |
| **Feed 12** | 120 | 120 | | |
| **Feed 13** | | 130 | | |
| **Feed 14** | | 120 | | |
| **Feed 15** | | 170 | | |
| **Feed 16** | | 140 | | |
| **Mean** | 106.3 | 129.4 | 111.9 | 81.3 |
| **SD** | 21.1 | 14.4 | 26.9 | 20.2 |
| | **% Methane in biogas / HRT** | | | |
| **1st HRT** | 52.2 | 54.4 | 56.6 | 54.2 |
| **2nd HRT** | 53.7 | 52.3 | 52.4 | |
| **3rd HRT** | 57.5 | 51.3 | | |
| **4th HRT** | | 50.5 | | |
| **Mean** | 54.5 | 52.1 | 54.5 | 54.2 |
| **SD** | 2.7 | 1.7 | 3.0 | 0 |
| | **% Volatile Solids Used / HRT** | | | |
| **1st HRT** | 67.2 | 60.7 | 55.3 | 53.4 |
| **2nd HRT** | 62.3 | 46.1 | 54.0 | |
| **3rd HRT** | 57.8 | 60.5 | | |
| **4th HRT** | | 84.1 | | |
| **Mean** | 62.4 | 62.8 | 54.7 | 53.4 |
| **SD** | 4.7 | 15.7 | 0.9 | 0 |
| | **$\log_{10}$ FDA / HRT** | | | |
| **1st HRT** | 6.2 | 4.5 | 6.0 | 5.9 |
| **2nd HRT** | 6.0 | 5.3 | 4.5 | |
| **3rd HRT** | 6.6 | 4.9 | | |
| **4th HRT** | | 4.9 | | |
| **Mean** | 6.3 | 4.9 | 5.3 | 5.9 |
| **SD** | 0.3 | 0.3 | 1.0 | 0 |
| | **Yield (mL methane/g of VS) / feed** | | | |
| **Mean** | 71.6 | 83.1 | 75.3 | 54.3 |
| **SD** | 15.0 | 8.2 | 19.0 | 13.5 |
| | **Productivity (mL methane/g of VS/day) / feed** | | | |
| **Mean** | 35.8 | 37.2 | 21.8 | 15.5 |
| **SD** | 7.5 | 7.1 | 5.4 | 2.2 |

HRT = Hydraulic Retention Time; SD = standard deviation.

Statistical differences in the means of the yield were found to be significant ($p < 0.05$) when using different temperatures to AD *L. hyperborea* (Figure 19). At a temperature of 25 ºC the mean yield was significantly smaller ($p < 0.05$) than the yield at 35 ºC. These two temperature levels represented, respectively, the lowest and the highest yields obtained. No other sample mean were found to be significantly different ($p > 0.05$). The AD at 30 ºC had very similar yield behaviour to the mini-fermenter incubated at 37 ºC showing a large variability (see SD of these respective yields on table 4).

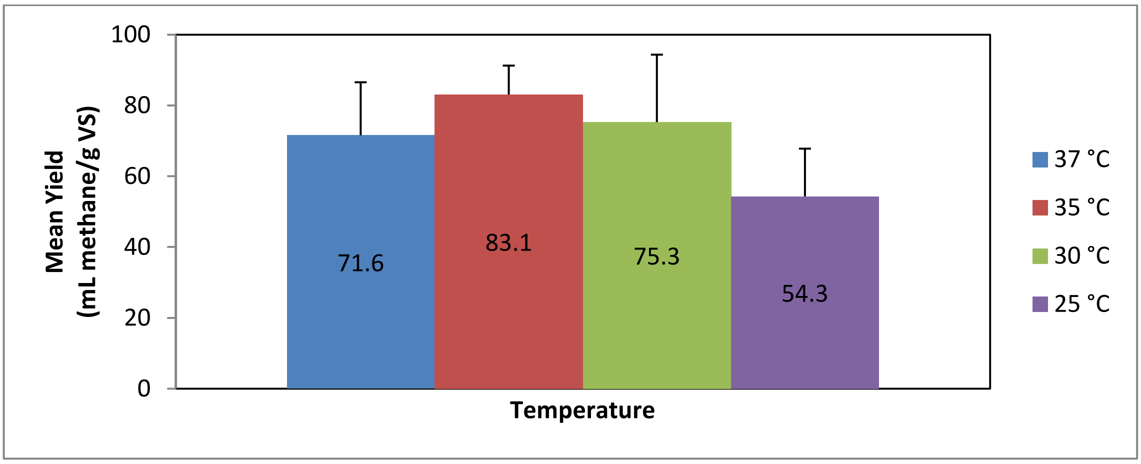

**Figure 19** Mean yield values of mini-fermenters at different temperatures. 37 ºC *n*=12, 35 ºC *n*=16, 30 ºC *n*=8, 25 ºC *n*=4. Error bars show standard error.

When analyzing the productivity, the null hypothesis was rejected ($p < 0.05$), meaning that at least one mean of productivity obtained when using different temperatures for AD was different (Figure 20). The Post-hoc test found that at a temperature of 37 ºC the productivity was significantly higher than the values obtained at 30 ºC or 25 ºC. Furthermore, AD at a temperature of 35 ºC resulted in a significantly higher productivity as compared to that of fermenters at 30 or 25 ºC ($p < 0.05$). Between 37 and 35 ºC or 30 and 25 °C no significant differences in productivity were found ($p < 0.05$). Clearly methane was produced significantly faster at temperatures higher than 30 ºC.

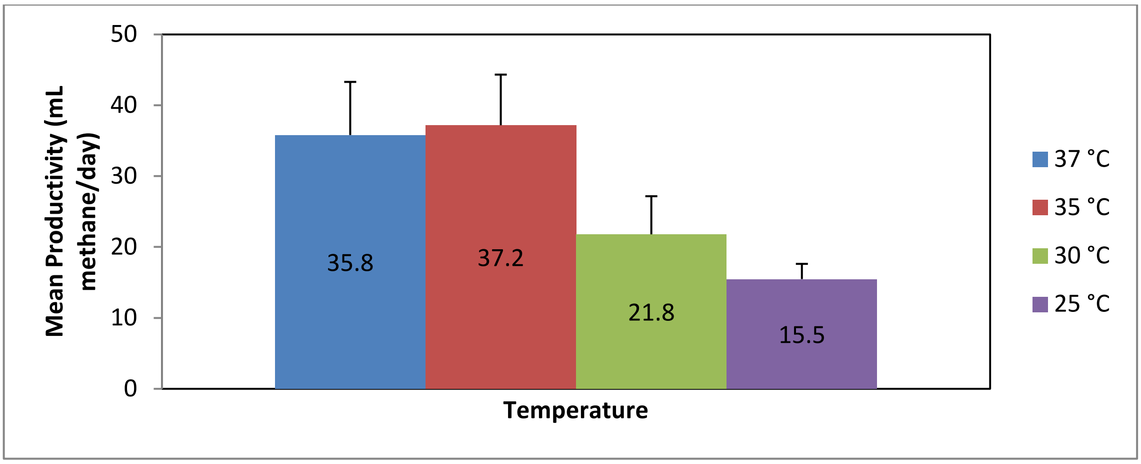

**Figure 20** Mean productivity values of mini-fermenters at different temperatures. 37 ºC *n*=12, 35 ºC *n*=16, 30 ºC *n*=8, 25 ºC *n*=4. Error bars show standard error.

## 4.4. Influence of feed rate

The purpose of this experiment was to find the best combination of feed concentrations given in terms of yield and productivity of methane while supporting viability of the inoculum throughout the experiment, *i.e.* the feed concentration should not cause any signs of toxicity to the inoculum due to high feed rate caused by excessive product feedback inhibition. Because some feed concentrations were high (10, 15, 20 % [ww/v]), biogas production continued for long periods after each feed. Only a maximum of 4 feeds were given. The 5% fed fermenter was followed just for 3 feeds (Table 5).

