Anaerobic digestion of the microalga *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+)

Biogas production and metagenome analysis

Dissertation

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Submitted by

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Para mi familia y amigos

To my friends and family

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Summary

Extensive use of fossil fuels has encouraged governments and research community to find alternative energy sources in order to reduce greenhouse gas (GHG) emissions and dependence on fossil fuels. One of these alternative energy sources is biogas, a mixture of gases, mainly CH_4 and CO_2, produced by the anaerobic fermentation of organic matter. One of the multiple substrates for the production of biogas are microalgae which are microorganisms that can transform sunlight and CO_2 into biomass.

In this work, a new approach for the production of biogas from the anaerobic fermentation of microalgae was investigated. Here we propose to perform the anaerobic digestion at alkaline conditions (pH~10; 2.0 M Na^+). This can have several advantages over traditional anaerobic digestion systems. One advantage is that due to the higher CO_2 solubility at alkaline conditions, the medium in the anaerobic reactor can act as a CO_2 scrubber resulting in biogas rich in methane.

To perform the anaerobic digestion at such alkaline conditions, sediments from alkaline lakes were used as inoculum. Several anaerobic reactors were set up to study the anaerobic digestion of *Spirulina*. These reactors were used to determine the optimal process parameters such as hydraulic retention time and organic loading rate. Several bottle necks were identified in this new approach, mainly related to ammonia inhibition and to poor granule formation. The produced biogas was rich in methane which made it suitable for the direct use in national gas grids or as fuel for vehicles.

Metagenome analysis was the applied method to taxonomically and functionally characterize the unique extremophilic microbial community present in the alkaline anaerobic reactors. The taxonomy profile of this particular anaerobic alkaline community was similar, at higher taxonomic levels, to known traditional anaerobic digester communities with Bacteria clearly dominating over Archaea. The main observed differences could be attributed to the type of bacteria and archaea found, mainly haloalkaliphilic. Uncultured haloalkaline bacteroidetes and other halotolerant bacteria such as *Halanaerobium* were among the most dominant bacteria, while among the methanogenic archaea a clear dominance of *Methanocalculus* was observed.

Genes related to the different strategies used by haloalkaline bacteria to cope with high pH were detected in the functional analysis. In the alkaline anaerobic reactor, the hydrogenotrophic methanogenesis pathway was the most prominent pathway while most genes of the acetoclastic pathway were practically absent.

Abbreviations

Aa/C	Amino Acid to Cellulases Ratio
AaM/CM	"Amino acid transport and metabolism" to "Carbohydrate transport and metabolism" ratio
AD	Anaerobic digestion
Alk-BT1	Alkaline Batch Test-1
Alk-BT2	Alkaline Batch Test-2
Alk-HRT	Anaerobic Alkaline reactor Alk-HRT
Alk-OLR	Anaerobic Alkaline reactor Alk-OLR
Alk-Opt	Anaerobic Alkaline reactor Alk-Opt
Alk-Sed-2	Anaerobic Alkaline reactor Alk-Sed-2
BD_{CH4} (%)	Percentage of Biodegradability
BMP	Biomethane Potential
BMP_{TH}	Theoretical Methane Potential
BOD_5	5 day Biological Oxygen Demand
BTU	British Thermal Units
CFB	Cytophaga-Flavobacteria-Bacteroides Group
COD	Chemical Oxygen Demand
COD_S	Soluble COD
COD_T	Total COD
COG	Cluster of Orthologous Groups
GHGs	Greenhouse gases
HRT	Hydraulic Retention Time
HTS	High Throughput Sequencing
LCA	Life Cycle Analysis (Production costs)
M-R	Maize Rye Dataset
Mtoe	Million tons of oil equivalents
NH_3-N	Ammonia Nitrogen
NH_4^+-N	Ammonium Nitrogen
NOG	Non-supervised Orthologous Groups
OLR	Organic Loading Rate
ORF	Open Reading Frame
ORP	Open Race Pond
P/C	Proteases to Cellulases Ratio
PBR	Photobioreactor
Pfam	Protein Domain Identifier
SAO	Syntrophic Acetate-Oxidizing bacteria
SBP	Specific Biogas Production

S-CSTR	Semi-continuous stirred tank reactor
SDI	Simpson's Diversity Index
SMP	Specific Methane Production
SPO	Syntrophic Propionate-Oxidizing bacteria
TN	Total Nitrogen
TS	Total Solids
TSA	Tryptone Soya Agar
VFA	Volatile Fatty Acids
VS	Volatile Solids

1. Introduction

Human emissions of greenhouse gases (GHGs) are currently considered the main cause of climate change. The global energy demand is growing rapidly and it is expected that by the year 2035 it will reach 739 quadrillion BTU that is over twofold the consumption from 1990 (Oncel, 2013). Today about 88% of this demand is covered by fossil fuels (Weiland, 2010). The rise in oil prices and the need to reduce the GHGs emissions have led governments to increase investment into the use of alternative energy sources. One of these alternative energy sources is biogas, a mixture of gases, mainly CH_4 and CO_2, produced by the anaerobic fermentation of organic matter.

1.1. The anaerobic digestion process

Anaerobic digestion (AD) is a natural process where the decomposition of organic matter takes place at anaerobic conditions to produce biogas, a mixture of mainly methane and carbon dioxide. First records of biogas usage date as early as the 10th century B.C., when biogas was used for heating bath water in Assyria, and records appear later in the 16th century in Persia (Abbasi et al., 2012; Lusk, 1998). In the 17th century, Van Helmont associated the production of flammable gas to the decay of organic matter, and later, in 1776, Volta correlated the amount of gas produced to the amount of decaying organic matter. In 1808 Sir Humphry Davy determined that methane was present in the gases produced during the AD of cattle manure (Abbasi et al., 2012; Deublein and Steinhauser, 2008; Gunnerson and Stuckey, 1986; Lusk, 1998). The first known anaerobic digestion plant was built in Bombay (India) in 1859, but it was not until 1895 that the use of AD plants reached Europe (Abbasi et al., 2012; Lusk, 1998). The production of biogas as an alternative energy supply was used quite extensively when energy supplies were reduced during and after World War II (Lusk, 1998). Today, it is possible to find urban, industrial or manure anaerobic digestion plants around the world, even though most of them have been built in the EU. The large implementation of the AD in the EU is a result of the financial support for projects in the field of alternative energy sources and energy savings as well as the implementation of the Landfill Directive (Directive 99/31/EC) (Astals Garcia, 2013). Today, over 13,000 biogas plants operate in Europe which produce over 10.1 million tons of oil equivalents (Mtoe) per year and these values are expected to grow