**Table 5** Results for the feed rate experiment (*n* = 15). Total biogas produced, % $CH_4$ present in biogas, %VS used, cellular viability measured by FDA assay and yield and productivity of four different feed concentrations (5, 10, 15, 20 % [ww/v]) are shown *per* feed with respective means and standard deviation.

| | **5%** | **10%** | **15%** | **20%** |
|---|---|---|---|---|
| | **Total Biogas Produced (mL)/feed** | | | |
| **1st Feed** | 270 | 505 | 525 | 1020 |
| **2nd Feed** | 115 | 655 | 525 | 510 |
| **3rd Feed** | 195 | 520 | 450 | 405 |
| **4th Feed** | - | 210 | 500 | 830 |
| **Mean** | 193.3 | 472.5 | 500.0 | 691.3 |
| **SD** | 77.5 | 187.6 | 35.4 | 284.1 |
| | **Solids Retention Time (days)/feed** | | | |
| **1st Feed** | 6 | 14 | 10 | 25 |
| **2nd Feed** | 6 | 17 | 12 | 19 |
| **3rd Feed** | 12 | 24 | 20 | 17 |
| **4th Feed** | - | 6 | 23 | 47 |
| **Mean** | 8.0 | 15.3 | 16.3 | 27.0 |
| **SD** | 3.5 | 7.5 | 6.2 | 13.8 |

**Table 5 (cont.)**

| | | | | |
|---|---|---|---|---|
| **% Methane in biogas/feed** | | | | |
| **1st Feed** | 64.3 | 58.0 | 56.8 | 52.9 |
| **2nd Feed** | 89.6 | 76.8 | 55.2 | 67.7 |
| **3rd Feed** | 66.8 | 59.3 | 78.3 | 58.5 |
| **4th Feed** | - | 71.2 | 86.5 | 82.4 |
| **Mean** | 73.6 | 66.3 | 69.2 | 65.4 |
| **SD** | 13.9 | 9.2 | 15.6 | 12.9 |
| **% Volatile Solids Used** | | | | |
| **1st Feed** | 51.3 | 59.7 | 69.51 | 89.4 |
| **2nd Feed** | 25.3 | 62.9 | 81.5 | 81.3 |
| **3rd Feed** | 48.7 | 60.3 | 82.6 | 79.2 |
| **4th Feed** | - | 62.3 | 73.5 | 68.8 |
| **Mean** | 41.8 | 61.3 | 76.5 | 79.7 |
| **SD** | 14.3 | 1.5 | 6.0 | 8.5 |
| **$\log_{10}$ FDA/feed** | | | | |
| **1st Feed** | 5.8 | 6.2 | 6.4 | 6.5 |
| **2nd Feed** | 5.1 | 5.3 | 6.2 | 5.9 |
| **3rd Feed** | 5.3 | 5.8 | 5.9 | 5.9 |
| **4th Feed** | - | 5.3 | 5.78 | 5.6 |
| **Mean** | 5.4 | 5.7 | 6.0 | 6.0 |
| **SD** | 0.4 | 0.5 | 0.3 | 0.4 |
| **Yield (mL methane/g of VS)/feed** | | | | |
| **1st Feed** | 85.6 | 72.0 | 48.9 | 66.4 |
| **2nd Feed** | 68.7 | 123.6 | 47.5 | 42.4 |
| **3rd Feed** | 86.9 | 102.8 | 78.3 | 39.5 |
| **4th Feed** | - | 49.9 | 96.1 | 114.0 |
| **Mean** | 80.4 | 87.1 | 67.7 | 65.6 |
| **SD** | 10.2 | 32.6 | 23.7 | 34.5 |
| **Productivity (mL methane/g of VS/day)/feed** | | | | |
| **1st Feed** | 14.3 | 5.1 | 4.9 | 2.7 |
| **2nd Feed** | 11.5 | 7.3 | 4.0 | 2.2 |
| **3rd Feed** | 7.2 | 4.3 | 3.9 | 2.3 |
| **4th Feed** | - | 8.3 | 4.2 | 2.4 |
| **Mean** | 11.0 | 6.3 | 4.2 | 2.4 |
| **SD** | 3.5 | 1.9 | 0.5 | 0.2 |

SD = standard deviation; (-) = not available.

Biogas volume produced per feed showed great variability depending on the fermenter observed (from 115 to 1020 mL biogas/feed). The solids retention time, *i.e.* the time each feed was retained inside the mini-fermenter until the day the fermenter no longer produced biogas, was sometimes of low variability others greatly variable. In general the days necessary to digest the seaweed feed given increased with increasing feed concentrations, as it would be expected. The percentage of $CH_4$ present in the biogas produced was generally high over the experimental period having means greater than 40%.

Statistically analyzing the means of %VS used, differences were found ($p < 0.05$; Figure 21). According to the Post-hoc test the mean % of VS used by the fermenter fed with 5% (ww/v) seaweed was significantly lower than the %VS used by the fermenter subjected to 15 or 20% (ww/v) seaweed feeds. In general, there is a clear pattern that can be taken from this experiment: as the feed concentration increases, the solids retention time (SRT) also increased, and so the inoculum seemed to use more time to digest a higher proportion of the VS available, therefore increasing the % of VS used.

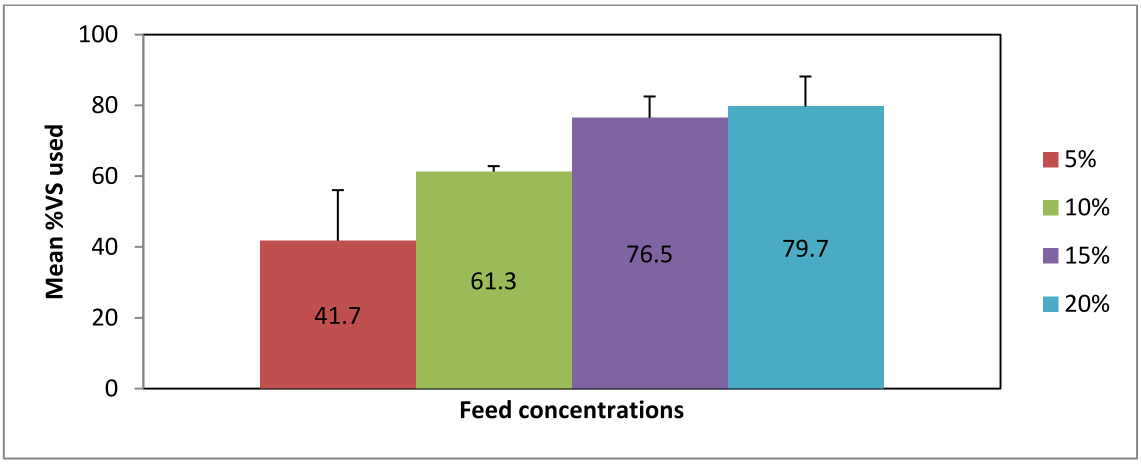

**Figure 21** Mean percentage of Volatile Solids used during the AD by the mini-fermenters at different feed concentrations. 5% *n*=3, 10% *n*=4, 15% *n*=4, 20% *n*=4. Error bars show standard error.

As they reflect the metabolic state of the inoculum and could therefore provide additional insight concerning the yield or productivity values, weekly $\log_{10}$ FDA fluorescence values were also determined. Upon analysis of Figure 22 it can be said that the inoculum of all mini-fermenters were metabolically active ($\log_{10}$ FU close to 6). The higher mean value of fluorescence was achieved by the 15% feed fermenter (6.0 ± 0.3 $\log_{10}$ FU). However, no statistical differences between the mean values of cellular viability between the mini-fermenters were found. So, other causes rather than toxicity from the higher concentrations of seaweeds might be the cause for the drop in the yield and productivity observed (Figure 23 and Figure 24) by the 15 and 20% fed mini-fermenters.