in the next years (EurObserv'er, 2012). The energy obtained in a biogas plant can be used to provide heat, electrical power, or transport. Substrates for anaerobic digestion plants can be obtained from agro food industries (sugar, potato, starch, cannery, fruit, vegetable, dairy, baker), beverage industries (beer, malting, wine, coffee), alcohol distilleries (sugarcane juice, sugarcane molasses, sugar beet molasses), pulp and paper industries (recycle paper, mechanical pulp, sulfite pulp, straw, bagasse), and other miscellaneous fields (chemical, pharmaceutical, sludge liquor, municipal sewage, landfill leachate, acid mine water, etc.) (Borja, 2011; Al Seadi et al., 2008).

1.1.1. Main parameters affecting the anaerobic digestion process

The anaerobic digestion process is a complex biological process where organic matter is degraded in the absence of oxygen which results in the production of methane and CO_2. Effective anaerobic digestion of a given substrate is influenced by multiple factors:

(i) Temperature: Anaerobic digestion can be performed at three different temperatures ranges, mesophilic (20 - 45°C), thermophilic (45 - 60°C) or psycrophilic (<20°C). The temperature directly affects the solubility of several compounds (CH_4, NH_3, volatile fatty acids, etc.) and the retention time needed to hydrolyze the substrate. Temperatures between 35 and 55°C are considered optimal (Borja, 2011; Al Seadi et al., 2008).

(ii) pH: The pH is one of the most important factors to take into account for effective digestion, because pH values too low or too high inhibit the anaerobic microbial community. The optimal operation pH range varies from 6.5 to 8.0 and the process is severely inhibited when the pH decreases below 6.0 or increases above 8.5. A drop or increase in the pH value mainly affects the methanogenic community as it is more sensitive to pH changes, whereas the fermentative bacteria are much more resistant. The pH in anaerobic reactors is mainly controlled by the bicarbonate buffer system. Therefore, the pH value inside digesters depends on the partial pressure of CO_2 and on the concentration of alkaline and acid components in the liquid phase (Borja, 2011; Al Seadi et al., 2008). This thesis investigates the possibility to perform AD at higher pH values with a pH-tolerant microbial consortium obtained from alkaline soda lakes (See section 1.3.3 for details).

(iii) HRT / SRT / OLR: Hydraulic retention time (HRT), Solid retention time (SRT) and Organic loading rate (OLR) also affect the process performance (Astals Garcia, 2013). HRT represent the average time that dissolved molecules stay inside the reactor, while SRT represent the average time that solid biomass remains inside the reactor; the OLR is the amount of substrate or organic matter introduced per day and digester volume into the system (Borja, 2011; Burke P. E, 2001; Al Seadi et al., 2008). The OLR can be increased / decreased by reducing / increasing the HRT or increasing / reducing the organic matter concentration of the digester feedstock. Both the HRT and the SRT are important as they set the amount of time available for bacteria to grow and subsequently convert the substrate into methane. In this sense, the HRT and SRT must be long enough so that the amount of microorganisms removed with the effluent (digestate) is not higher than the amount of microorganisms reproduced. Too short HRT or SRT can result in the washout (loss) of active biomass while an excessively long HRT / SRT can result in accumulation of toxic compounds. Both situations result in a decrease of the biogas production rate. Too high OLR can result in the overload of the reactor which leads to the accumulation of toxic compounds and results in poor biogas production (Astals Garcia, 2013; Borja, 2011; Burke P. E, 2001; Kwietniewska and Tys, 2014; Al Seadi et al., 2008). Therefore, the HRT, the SRT and the OLR need to be optimized together to maximize the degradation of the substrate and obtain the highest possible biogas production rate.

(iv) VFA: Volatile fatty acids (VFAs), which typically include acetate, propionate, butyrate and valerate, are the main intermediates of the AD process produced during acidogenesis. Therefore, their concentrations are often monitored as a proxy for digester performance and stability (Astals Garcia, 2013; Al Seadi et al., 2008). For example, accumulation of VFAs reflects an uncoupling between acid formers and methanogens, and can result from stress caused by hydraulic or organic overloading, sudden temperature variations, or the presence of toxic or inhibitory compounds. In most cases, accumulation of VFAs results in a drop of pH-value which in turn reduces the biogas production rate (Borja, 2011; Al Seadi et al., 2008).

(v) Ammonium / Ammonia: Ammonium (NH_4) and ammonia (NH_3) are produced by the degradation of proteins and other nitrogenous compounds. Ammonium is an important source of nitrogen for bacteria and beneficial to the

process (Kwietniewska and Tys, 2014). However, the free form of ammonia (FA), NH_3, is toxic for the methanogenic archaea and its accumulation results in digester failure (Chen et al., 2008; Kwietniewska and Tys, 2014; Pind et al., 2003; Al Seadi et al., 2008). The concentration of free NH_3, according to Eq. 1, is influenced by the pH and the temperature. Therefore, an increase in the pH would result in an accumulation of FA and an increase in toxicity (Chen et al., 2008).

$$\text{N-NH}_3 = \frac{\text{TAN} * 10^{\text{pH}}}{e^{\left(\frac{6344}{273.15+\text{T}}\right)} + 10^{\text{pH}}}$$ Eq. (1) (Anthonisen et al., 1976)

Free ammonia seems to be the main cause of inhibition because it is freely, passively membrane permeable and causes proton imbalance and / or potassium deficiency (Kwietniewska and Tys, 2014).

(vi) Other parameters: Several other parameters affect the AD process. Ions such as sodium (Na^+), calcium (Ca^{2+}), potassium (K^+) and magnesium (Mg^{2+}) are known to be important for an effective digestion and their absence or excessive accumulation can have negative effects on the biogas production (Chen et al., 2008). Addition of micronutrients and / or vitamins is also important for a correct performance (Al Seadi et al., 2008).