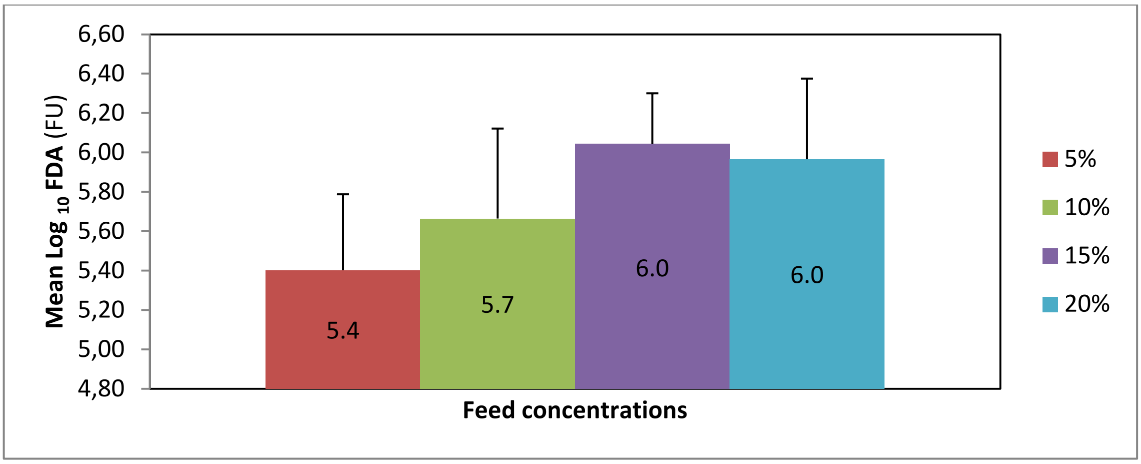

**Figure 22** Cellular viability of mini-fermenters at different feed concentrations. 5% *n*=3, 10% *n*=4, 15% *n*=4, 20% *n*=4. FDA = fluorescein diacetate. Error bars show standard error.

When using different feed concentrations for AD no significantly different methane yields were obtained ($p > 0.05$; Figure 23). It should be noted, however, the great similarity of the yield values obtained for the 15%- and the 20%-fed fermenter. When comparing these results with the one obtained in the diluent experiment for the 2% distilled water fed fermenter (see section 4.2, page 22), no other conclusion can be elaborated, because no significant differences were found; the mean yield obtained for the 2% fed fermenter was 71.4 ± 11.8 mL $CH_4$/g VS, which would be in between of the previously mentioned results (Figure 23).

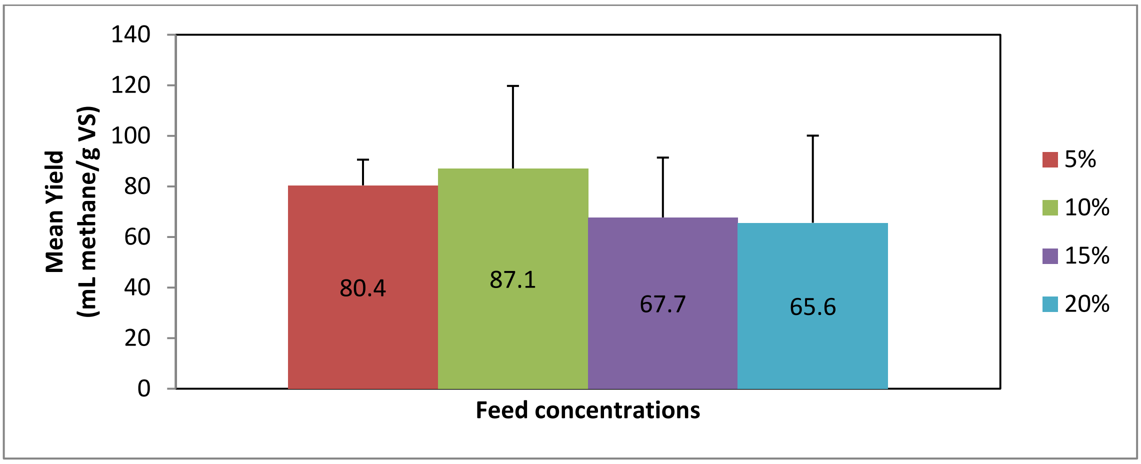

**Figure 23** Mean yield values of mini-fermenters at different feed concentrations. 5% *n*=3, 10% *n*=4, 15% *n*=4, 20% *n*=4. Error bars show standard error.

Analyzing the productivity it was confirmed that differences existed between the means of the differently fed fermenters ($p < 0.05$; Figure 24). Having a fermenter fed with a concentration of 5% was significantly more productive than the one fed with a concentration of 20%. There were no differences in the means productivity values between the other possible combinations, except when the significance level was higher ($\alpha = 0.10$). In that case, also the

10% fed fermenter showed a significantly higher productivity than the 20% one ($p < 0.10$). Once more, when comparing these results with the one obtained in the diluent experiment for the 2% distilled water fed fermenter (see section 4.2, page 22), it is possible to conclude that the 2% fed fermenter was less productive (8.9 ± 1.4 mL $CH_4$/g VS/d) than the 5% one, but more productive than the rest of the other fermenters (Figure 24).

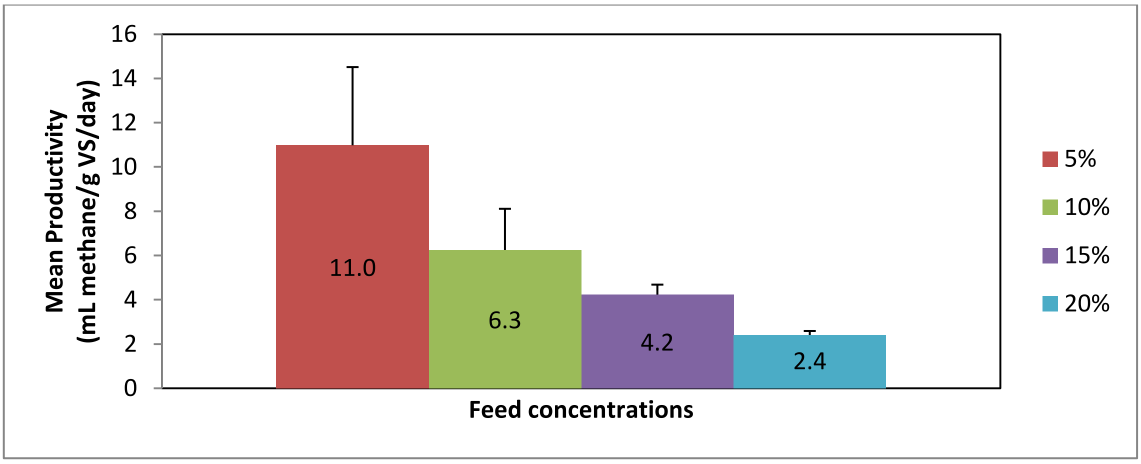

**Figure 24** Mean productivity values of mini-fermenters at different feed concentrations. 5% *n*=3, 10% *n*=4, 15% *n*=4, 20% *n*=4. Error bars show standard error.

### 4.5. Pre-treatment of seaweed

As the point of the following experiment was to find whether seaweed biodegradability increased upon pre-treatment, the %VS was determined. If a pre-treatment caused a significant alteration in seaweed digestibility, the microorganisms present in the inoculum would be able to access/consume more of the organic matter available, therefore increasing the % VS used. It should be noted that the pre-treatments with alkali, acid and heating were followed by AD for 4 HRT. However, as there were missing data regarding some methane values at the $3^{rd}$ HRT, all the data related with this HRT had to be discarded (Table 6). The period of the experiment was 44 days, where mean biogas volumes produced ranged between 75 and 210 mL per HRT. Methane percentages in the biogas were in general low, especially when compared with previous experiments (*e.g.* feed rate experiment in the previous section 4.4). Cellular viability was on average good in all fermenters, apart from two lower values of $\log_{10}$ FDA, *e.g.* 4.6 units in the $2^{nd}$ HRT of the untreated seaweed fed fermenter and 5.0 units in the $4^{th}$ HRT of the autoclaved fed fermenter. However, no significant differences were found between the means of $\log_{10}$ FDA FU of the inoculum subjected to different feed pre-treatments ($p > 0.05$).

**Table 6** Results for the feed pre-treatment experiment (*n* = 17). Total biogas produced, % $CH_4$ present in biogas, %VS used, cellular viability measured by FDA assay and yield and productivity obtained for each different treatment (untreated, alkali [NaOH], acid [HCl], heated and autoclaved) during four HRTs are shown with respective means and standard deviation.

| | **Untreated** | **NaOH** | **HCl** | **Heated** | **Autoclaved** |
|---|---|---|---|---|---|
| **Total Biogas Produced (mL)/HRT** | | | | | |
| **1st HRT** | 210 | 125 | 170 | 90 | 145 |
| **2nd HRT** | 165 | 105 | 125 | 95 | 135 |
| **3rd HRT** | 160 | 90 | 75 | 85 | 125 |
| **4th HRT** | 180 | | | | 180 |
| **Mean** | 178.8 | 106.7 | 123.3 | 90.0 | 146.3 |
| **SD** | 22.5 | 17.6 | 47.5 | 5.0 | 23.9 |
| **Methane %/HRT** | | | | | |
| **1st HRT** | 20.8 | 48.8 | 9.3 | 64.8 | 43.3 |
| **2nd HRT** | 14.2 | 34.7 | 8.3 | 26.8 | 20.9 |
| **3rd HRT** | 12.1 | 10.4 | 53.9 | 40.8 | 45.5 |
| **4th HRT** | 3.2 | | | | 76.7 |
| **Mean** | 12.6 | 31.3 | 23.8 | 44.1 | 46.6 |
| **SD** | 7.3 | 19.4 | 26.1 | 19.2 | 22.9 |
| **Volatile Solids Used %** | | | | | |
| **1st HRT** | 52.5 | 57.1 | 59.6 | 56.7 | 66.5 |
| **2nd HRT** | 64.6 | 84.0 | 72.0 | 58.0 | 64.2 |
| **3rd HRT** | 61.3 | 90.0 | 73.0 | 63.0 | 59.2 |
| **4th HRT** | 61.7 | | | | 56.3 |
| **Mean** | 60.0 | 77.0 | 68.2 | 59.2 | 61.5 |
| **SD** | 5.2 | 17.5 | 7.5 | 3.3 | 4.6 |
| **$\log_{10}$ FDA/HRT** | | | | | |
| **1st HRT** | 5.3 | 5.9 | 4.9 | 5.5 | 5.1 |
| **2nd HRT** | 4.6 | 5.4 | 5.9 | 5.7 | 5.1 |
| **3rd HRT** | 5.6 | 5.1 | 6.5 | 5.3 | 5.6 |
| **4th HRT** | 5.4 | | | | 5.0 |
| **Mean** | 5.2 | 5.5 | 5.8 | 5.5 | 5.2 |
| **SD** | 0.4 | 0.4 | 0.8 | 0.2 | 0.3 |
| **Yield (mL methane/g of VS)/HRT** | | | | | |
| **1st HRT** | 18.2 | 25.4 | 6.6 | 24.3 | 26.2 |
| **2nd HRT** | 9.8 | 15.3 | 4.2 | 10.7 | 11.8 |
| **3rd HRT** | 8.0 | 3.8 | 13.5 | 14.5 | 23.7 |
| **4th HRT** | 1.9 | | | | 57.5 |
| **Mean** | 9.5 | 14.8 | 8.1 | 16.5 | 29.8 |
| **SD** | 6.7 | 10.9 | 4.9 | 7.0 | 19.5 |
| **Productivity (mL methane/g of VS/day)/HRT** | | | | | |
| **1st HRT** | 2.0 | 2.8 | 0.7 | 2.7 | 2.9 |
| **2nd HRT** | 1.1 | 1.7 | 0.5 | 1.2 | 1.3 |
| **3rd HRT** | 0.9 | 0.4 | 1.5 | 1.6 | 2.6 |
| **4th HRT** | 0.2 | | | | 6.4 |
| **Mean** | 1.1 | 1.7 | 0.9 | 1.8 | 3.3 |
| **SD** | 0.7 | 1.2 | 0.5 | 0.8 | 2.2 |

HRT = Hydraulic Retention Time; SD = standard deviation.

Regarding the mean yields of methane obtained, there were no significant differences ($p > 0.05$) in subjecting the seaweeds to different pre-treatments before AD (Figure 25). The higher yield obtained was however for the autoclaved pre-treatment of the seaweeds followed by the pre-treatments: heated, alkali, untreated and acid.

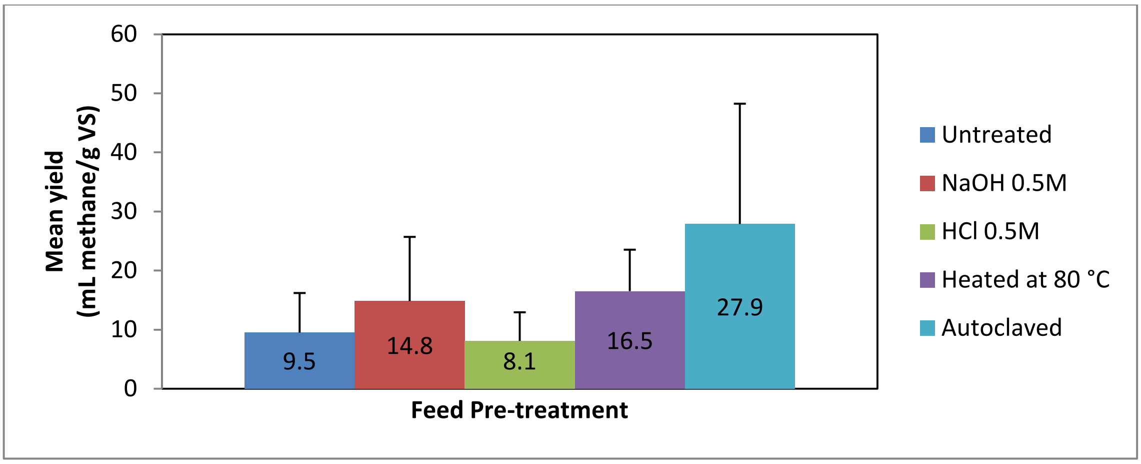

**Figure 25** Mean yield from mini-fermenters with different feed pre-treatments. Untreated *n*=4, NaOH 0.5M *n*=3, HCl 0.5M *n*=3, Heated *n*=3, Autoclaved *n*=4. Error bars show standard error.

Moreover, for the productivity obtained there were also no significant differences between the different pre-treatments applied ($p > 0.05$; Figure 26). Although, showing great variability, the highest value for the productivity was observed in the autoclaved pre-treatment, followed by the same hierarchical sequence as the yield results (heated, alkali, untreated and acid).