The above list enumerates the most important factors and parameters affecting the anaerobic digestion process. For a deeper understanding, multiple reviews exist that explain the AD process and its parameters in greater details (Borja, 2011; Burke P. E, 2001; Deublein and Steinhauser, 2008; Kwietniewska and Tys, 2014; Pind et al., 2003; Al Seadi et al., 2008).

1.1.2. Adding value through anaerobic digestion

One of the main advantages of the anaerobic digestion process is that it can be fueled with multiple organic substrates, from manure to wheat straw to microalgae. Production of biofuels (such as bioethanol or biodiesel) or other products from biomass only makes use of a fraction of the biomass. The remaining biomass is often a good substrate for methane production (Ahring, 2003). For example, the anaerobic co-digestion of glycerol, a by-product of the biodiesel production process, with manure not only boost the biogas production (Astals et al., 2012) but can also contribute to reduce the biodiesel production cost by adding value to a waste. In this

sense, the use of residual algal biomass, obtained during the production of biofuels, can also contribute to reduce the algal production costs while providing energy (de Boer et al., 2012; Kwietniewska and Tys, 2014).

1.2. Microalgae as substrate for anaerobic digestion

Commercially operated biogas plants use organic wastes such as wastewater, the organic fraction of solid urban waste, agroindustrial waste (manure) or biomass to produce methane which then can be converted into heat, electricity or used directly as natural gas (Angelidaki et al., 2003; Holm-Nielsen et al., 2009; Weiland, 2010). In past recent years however, biogas production plants are also being fuelled with whole-crop silage, the so called energy crops, which to date contribute over 50 % of the total biogas production (Weiland, 2010)**.** The use of these energy crops presents several long-term disadvantages and drawbacks: (i) they compete for arable land with traditional crops, (ii) they use large quantities of fresh water and (iii) they require fertilization (Karp and Richter, 2011). On the other hand, microalgae are microorganisms that can transform sunlight and atmospheric CO_2 into biomass (Eq. 2) without the drawbacks associated with energy crops.

[a] CO_2 + [b] nutrients + sunlight → [c] O_2 + CH_2O (biomass) Eq. (2) (Davis et al., 2011)

Microalgae have several advantages over energy crop biomass; (i) they have a higher photon conversion efficiency which can result in a higher biomass yield per hectare, (ii) they also have a higher CO_2 absorption capacity compared to energy crops, (iii) they can be grown in liquid medium including salt and waste water streams, (iv) algae can be produced on marginal land which does not compete with arable land and their production is not seasonal, (v) they have minimal environmental impact such as deforestation, and (vi) they require less fertilizers and pesticides (de Boer et al., 2012; Clarens et al., 2010; Gouveia, 2011; Hannon et al., 2010; Wiley et al., 2011).

One of the first attempts to use algal biomass as substrate for AD was performed by Gouleke et al., 1957 who digested a mixture of *Chlorella sp*. and *Scenedesmus sp.* biomass. Later, in the 70ties and 80ties, Foree and McCarty, 1970; Samson and LeDuy, 1982; Samson and LeDuy, 1986; Varel et al., 1988 continued with the study of the anaerobic digestion of algae, mainly the cyanobacteria *Spirulina*. However, the circumstances at that time, cheap oil prices (Mouawad, 2008) and less environmental

concerns led to a loss of interest. The need to use non-fossil energy sources, the biorefinery concept and the oil price spike in 2008 has brought back the attention to using algal biomass for the production of biofuels (de Boer et al., 2012; El-Mashad, 2013; Mussgnug et al., 2010; Ras et al., 2011; Sialve et al., 2009).

Algal biomass can be used for several purposes. Next to using algal biomass as source for renewable energy by the production of biodiesel or biogas (de Boer et al., 2012), algae can also be a source for value added products such as proteins, polysaccharides, pigments, animal feed, nutritional dietary products, etc. (Gouveia, 2011).

To date, the production of biofuels from microalgae is not economically feasible mainly because with the current technology and developments, the energy balance between the in-put and the output is still negative, that is, more energy is consumed than produced (de Boer et al., 2012; Clarens et al., 2010). Several factors related to algae production and harvest, extraction and conversion of oils make the process of obtaining biofuels from algae still expensive. Slade et al., 2013 compared the production of algal biomass in open raceway ponds (ORP) with the production of algae using a photobioreactor system (PBR). According to Slade and Bauen, 2013, the cost of 1.0 Kg of algae ranges from 1.8 € in the ORP system to 10.0 € in the PBR system. These costs do not yet include the extraction and conversion of algae into fuels. According to a Life Cycle Analysis (LCA) performed by Davis et al., 2011, the selling price of 1.0 gallon (3.78 L) of biodiesel would be located at approximately 7.0 € for the ORP and 15.0 € for the PBR system which is markedly higher than the average current price of 3.0 € per gallon[1]. The main production costs in the open raceway pond are associated to fertilizer, CO_2, algae harvest, compacting, drying and oil extraction and in the photobioreactors system to electricity, drying and oil extraction (Slade and Bauen, 2013; de Boer et al., 2012; Davis et al., 2011). Direct digestion of the algal biomass into methane can have several advantages over the production of biodiesel from microalgae: (i) nutrients, P and N can be recirculated from the anaerobic digester to the algae culture system (de Boer et al., 2012; Collet et al., 2011; Oncel, 2013); (ii) for anaerobic digestion, solids with a higher water content can be used, therefore reducing the harvesting and the drying costs (Collet et al., 2011; Torres et al., 2013; Wiley et al., 2011); (iii) with the direct digestion of algae

[1] Prices were calculated based on the US market and converted to Euros.

there is no need to use solvents for lipid extraction (de Boer et al., 2012); (iv) CO_2 generated during the AD process can be recycled to the algae (de Boer et al., 2012; Slade and Bauen, 2013; Wiley et al., 2011); (v) there is no need to use high lipid production strains (Torres et al., 2013; Wiley et al., 2011) and, (vi) the possibility to use algae adapted to extreme conditions which reduces contamination (Hannon et al., 2010). With these advantages, it is well possible that the direct anaerobic digestion of algae can become a suitable source of renewable energy.