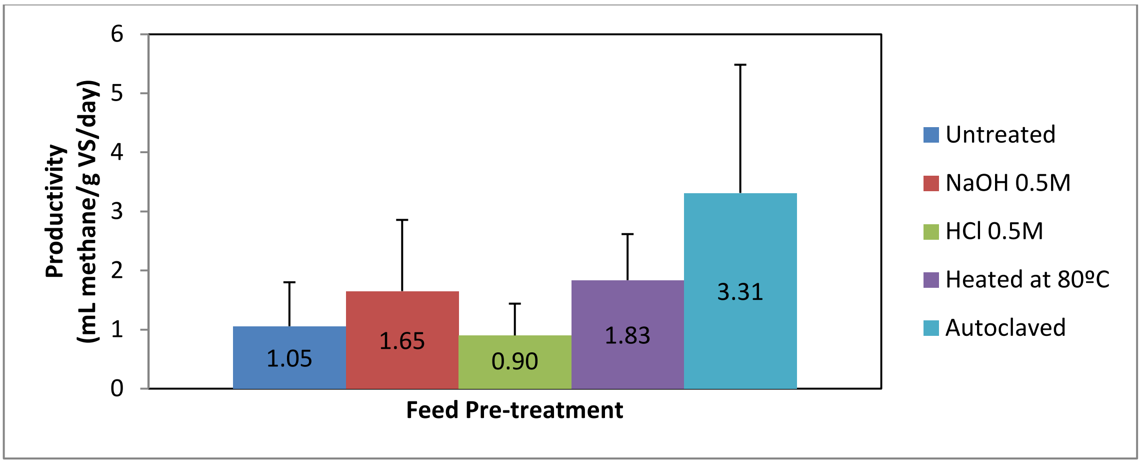

**Figure 26** Productivity from mini-fermenters with different feed pre-treatments. Untreated *n*=4, NaOH 0.5M *n*=3, HCl 0.5M *n*=3, Heated *n*=3, Autoclaved *n*=4. Error bars show standard error.

When comparing the means of the %VS used by the inoculum fed with different pre-treated seaweeds (Figure 27), no significant differences existed between the untreated, alkali, acid, heated and autoclaved pre-treatment ($p > 0.05$). Despite this conclusion, it can be said that the alkali treatment of the seaweeds allowed, in average, higher consumption of VS by the inoculum, compared to the heat treatment. Intermediate mean values were found for the acid, autoclaved and untreated feed treatments with.

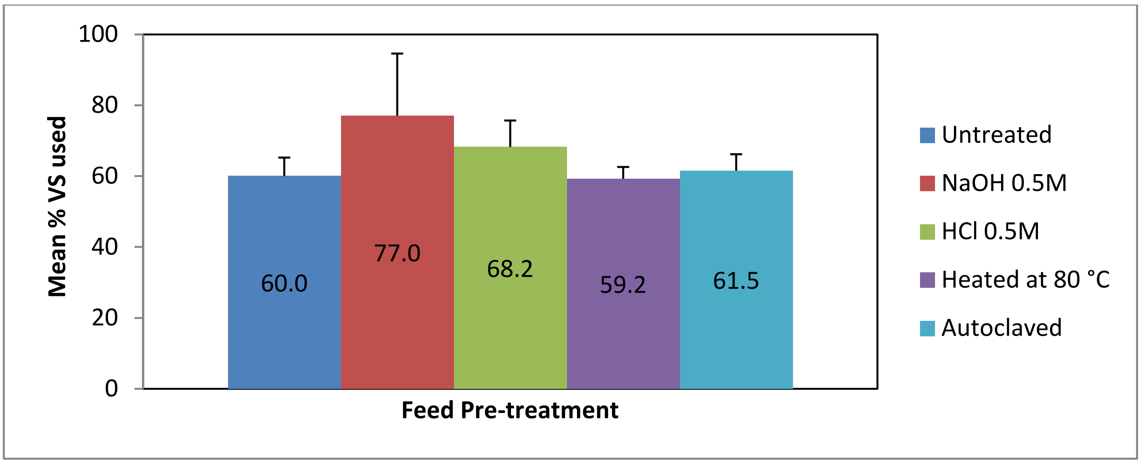

**Figure 27** Mean percentage of Volatile Solids used during the AD from mini-fermenters with different feed pre-treatments. Untreated *n*=4, NaOH 0.5M *n*=3, HCl 0.5M *n*=3, Heated *n*=3, Autoclaved *n*=4. Error bars show standard error.

In summary, since no significant differences were found it is not possible to indicate that a given pre-treatment is preferable to another or to the absence thereof. However, it should be pointed out that these results suggest that a balance of methane produced plus the %VS used should be the way of selecting the best pre-treatment in future experiments. For instance, if the mini-fermenter fed with the autoclaved seaweed did not have such a high variance in productivity, it may have been marked as the best pre-treatment of seaweed since its %VS used were also high (see values on Table 6).

## 4.6. Continuous or occasional stirred fermentation

During 4 HRT a continuously stirred mini-fermenter was fed and monitored by means of several parameters (Table 7). Data for the occasionally stirred fermenter was taken from the previous experiment (see data from the 2% autoclaved fed fermenter in section 4.5). Analysing the means of the different parameters determined it is possible to find a great similarity between the two stirring methods analysed.

**Table 7** Results for the stirred experiment (*n* = 8). Total biogas produced, % $CH_4$ present in biogas, %VS used, cellular viability measured by FDA assay and yield and productivity of the two different stirring methods during four HRTs are shown with respective means and standard deviation.

| | **Continuously stirred** | **Occasionally stirred** |
|---|---|---|
| | **Total Biogas Produced (mL)** | |
| **1st HRT** | 250 | 145 |
| **2nd HRT** | 140 | 135 |
| **3rd HRT** | 130 | 125 |
| **4th HRT** | 90 | 180 |
| **Mean** | 152.5 | 146.3 |
| **SD** | 68.5 | 23.9 |

**Table 7 (cont.)**

| | | |
|---|---|---|
| **Methane %** | | |
| **1st HRT** | 45 | 43 |
| **2nd HRT** | 69 | 21 |
| **3rd HRT** | 60 | 45 |
| **4th HRT** | 17 | 77 |
| **Mean** | 47.6 | 46.6 |
| **SD** | 22.6 | 22.9 |
| **Volatile Solids Used %** | | |
| **1st HRT** | 60 | 66 |
| **2nd HRT** | 65 | 64 |
| **3rd HRT** | 73 | 59 |
| **4th HRT** | 74 | 56 |
| **Mean** | 67.9 | 61.5 |
| **SD** | 6.6 | 4.6 |
| **$\log_{10}$ FDA** | | |
| **1st HRT** | 6.7 | 5.1 |
| **2nd HRT** | 6.0 | 5.1 |
| **3rd HRT** | 5.5 | 5.6 |
| **4th HRT** | 5.8 | 5.0 |
| **Mean** | 6.0 | 5.2 |
| **SD** | 0.5 | 0.3 |
| **Yield (mL methane/g of VS)** | | |
| **1st HRT** | 46 | 26 |
| **2nd HRT** | 40 | 12 |
| **3rd HRT** | 32 | 24 |
| **4th HRT** | 6 | 58 |
| **Mean** | 31.4 | 29.8 |
| **SD** | 17.5 | 19.5 |
| **Productivity (mL methane/g of VS/day)** | | |
| **1st HRT** | 5.2 | 2.9 |
| **2nd HRT** | 4.5 | 1.3 |
| **3rd HRT** | 3.6 | 2.6 |
| **4th HRT** | 0.7 | 6.4 |
| **Mean** | 3.5 | 3.3 |
| **SD** | 2.0 | 2.2 |

HRT = Hydraulic Retention Time; SD = standard deviation.

Due to these similarities there was no significant difference between the yields of a mini fermenter that was stirred continuously or just occasionally ($p > 0.05$), although a trend towards higher yields in the continuously stirred fermenter could be observed, except on the last HRT (Fig. 28). The continuously stirred fermenter seemed to decrease its yield along the time of the experiment while the occasionally stirred fermenter suffered some oscillations.

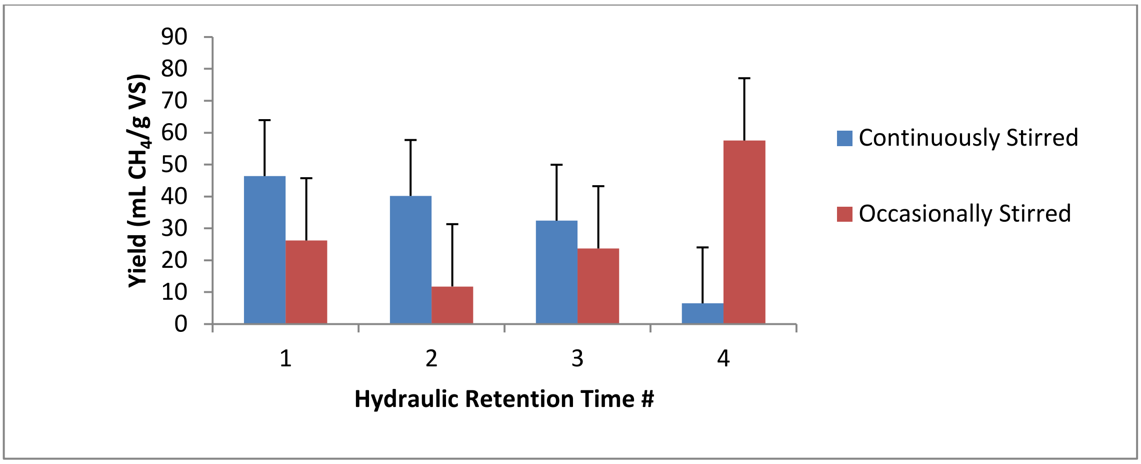

**Figure 28** Yield of mini-fermenters with different stirring treatments during four HRT. Continuously stirred *n*=4, Occasionally stirred *n*=4. Error bars show standard error.

There were no significant differences between the productivity of both mini-fermenters ($p > 0.05$). In the figure below (Figure 29) the productivity variations along the 4 HRT can be observed, which mirrors a pattern similar to that of the previous figure.

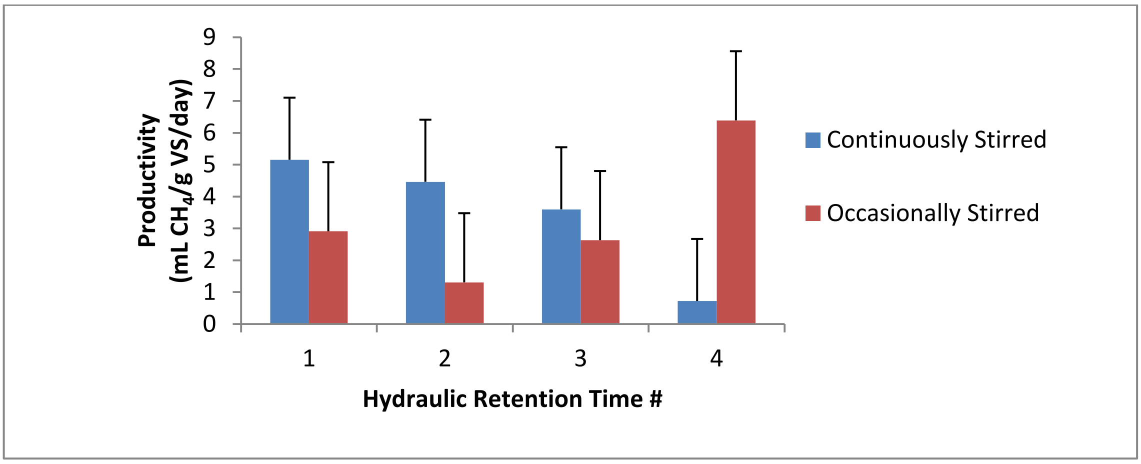

**Figure 29** Productivity of mini-fermenters with different stirring treatments during four HRT. Continuously stirred *n*=4, Occasionally stirred *n*=4. Error bars show standard error.

No significant differences between the %VS used in both the mini-fermenters were found, either ($p > 0.05$). The mean values were shown in Table 7, and in Figure 32 the % of VS used at each HRT are shown to differ very little throughout the experiment.

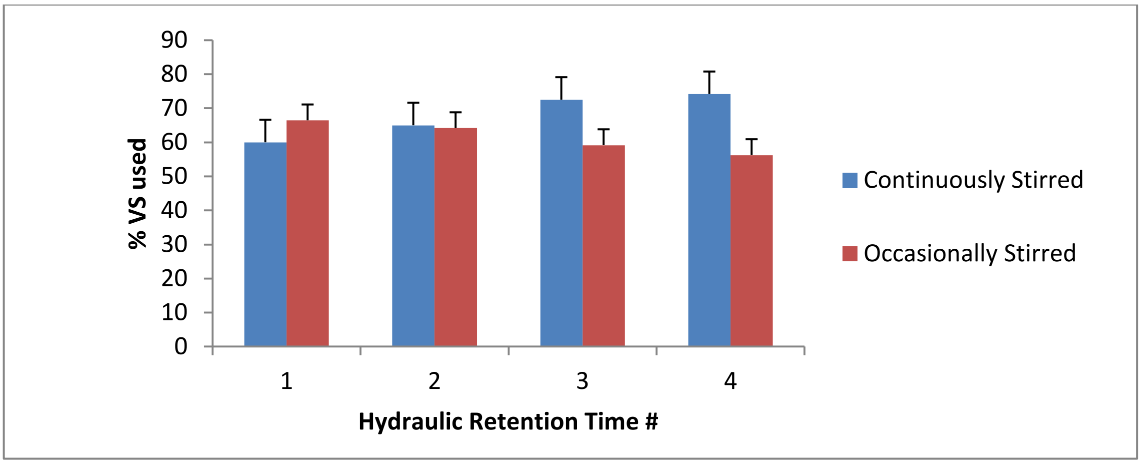

**Figure 30** Percentage of VS used during the AD by mini-fermenters with different stirring treatments during four HRT. Continuously stirred *n*=4, Occasionally stirred *n*=4. Error bars show standard error.

The only significant difference in this experiment was for the cellular viability ($p < 0.05$), and so it seems that having a continuously mixing of the inoculum promotes the cellular viability of the inoculum.

## 5. Discussion

Commonly buffer solutions, such as phosphate buffer solution (PBS) are used to keep pH values constant in a solution (*e.g.* Williams *et al.*, 2012). PBS may act as pH buffer, as well as a supply of phosphate increasing the AD efficiency of the inoculum when compared to other diluents. However, if distilled water or even seawater were as good as, or better than, PBS further studies could be carried out with less expensive diluents. From this study it was clear that distilled water can replace PBS as a diluent for AD of *L. hyperborea* without adversely affecting methane productivity, culture viability and usage of VS. Moreover, distilled water productivity was significantly higher than seawater production and although methane production was quickly reduced in the seawater diluent it still did produce methane at the same percentage in the biogas as in the other diluents. If a supply of fresh water is unavailable or prohibitively expensive, then using seawater as a diluent might be an alternative, which would however result in a 30% reduction in methane productivity. Using a lower percentage of seawater mixed with fresh water could be investigated in future experiments.

The shelf life of brown seaweeds is fairly long, taking hours or even days at ambient temperature without deterioration, due to their polyphenol contents. These compounds enable them to survive microbial degradation, a useful property for conventional manufacturing or extraction purposes. However, for biogas production, this resistance to fermentation is an inhibitory factor (SEI, 2009). If supply of seaweed for AD is transient-seasonal or dependent upon beach cast, then it may be necessary to store seaweed long term, either to overcome glut, when too much biomass is available for digester space, or to even out supply for when no fresh algae are available. Additionally, as the sugar content in brown seaweeds varies seasonally (Horn, 2000), it would therefore be advantageous to harvest the seaweeds when sugar content is highest.