As identified by the majority of the LCA studies, the supply of CO_2 to the algae represents a high percentage of the production costs (Davis et al., 2011; Slade and Bauen, 2013). This high energy consumption, especially in the ORP, can be attributed to the low dissolution rate of atmospheric CO_2 in water, which results in a low CO_2 fixation efficiency (Slade and Bauen, 2013; Wiley et al., 2011). The CO_2 loss due to evaporation in the PBR is minimal, as the system is closed and CO_2 can be directly pumped into the medium with minimal losses, increasing the CO_2 fixation efficiency (Slade and Bauen, 2013). This problem, high CO_2 consumption due to low fixation efficiency, could be overcome by growing algae at alkaline conditions. At alkaline conditions, that is, high pH, the dissolution capacity of CO_2 is greater therefore more CO_2 becomes available for the cells to grow (Eq. 2).

Compared to a traditional AD system, there are several additional advantages of also performing the anaerobic digestion of the algal biomass at alkaline conditions. The high pH and alkalinity of the sludge from the anaerobic digester can act as a CO_2 scrubber, capturing the produced CO_2 and obtaining biogas rich in methane which, with minor upgrade, could directly be used as biomethane (Persson et al., 2006; Weiland, 2010). Alkaline sludge rich in carbonate can be recirculated to the algae cultivation pond / photobioreactor reducing the amounts of CO_2 needed. Another possible advantage is that the alkaline conditions favor the precipitation of nitrogen and phosphorous which can be recirculated to the culture tank as a supply of nutrients for growth (Collet et al., 2011; Wang et al., 2013). And, as stated by other authors, cultivating algae at extreme conditions also reduces risk of contamination by invasive species (Hannon et al., 2010; Wiley et al., 2011). Therefore, combining both approaches, alkaline culture of algae and subsequent alkaline digestion of the algal biomass can be an interesting and attractive way to obtain energy from algae.

To put this new approach into practice, two main issues need to be addressed. The first issue is related to the selection and cultivation of an algal strain that is able to grow at such extreme conditions. The second issue is the identification of a suitable microbial community capable of performing the AD at alkaline conditions. Concerning the selection of a suitable algal strain, several algae are known to be able to grow in environments with high pH and high salt concentrations (Hannon et al., 2010). For example, several cyanobacteria, e.g. *Spirulina*, are known to grow and thrive in alkaline or soda lakes and in hypersaline habitats (Grant, 2006; Jones and Grant, 1999; Kompantseva et al., 2009; Sorokin et al., 2014). Other species such as *Dunaliella viridis* and *Chlorella minutissima* are known to tolerate alkaline conditions (Ballot et al., 2004; Melack, 1981). The selection and cultivation of a specific microalga at alkaline conditions is beyond the scope of this doctoral thesis, which addresses the second issue, a high-pH adapted anaerobic microbial community. Such communities exist naturally in alkaline environments such as soda lakes. Alkaline or soda lakes are natural occurring environments with a high concentration of carbonates and pH values up to 12 (Grant, 2006; Jones et al., 1998). Several studies have already shown that methanogenesis occurs in such environments which indicates the presence of an active methanogenic community (Nolla-Ardèvol et al., 2012; Sorokin et al., 2004).

1.3. Extreme alkaline (soda) lakes

Alkaline lakes or soda lakes are naturally occurring high pH and high salt habitats with pH up to 12 and high salt concentration (in some lakes up to 7 M Na^+) which, despite their extreme conditions, are among the most eutrophic / prolific habitats.

1.3.1. Characteristics of soda lakes

Soda lakes and soda deserts represent the major types of naturally occurring highly alkaline environments, in which the micro flora is subjected to extreme conditions. Soda lakes can be found in dry or cryoarid areas of America, Africa and Asia among others (Baumgarte, 2003; Kompantseva et al., 2009; Sorokin et al., 2014). One of the main characteristics of alkaline lakes is the almost complete absence of Ca^{2+} and Mg^{2+} which is a key feature for the generation of high alkalinity. Concentration of the ions in the groundwater through evaporation leads to a shift in the carbon dioxide / bicarbonate / carbonate equilibrium in favor of carbonates. The carbonate,

$CO_2/HCO_3^-/CO_3^{2-}/OH^-$, equilibrium is responsible for the high alkalinity observed in these lakes. Alkalinity develops by a shift in the equilibrium towards a preponderance of CO_3^{2-} in the absence of significant amounts of Ca^{2+} (which would precipitate CO_3^{2-} from the solution as $CaCO_3$) (Duckworth et al., 1996). An increase in carbonate ion concentration results in the precipitation of insoluble carbonates of, first, calcium, and, then, magnesium, removing these from solution and allowing the more soluble carbonates of sodium and potassium to accumulate. As a result, and due to the presence of Na^+ and Cl^- in the surroundings, an alkaline soda / salt brine with pH values up to 12 develops, creating a highly alkaline environment (Baumgarte, 2003; Grant, 2006; Jones et al., 1998; Sorokin and Kuenen, 2005b). In contrast to other alkaline environments, such as low-salt alkaline springs, soda lakes maintain a stable alkaline pH due to the high buffering capacity of the soluble carbonates. This particularity of double extreme conditions, high pH and high salinity, make soda lakes a unique ecosystem (Foti et al., 2008).

1.3.2. Soda lakes of the Kulunda steppe (Russia)

The best known and studied soda lakes are those located in the East African Rift valley in Kenya (Baumgarte, 2003; Grant, 2006). In this project however, the interest is set on the Central Asian soda lakes of the Altai province (Russia), in the south region of the Kulunda steppe (Figure 1.1).

The characteristics of this cryoarid region, such as the geological terrain structure, the poor river network, the extremely continental dry climate, permafrost, and seasonally frozen ground led to the formation of numerous small soda lakes, which have the peculiarity of relatively low water mineralization resulting in shallow brackish lakes (Kompantseva et al., 2007; Kompantseva et al., 2009). Furthermore, the lakes are subjected to unstable water and temperatures regimens between -40°C and +40°C, causing frequent fluctuations of the water level and salinity (Foti et al., 2008). These water fluctuations results in salinity levels of up to 400 mg L^{-1} while the pH ranges from 8 to 10.6 (Sorokin et al., 2014).