Regarding the obtained results on the methods to ‘preserve/ ensile’ seaweed stock, further research is encouraged, since no storage method tested was found to be efficient in preserving the seaweeds from contaminations. Nevertheless, it is believed that the fungal growth observed in the differently stored seaweed may not in fact alter the digestibility of seaweeds for AD since the ratio of VS/TS and the sugars present did not change significantly after the storage period. The use of those seaweeds as feedstock for AD should be investigated about their methane productivity in comparison with non-stored fresh seaweed. Lastly, gassing with a mixture of an anaerobic gas proved not to be enough to stop fungal growth and spoilage of seaweeds stored in bags, under ambient conditions. When comparing results with other authors, Tanjore *et al.* (2012) also described contaminations by fungal organisms after 7 days of ensilage in a double plastic bag stored in the refrigerator for corn stover. Moreover, a decrease in sugar concentration was also observed. Alternative storage experiments for different biomass sources that may be useful in further research are, for example, a natural hydrolysis process (percolation), where seaweeds (*Ulva*) were left in large containers for a long period at 4 ºC, improving thereby the methane yield by 45 % (Carpentier, 1986). A second procedure would be to leave freshwater green algae in a tank for a few days with a water-sprinkling system, so that the percolated water would produce a methanisable effluent (Guiry & Blunden, 1991). Lastly, it has been suggested a third method in which brown seaweeds are stored at 22 ºC in polyethylene boxes or sealed containers flushed with nitrogen with addition of formaldehyde (2 % w/w) to prevent microbial decomposition and stimulate AD (Moen, 1997). However, the latter procedure has an important drawback since formaldehyde is not environmentally friendly and is possibly carcinogenic.

Concerning the optimal temperature to ferment *L. hyperborea*, according to some authors, even small changes in temperature (± 2 ºC) can cause a substantial decrease in AD activity (Deublein & Steinhauser, 2008). Optimal temperatures vary along the different steps of the process; hydrolysis/acidogenesis have an optimal temperature range between 25-35 ºC and methane formation has an optimal temperature range between 32-42 ºC (mesophilic process) or 50-58 ºC (thermophilic process) (Deublein & Steinhauser, 2008). Over the years, different temperatures were tested for different sources of biomass (*e.g.* Chynoweth, 1987 and Horn, 2000). In the present work 37 or 35 ºC were the most productive temperatures. From an industrial point of view, however, 35 ºC may be a better option, as it gave less variability in productivity and also because it will reduce the energy costs of the fermenter/reactor operation without compromising the biogas production. Interestingly, the observed increase in temperature with decrease in HRT is in agreement with the results reported by Krishania *et al.* (2012) for AD of agriculture and food wastes, and also cattle manure. Moreover, biomass productivity increased with decreasing HRT, which is also in agreement with Valigore *et al.* (2012). Considering the two microorganisms groups of AD – mesophilic (30-50 ºC) and psychrophilic (15-30ºC) – and the days the solids stay inside the bioreactor (residence time of 17-45 and 60 days for, respectively, mesophilic and psychrophilic AD) (Deublein & Steinhauser, 2008), it is easier to understand the period that each mini-fermenter took to complete each SRT or, in this case, each HRT. A clear segregation of productivity was apparent, where mesophilic temperatures (37, 35 and 30 ºC) took less time (8, 9 and 14 days) to complete an HRT. For example, the 25 ºC mini-fermenter had just one HRT because the fermenter was taking a very long time to reach the 2nd HRT – more than 28 days – being thus a very slow biogas producer. The segregation in productivity depending on the temperatures (37 and 35 ºC *versus* 30 and 25 ºC, Figure 20) of this seaweed is also in agreement with Deublein & Steinhauser (2008) who stated that microbial metabolism processes in AD range between an optimal temperature of 25-35 ºC for hydrolysis/acidogenesis phases and 32-42 ºC for methane formation. Hence, 35 and 37 ºC are both suitable temperatures for the different phases of AD. Comparing the yields (mL $CH_4$/g VS) obtained for *L. hyperborea* at 35 and 25 ºC with the yields obtained by Vanegas & Bartlett (2013) for *L. digitata* at 35 and 20 ºC, they similarly produce more methane at higher temperatures and less at lower temperatures (83 mL $CH_4$/g VS for *L. hyperborea* at 35 ºC and 161 mL $CH_4$/g VS for *L. digitata*, *versus* 54 mL $CH_4$/g VS for *L. hyperborea* at 25 ºC and 93 mL $CH_4$/g VS for *L. digitata* at 20 ºC). Differences in the yields around the same temperatures possible derive from the fact that they were different species with different carbohydrates concentrations and/or components.

Although using a high temperature gives a good productivity in a short HRT, there are aspects to consider regarding the life cycle of the microorganisms, especially the methanogenes. The (re)generation time is a parameter of great importance for HRT. For example, an acidogenic bacterium such as *Bacteroides* regenerate in less than 24 h and

*Clostridia* in 24 – 36 h and an acetogenic bacteria in 80 – 90 h, but a methanogenic bacteria like *Methanosarcina barkeri* takes 5–16 days and *Methanococcus ca*. 10 days (Deublein & Steinhauser, 2008). Considering this, to avoid microorganism washing out from the bioreactor at every feed, HRT should take at least 10–15 days, which was not the case in some of these studies and may explain a decrease in productivity observed as the experiments progressed. This event of microorganisms washout during *L. hyperborea* AD was recently found by others (Hinks *et al.*, 2013) to cause inhibition in methanogens growth, caused by nutrient deficiency or antimicrobial compounds.

The recovery experiment (to 37 ºC) showed the possibility of decreasing the digester temperature to 25 ºC with no subsequent harm to the inoculum. This situation is helpful if a reduction in the amount of seaweed available for AD occurs due to, for example, biomass seasonal fluctuations. The reduction in temperature increases the SRT and so decreases the yield over time, *i.e.* microorganisms take more time to digest the seaweeds given and do not need to be fed often. However, they continue to produce biogas (in small amounts). The metabolism of the microorganisms is reduced and the productivity becomes lower.

Regarding the feed rate, it is known that it depends on the concentration of seaweed given and how often it is supplied to the fermenter. If solids are not retained in the fermenter long enough, then all digestible solids will not be utilized and maximum methane production will not be achieved. The solids retention time (SRT) also depends upon the concentration of the solids — the higher the feed concentration the longer the SRT required. For commercial seaweed AD both short digestion times and high methane yields are important considerations. Semi-continuous culture experiments were designed to maximise feed rate and minimise SRT whilst giving maximum methane productivity. Considering the results of the experiment, pH data (not shown) recorded an abrupt decrease in pH after each feed, especially in the high % concentrations feeds (*e.g.* 20% feed mini-fermenter reached the 4.9 pH units, the 15% one reached pH 5.6 and the 10% reached pH 5.7). With such variations in pH values, it was probable that a bottleneck effect had affected the methanogens since if the fermenter pH drops below 6.5 they can die. Every time a feed was given a decrease in the inoculum buffering capacity took place, due to high hydrolysis (and acidogenesis) activity (pH optima between 5.2 – 6.3 units) to degrade the high amount of organic matter given. This degradation process led to a decrease in pH by the accumulation of VFA, which also affected the biogas produced (Krishania *et al.*, 2012). In order to counteract this event, each time a feed was given, the pH was adjusted to higher values (7.4-7.6) since methane formation has its optima range of pH values between 6.7 – 7.5 units. In the end of each HRT, there was no need at all to adjust the pH in the fermenters of higher feed %, possibly due to the counteracting event of ammonia accumulation from protein degradation, which increases the pH (Krishania *et al.*, 2012). Similarly to what happened in the temperature experiment, the longer the SRT the higher methane percentage was obtained (*e.g.* 20% feed fermenter produced 53% $CH_4$ at the end of feed 1 [SRT = 25 days] but increased to 82% $CH_4$ at the end of feed 4 [SRT = 47 days]). This is also related to the increase of SRT and HRT, once methanogens have time to grow, producing more methane per feed. That is also possibly why the FDA value of the 15 and 20% feed fermenters were higher, because their SRT ranged between 10 – 47 days.

In conclusion and taking everything into consideration, it can be said that the best concentration (5% [ww/v]) of *L. hyperborea* to be given as feed should not be the most productive, but a similar high producer – the 10% (ww/v) concentration. This choice happens once the SRT respect the time needed for methanogens to grow, no feedback inhibition seems to be strong enough to harm the inoculum and the productivity is not significantly different from the 5% feed concentration one.

The biodegradability of the seaweed influences the accessibility of microorganisms in the inoculum to digest them. Pre-treatment processes to increase the seaweeds biodegradability can be helpful for AD. In previous experiments autoclaving the seaweeds before using them as feed was an important step to eliminate contamination of the inoculum with exogenous microbiota, which otherwise could have compromised the survival of the microorganisms and the metagenomic analysis of the inoculum (A.D. Sutherland, personal communication). However, autoclaving could also affect polymer access for AD (it was shown to release total sugars, A.D. Sutherland, personal communication) and would be commercially expensive to do in an industrial way.

Previous pre-treatments on various biomass known to work include: physical ultrasonic disintegration of biosolids (Farooq *et al.*, 2009), physical milling (Krishania *et al.*, 2012), chemical disintegration of municipal waste activated sludge using NaOH (Lin *et al.*, 1999; Gaspar *et al.*, 2007), use of HCl for lignocellulosic materials (Taherzadeh & Karimi, 2008; Ward *et al*., 2008), enzymatic digestion of sisal leaf decortications residues (Mshandete *et al.*, 2005) and thermal disintegration (Ferrer *et al.*, 2008). A summary of additional pre-treatments was made by Taherzadeh & Karimi (2008)**.** Concerning the pre-treatment of *L. hyperborea*, the low percentage of methane produced in the biogas in this work was probably due to the experimental design. It was set that one HRT should happen at every 4th feed.

Since at each feed 50 mL from the 200 mL mini-fermenter were removed and replaced by new feed, and taking into consideration the fermenter was fed every second day, each HRT took only 8 days. However, methanogens have been reported to need at least 10 to 15 days to grow (Deublein & Steinhauser, 2008), and so the use of a short SRT may explain why the % methane continued to decrease during the experiment. This assumption is supported by the fact that %VS used remained more or less the same, an expected result since hydrolytic bacteria have lower generation times and would thus be unaffected by the short SRT.

Structural changes to terrestrial biomass upon pre-treatment were discussed by Taherzadeh & Karimi (2008). Briefly, alkali pre-treatment is said to be the most effective pre-treatment method because it increases saccharification, breaks the ester bonds between lignin, hemicelluloses and cellulose, avoiding the breakup of hemicelluloses polymers. Besides, alkali, the acid pre-treatment is also important to degrade lignocellulosic materials by improving enzymatic hydrolysis (at high temperatures) and promoting the hydrolysis of fermentable sugars. Since the biomass used in this study was of marine origin, with no lignin and low content of cellulosic materials, a variation in the degradability of the seaweed was not expected when these pre-treatments were applied, unless some other polysaccharides (*e.g.* alginate) were affected, thereby increasing the general degradability of the seaweed. The heated pre-treatment is the softer of the previous treatments, rupturing the chemical bonds of the cell wall and membrane and making the proteins more accessible for AD (Hanjie, 2010). Comparing the results obtained in the present study, it is possible to see a consistent trend in which the alkali pre-treatment resulted in highest %VS, followed by the pre-treatments with acid, autoclave, and heat with the negative control being slightly better than the latter. However, this trend could not be shown to be statistically significant due to the high variance of the results. Experiments with a higher number of replicates could solve this question in the near future.

With reference to the stirring experiment, continuous stirring may (Kaparaju *et al.*, 2008; Hoffmann *et al.*, 2008; Karim *et al.*, 2005; Deublein & Steinhauser, 2008) or may not (Tian *et al.*, 2013; Ong *et al.*, 2002; Chynoweth *et al.*, 1987) improve productivity as previous studies have shown. In this study, no differences were found in the parameters determined, except for the cellular viability where stirring seems to increase the metabolic activity of the inoculum. Stirring in a fermenter could have led to better productivities because of a higher degree of surface contact between microorganisms and seaweeds, which minimises dead space; eliminates possible scum formation, releases trapped bubbles of biogas, and evens out environmental factors. However, it seems that, in this case, probably due to the small volume of the bioreactor, there were no such gradients to be eliminated, and thus a little mixing at each feed process was enough to keep a good productivity. This finding is in agreement with Lema *et al.* (1991) who stated that in AD vigorous mixing can interfere with interspecific hydrogen transferences since the bacteria present cannot aggregate. However, stirring a fermenter continuously seems to be a good strategy to increase its productivity only if the feed concentration is high — *e.g.* 10 or 15% feed concentration (Karim *et al.*, 2005). Kaparaju *et al.* (2008) found that low residues/inoculum ratio, i.e. 50 mL of residues (seaweeds) to 150 mL of inoculum could benefit from a gentle mixing. Further investigation could be done on stirring experiments to try to understand the productivity oscilations observed here. Perhaps the microbial population suffered bottleneck effects due to the consistent dilution rate and possible breakdown of symbioses among microorganisms and between microorganisms and seaweeds. Tian *et al.* (2013) previously investigated differences in the population of a stirred and a non-stirred fermenter. It seems that in the non-stirred fermenter a higher and diverse microbial community could be observed, including phylotypes of *Methanoculleus* and *Methanosarcina* as dominant methanogens, whereas in the stirred fermenter *Methanosaeta* was the only methanogen present. Conversely, the stirred fermenter contained a hydrogen-producing bacterial phylotype *Petrotoga* in high proportion. Taken together, further molecular studies regarding the population that contribute to the AD of *L. hyperborea* during stirring and non-stirring are warranted.

# 6. Conclusion

Seaweeds are promising feedstocks for biofuel production, which can replace the need for edible feedstocks that are used for the production of first generation of biofuels. This due to the fact that their harvest can be more environmentally sustainable, as they do not compete with food crops for arable land.

The optimization of AD of *L. hyperborea* biomass here researched revealed that using distilled water as diluent was an effective and cheaper alternative to the use of PBS used in earlier experiments by Dr. A.D. Sutherland. However, methodologies for optimal storage should be further investigated, as the use of plastic bags with an anaerobic mixture of gases to store seaweeds was not enough to maintain the environment anaerobic. Preserving the seaweeds in the refrigerator was a better method than exposing the biomass to room temperature. Higher temperatures of incubation were found to produce higher quantities of methane/g VS/day than at 25 ºC and higher feed concentrations were shown to take more time to be digested, thus lowering productivity. Pre-treatment of the biomass to increase the biodegradability of the seaweed did not significantly improve the %VS consumed by the inoculum. However, it was observed a trend of higher %VS when the samples were pre-treated with alkali. Lastly, continuously stirring such a small volume fermenter (200 mL) did not improve its productivity and therefore a minimal occasional mixing is preferable since it saves energy and costs less.

Other conclusions to keep in mind for the near future concern the maintenance of fermenters: during the first days of fermentation, pH has such a limiting role that it should be carefully checked and adjusted to the right levels in order to prevent the loss of viability of the methanogens and reduce the methane rate of productivity; moreover, special considerations in the dilution rate should be made to avoid the wash-out of the methanogens.

Taken together, the results of this dissertation are of value for a future design of a larger scale fermenter system. More reliable sensory equipment to monitor the fermentation process should be considered as an investment for the good optimal performance of the process.

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