Figure 1.1 Central Asian soda lakes
Geographic location of the soda lakes from the Kulunda steppe (Russia). Soda lake photograph courtesy of Dr.Dimitry Sorokin

1.3.3. Microbial diversity of soda lakes

Despite the extreme environmental conditions present in soda lakes, these habitats are among the most productive natural ecosystems (Grant et al., 1990; Jones and Grant, 1999; Kompantseva et al., 2009; Melack, 1981). For example, in the soda lakes of the East African Rift, the photosynthesis rate can exceed 10 g C m^{-2} day^{-1}. Lakes in the Altai region are also highly productive with rates of photosynthesis up to 1.32 g C m^{-2} day^{-1} (Kompantseva et al., 2009). The presence of sodium carbonate in variable combinations with sodium chloride and sodium sulfate creates a unique, buffered haloalkaline habitat appropriate for a stable development of obligate (halo)alkaliphilic microorganisms growing optimally at pH around 10 (Sorokin and Kuenen, 2005a). Among these highly active (halo)alkaliphilic organisms multiply types of bacteria and archaea and some cyanobacteria (*Spirulina*, *Phormidium*) and microalgae (*Dunaliella viridis, Chlorella sp*) can be found (Ballot et al., 2004; Kompantseva et al., 2009). For example, cyanobacterial mats, mainly composed of *Spirulina*, have been reported to colonize such alkaline lakes (Grant, 2006; Kompantseva et al., 2009). Also a high number of crustaceans, mainly Artemia sp, have been detected (Kompantseva et al., 2009).

In the bacterial domain, multiple extremely halotolerant and alkaliphilic groups have been detected. Most of the identified alkaliphilic microorganisms belong to the phylum Firmicutes, but members of other phyla such as Actinobacteria, Proteobacteria, Bacteroidetes, Thermotogae and Spirochaetes have also been widely detected (Yumoto et al., 2011). Among the Proteobacteria, members belonging to the Gammaproteobacteria class (e.g. *Ectothiorhodospira* and *Halomonas*), and Alphaproteobacteria of the family Rhodobacteraceae are known to be present in soda lakes (Duckworth et al., 1996; Kompantseva et al., 2010). Gram-positive Bacilli such as *Alkalibacillus*, *Halolactobacillus* and Bacilli of the Family VII Insertae Sedis have also been isolated (Duckworth et al., 1996; Yumoto et al., 2011). Among the Clostridia, members of the genus *Alkaliphilus*, *Natronincola* and *Tindallia* have been identified in multiple soda lakes (Humayoun et al., 2003; Kevbrin et al., 2013; Wani et al., 2006; Yumoto et al., 2011; Zhilina et al., 2009). Likewise, other Clostridia such as *Natranaerobius* and *Anaerobranca* are also known to be part of the microbial community of soda lakes (Humayoun et al., 2003; Mesbah and Wiegel, 2009; Yumoto et al., 2011). Sorokin and Kompantseva have conducted extensive research on the presence of chemolithoautotrophic sulfur-oxidizing bacteria in soda lakes (Kompantseva et al., 2010; Sorokin et al., 2004; Sorokin and Kuenen, 2005a; Sorokin and Kuenen, 2005b). Further information can be found in the different reviews on the halotolerant and alkaline microbial communities from soda lakes that have been published (Duckworth et al., 1996; Grant, 2006; Kivistö and Karp, 2011; Sorokin et al., 2014; Yumoto et al., 2011).

The main groups of anaerobes, such as fermentative, acetogenic, methanogenic and sulfate-reducing bacteria are represented in soda lakes by unique haloalkaliphilic species (Sorokin and Kuenen, 2005b). Methanogenesis has also been reported and extensively studied in soda lakes. Members of the orders Methanomicrobiales, Methanobacteriales and Methanosarcinales are present in these extreme habitats (Grant et al., 1999; Grant, 2006; Mesbah et al., 2007a; Oren, 2008). Methanogenic archaea isolated to date are mainly methylotrophic, utilizing a variety of one carbon (C1) compounds including methanol and methylamine. C1 compounds are likely to be abundant in soda lakes, probably derived from the anaerobic digestion of cyanobacterial mats. *Methanosalsum* and *Methanolobus* are two of the methylotrophic methanogens present in such lakes (Grant, 2006). This suggests that methane in soda lakes is mainly formed via the methylotrophic pathway with some

contribution of the hydrogenotrophic pathway, while the acetoclastic methanogenesis is practically absent (McGenity, 2010; Sorokin et al., 2014). Despite the dominance of methylotrophic methanogens, Zhilina et al., 2013 recently isolated and identified the first truly alkaliphilic methanogen, *Methanocalculus natronophilus*, isolated from sediments of the Tanatar lake system. This *Methanocalculus* can grow at pH values between 8.0 and 10.2, with an optimum pH of 9.9 – 9.5, and it requires between 0.5 and 1.6 M of carbonates and between 0.9 and 3.3 M of Na^+. *M. natronophilus* is a hydrogenotrophic methanogen and cannot use acetate as substrate for methanogenesis but it requires acetate for growth as carbon source. In addition to detecting the presence of multiple methanogens and their isolation, several studies have already shown the possibility of methane production from soda lakes sediments. In this sense, the presence of active haloalkaline methanogens in soda lake sediments from the Kulunda Steppe has already been demonstrated (Nolla-Ardèvol et al., 2012), while van Leerdam et al., 2008 were able to operate an anaerobic reactor inoculated with sediments from several soda lakes.

1.3.4. Biotechnological applications of haloalkaliphiles

Extremophiles have multiple biotechnological applications mainly related to the production of enzymes capable of working under extreme conditions, from thermotolerant to psycrophilic and acidophilic enzymes (Berlemont and Gerday, 2011). The biotechnological application of halotolerant and alkaliphilic microorganisms has also been widely studied (Grant et al., 1990; Horikoshi, 1999; Kivistö and Karp, 2011; Margesin and Schinner, 2001; Sarethy et al., 2011). The main biotechnological applications of alkaliphiles are related to the use of enzymes such as alkaline proteases, amylases and lipases as detergent additives (Grant et al., 1990; Horikoshi, 1996; Sarethy et al., 2011), alkaline proteases for leather tanning (Grant et al., 1990; Sarethy et al., 2011), chitinases for the treatment of seafood waste (Sarethy et al., 2011), alkaline proteases for the recovery of silver from X ray films (Horikoshi, 1996) and thermo stable alkaline xylanases for the enzymatic debleaching in pulp-mill industries (Horikoshi, 1996) among many others. Alkaliphiles are also used for the industrial production of cyclodextrins (Horikoshi, 1996) and of multiple metabolites such as carotenoids, siderophores, antibiotics and enzyme inhibitors (Horikoshi, 1996; Sarethy et al., 2011).

In the case of Halophiles, to date no direct industrial applications have been fully developed (Kivistö and Karp, 2011). However, multiple future possible applications are under exploration: biopolymers, biosurfactants and bioplastics; multiple enzymes; possible biological waste treatment of high salt and heavy metal containing wastewaters and production of H_2; bacteriorhodopsin for the application in holography, artificial retina and 3D computer memory. Halotolerant bacteria are also being used for the detoxification of chemical warfare agents (Kivistö and Karp, 2011; Margesin and Schinner, 2001). Despite all the above mentioned applications, to date, only the study of van Leerdam et al., 2008 explored the possibility of using haloalkaline extremophiles for the production of biogas.

1.4. Microbial community of the anaerobic digestion process

The anaerobic digestion is a complex biological process where multiple bacteria collaborate to degrade organic matter in the absence of oxygen with the release of methane. A good knowledge of the different players and understanding of their relationships can contribute to optimize the production of methane.

1.4.1. Microbiology of the anaerobic digestion process

The anaerobic degradation of organic matter, also known as methanogenesis, is a four step biological process in which a consortium of microorganisms transforms complex molecules into methane and carbon dioxide. The four steps of the methanogenesis involve (i) hydrolysis of macromolecules, (ii) acidogenesis and formation of volatile fatty acids, (iii) acetogenesis or formation of acetate and finally (iv) the methanogenesis or transformation of acetate, H_2 and CO_2 into CH_4 (Figure 1.2).

(i) Hydrolysis: This initial step involves the hydrolysis of complex organic polymers such as carbohydrates, lipids, nucleic acids and proteins into glucose, glycerol, purines, pyridines and amino acids (Gerardi, 2003; Al Seadi et al., 2008). Generally, this hydrolysis step is conducted by multiple hydrolytic enzymes, proteases, cellulases, pectinases, amylases and chitinases released by hydrolytic bacteria (Anderson et al., 2003; Pavlostathis, 2011; Al Seadi et al., 2008).

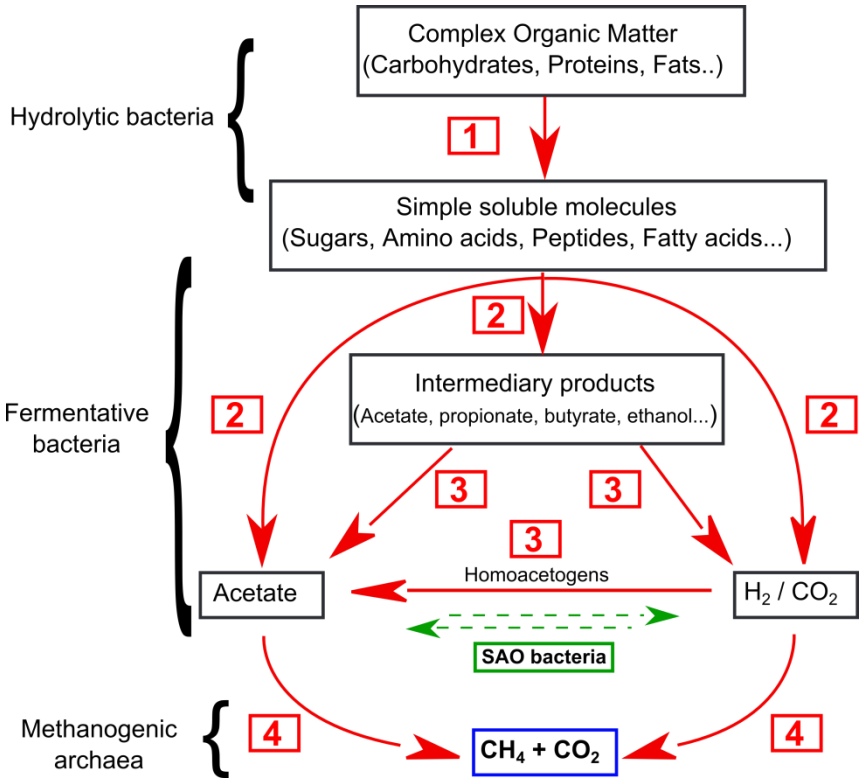

Figure 1.2 The anaerobic digestion process.
Microbiology and steps of the anaerobic digestion process:
1 Hydrolysis; **2** Acidogenesis; **3** Acetogenesis; **4** Methanogenesis.
*SAO: Syntrophic Acetate Oxidizing bacteria. See text for details.

The bacteria responsible for this initial hydrolysis step are mainly *Clostridium, Bacteroides, Acetivibrio, Bifidobacterium, Lactobacillus, Streptococcus* and others (Anderson et al., 2003; Borja, 2011). The result of this hydrolysis step is the production of simple soluble organic compounds such as volatile acids, alcohols and other monomers (Borja, 2011; Gerardi, 2003).

(ii) Acidogenesis: In this second step, the products of hydrolysis such as long-chain fatty acids and alcohols are converted into intermediate products like volatile fatty acids (mainly acetate, propionate, butyrate), hydrogen, CO_2, and ammonia (Anderson et al., 2003; Pavlostathis, 2011; Al Seadi et al., 2008). The acidogenesis is performed by many different fermentative genera such as *Clostridium, Bacteroides, Lactobacillus* and others like *Butyribacterium, Propionibacterium, Eubacterium, Desulfobacter, Bacillus* and *Escherichia* (Anderson et al., 2003).

(iii) Acetogenesis: The products from the acidogenesis step which cannot be directly transformed into methane by methanogenic bacteria, such as long and short chain fatty acids, other than acetate, as well as lactate, methanol, and ethanol are

converted into acetate, H_2 and CO_2, the three main substrates for methanogens (Anderson et al., 2003; Pavlostathis, 2011; Al Seadi et al., 2008). Two groups of acetogenic bacteria can be distinguished on the basis of their metabolism. The first group, the obligate hydrogen producing acetogens produce acetic acid, CO_2 and H_2 from propionate, butyrate, valerate and alcohols. So far, only a limited number of bacteria of this first group have been isolated and identified, *Syntrophomonas wolfei* and *Syntrophobacter wolinii*, which oxidize butyrate and propionate, respectively (Anderson et al., 2003). The second group, the homoacetogens are responsible for the conversion of H_2 and CO_2 to acetate (Anderson et al., 2003; Pavlostathis, 2011). Homoacetogenic bacteria include *Acetoanaerobium, Acetogenium, Butyribacterium, Clostridium, Eubacterium,* and *Pelobacter* (Borja, 2011).

(iv) Methanogenesis: The final step in the anaerobic digestion process is the production of methane (CH_4) by methanogenic Archaea (Figure 1.2). These can be acetoclastic methanogens, which use acetate, or hydrogenotrophic methanogens, which use H_2 and CO_2. In addition, some methanogens can also use methylamines, methanol, formate, and carbon monoxide (CO) (Anderson et al., 2003; Pavlostathis, 2011). Acetate is regarded as the most important precursor of methane production which can account for up to 70% of the produced methane. In spite of this fact, only two methanogenic genera are known acetoclastics, *Methanosaeta* and *Methanosarcina* both of the order Methanosarcinales (Anderson et al., 2003; Demirel and Scherer, 2008). The hydrogen partial pressure is an important parameter, which defines process stability or upsets in an AD process. Therefore, and even though generally only about 30% of the produced methane is produced by hydrogenotrophic methanogens, the activity of this type of methanogens is crucial for a stable and efficient process performance (Anderson et al., 2003; Demirel and Scherer, 2008). These methanogens reduce carbon dioxide, formate, methanol and methylamines, using the hydrogen produced during the acidogenesis and acetogenesis steps (Anderson et al., 2003). Hydrogenotrophic methanogens can be found in all known methanogenic orders, Methanobacteriales, Methanococcales and Methanomicrobiales (Demirel and Scherer, 2008). It is worth noting that under certain circumstances, for example in alkaline lakes, the main methane producers are the methylotrophic methanogens, which use methylated compounds as substrate. In soda lakes hydrogenotrophic and acetoclastic methanogenesis are almost undetectable (Sorokin et al., 2014).

Methanogenesis is the critical step in the entire anaerobic digestion process, and it is regarded as the limiting step as it is the slowest biochemical reaction of the process (Anderson et al., 2003; Al Seadi et al., 2008). Methanogenesis is severely affected by the operational conditions and any disturbance in the process results in its inhibition. The two main factors affecting methanogenesis are acetate and free ammonia, and their accumulation results in the inhibition of mainly the acetoclastic methanogens (Angelidaki and Ahring, 1993; Calli et al., 2005; Koster and Lettinga, 1988).

An interesting phenomenon during the anaerobic digestion, which under certain circumstances plays a key role in the production of methane, is the syntrophic association between bacteria and methanogenic archaea (Hattori, 2008; Müller et al., 2013; Schink, 2006). Syntrophy is a specific case of symbiotic relationship that occurs between two metabolically different types of microorganisms that depend on each other for the degradation of a substrate. This syntrophic degradation involves the transfer of one or more metabolic intermediates between the two organisms (Schink, 2006). The most important syntrophic relationship in the AD process is the one established between the syntrophic acetate oxidizers (SAO) and the hydrogenotrophic methanogens (Hattori, 2008; Müller et al., 2013). SAO normally grow as lithotrophs or heterotrophs, producing acetate through the Wood–Ljungdahl pathway, however, when they grow in syntrophy with hydrogenotrophic methanogens, they reverse this pathway and are capable of consuming acetate by oxidizing it to hydrogen and carbon dioxide (Figure 1.2) (Hattori, 2008; Müller et al., 2013). The oxidation of acetate to methane by the syntrophic association between SAO and hydrogenotrophic methanogens generally takes place when the acetoclastic methanogens are inhibited due to ammonia accumulation. Other factors affecting this relationship are acetate concentration, operational parameters, and microbial community structures (Ahring, 2003; Hattori, 2008; Müller et al., 2013). If the hydrogenotrophic methanogens are inhibited or absent and the available H_2 is not consumed, the syntrophic relation does not develop (Garcia et al., 2000). So far a number of SAO bacteria have been identified, mainly belonging to the genera *Syntrophobacteri, Syntrophomonas, Syntrophospora* and *Clostridium* (Garcia et al., 2000).

Taken together, the anaerobic digestion of organic matter is a complex process with multiple functionally interconnected microbial populations that play specific roles, each of them crucial for the effective conversion of complex substrates into methane.

1.4.2. Metagenomics to study the anaerobic digestion process

Next Generation Sequencing technologies have allowed the study of complex communities by sequencing at lower costs and higher throughput than the Sanger based sequencing technology (Scholz et al., 2012). The lower sequencing costs have boosted metagenomics, which has become a common way to study the taxonomy, gene composition and gene function of microbial communities (Simon and Daniel, 2011).

The anaerobic digestion of organic matter is a complex process which involves the participation of numerous types of bacteria and archaea (Schlüter et al., 2008; Wirth et al., 2012). Elucidating the microbial composition of an anaerobic digester and understanding the function and relationships of the different microorganisms involved in the process could help to improve the biogas production. So far, a number of metagenome studies from several biogas producing plants and lab scale anaerobic reactors have been performed (Jaenicke et al., 2011; Li et al., 2013; Schlüter et al., 2008; Wirth et al., 2012). However, few metagenomic approaches have been used to study the microbial community of alkaline lakes or hypersaline environments. For example, Lanzén et al., 2013 studied the prokaryotic diversity in a Kenian soda lake using the GS-FLX Titanium sequencing platform. To date, the only known work in which a metagenomic approach was applied to study an anaerobic microbial community at high pH was recently performed by Wong et al., 2013, in which they pretreated waste activated sludge at pH 10 which was subsequently used as inoculum for an anaerobic digester.

Several High Throughput Sequencing (HTS) techniques are available for the sequencing of large metagenomes which use different approaches to produce the sequenced reads (Logares et al., 2012; Metzker, 2010). The sequencing of reads with Roche's 454 platform produces reads up to 800 bp in length and is based on single-nucleotide addition. Incorporation of specific nucleotides is detected as a light signal. Illumina is another commonly used sequencing platform, where sequencing is done by the addition of nucleotides one at the time which are labeled with different

fluorophores. This results in reads up to 500 bp long. The SOLiD platform sequencing is done by ligation which can produce reads with a length of 75 bp, while the IonTorrent PGM, which measures the protons that are released when a nucleotide is incorporated to a DNA strand, can produce reads with a length of up to 400 bp.

Two main approaches can be used to obtain information from the metagenomic reads. In the first approach, short reads are directly compared against DNA or protein coding sequences in databases using alignment tools such as BLAST. In the second approach reads are first assembled into long contigs and all subsequent analyses are based on these assembled contigs (Liu et al., 2013; Teeling and Glöckner, 2012). The analysis in the second approach can be performed via sequence homology where contigs are compared to reference sequence databases using BLAST, or via a sequence compositional analysis, where the classification takes place by matching, for example, the tetranucleotide frequency found within each contig to the genome(s) with the most similar frequency. Since the tretranucleotide frequencies are relatively conserved between taxa, it is possible to use this for classification purposes (Logares et al., 2012; Strous et al., 2012). Obtained reads and/or contigs can be used to perform taxonomic classification or functional analysis using one or several of the multiple available tools and pipelines (Simon and Daniel, 2011).

2. Challenges

The production of biogas via the anaerobic digestion of biomass is a commonly used technology for energy production, where the obtained biogas can be used for the production of electricity and heat or as transport fuel (Persson et al., 2006; Weiland, 2010). The production of biogas from microalgal biomass is one of the multiple approaches to obtain energy from this specific type of biomass. Microalgal biomass can be used directly or after extraction of high value products such as oil. The use of whole algal biomass as substrate for biogas has the advantage that both processes, the algal production and the anaerobic digestion, can be directly coupled, which results in the reduction of processing costs (de Boer et al., 2012; Clarens et al., 2010; Hannon et al., 2010). A main issue during algal cultivation is the need to supply carbon dioxide in order to obtain high biomass density which results in relatively high production costs associated with the use and loss of carbon dioxide (Slade and Bauen, 2013; Wiley et al., 2011). A possible solution to eliminate this bottleneck would be the cultivation of microalgae at elevated pH as this contributes to an increase of CO_2 solubility and availability for the microalgae. In order to directly couple the algal production with the anaerobic digestion process, and to reduce processing costs, the latter has to be performed also at high pH.

The anaerobic digestion process is currently performed, independent of the biomass used, under a strict pH range due to the pH sensitivity of methanogens (Chen et al., 2008). Several studies have shown that sediments from soda or alkaline lakes contain active methanogens capable of producing methane at elevated pH (Nolla-Ardèvol et al., 2012; Sorokin et al., 2004). Moreover, a recent study has demonstrated the possibility of performing anaerobic digestion at high pH by inoculating the reactor with sediments from alkaline lakes containing active methanogens (van Leerdam et al., 2008). Producing methane in an anaerobic high pH reactor would make it possible to couple this process to the production of algal biomass also at high pH conditions. The combination of these two processes could contribute to the overall reduction of the production costs and make the use of microalgae as energy source an economically viable option.

3. Objectives

Microalgae are microorganisms that can transform sunlight and CO_2 into biomass and their suitability as substrate for the production of biogas has already been shown. One of the bottlenecks in algae cultivation is the low absorption rate of CO_2 which results in high production costs. These could be partially reduced by cultivating algae at high pH. Performing the anaerobic digestion of microalgae at high pH as well could be beneficial as: (i) the anaerobic reactor would function as a CO_2 scrubber, leading to biogas rich in methane which, with minor upgrade, could directly be used as biomethane, and (ii) the dissolved CO_2 along with phosphorous and other nutrients could be directly recycled to the photobioreactor and used for algae growth. Therefore, the combination of algal biotechnology and anaerobic digestion at high pH might be economically favorable, and such a combined system could be a possible source for renewable energy.

In this work, the study of the anaerobic digestion of the microalga *Spirulina* at alkaline conditions, pH~10 and 2.0 M Na^+, will be addressed. To do so, sediments from soda lakes, which are natural alkaline habitats, will be adapted to perform the anaerobic digestion at controlled alkaline conditions. One anaerobic reactor will be set to determine the optimal operational conditions, such as hydraulic retention time and organic loading rate, in order to obtain the highest possible biogas yield. Subsequently a reactor will be operated at the identified optimal parameters.

In addition to the study of the anaerobic digestion, DNA will be extracted from the alkaline anaerobic reactor and sequenced in order to obtain the metagenome of the haloalkaline microbial community. This will be analyzed both taxonomically and functionally, with the aid of several bioinformatics tools, to study the haloalkaline microbial community present in the reactor.

In parallel to this work, multiple biomethane potential tests will be set up to study the anaerobic digestion at alkaline conditions of other substrates of interest such as wheat straw and fresh algal biomass.

To serve as a control and comparison system, an anaerobic reactor will be set up to study the production of biogas from the digestion of *Spirulina* at mesophilic pH conditions. As for the alkaline system, the metagenome of the microbial population in the mesophilic digester will also be studied.

4. General discussion

The work presented here is dedicated to demonstrate the possibility to produce biogas form microalgae both, at mesophilic and haloalkaline conditions. Most importantly, the anaerobic digestion (AD) of the microalga *Spirulina* was performed at high pH, pH~10, and high sodium content, 2.0 M Na^+. To do so, sediments from Central Asian soda lakes, a natural alkaline environment, were used to inoculate an anaerobic reactor which was continuously fed with *Spirulina*.

Anaerobic digestion of microalgae at alkaline conditions can have two main advantages over digestion at mesophilic conditions. At alkaline conditions, most of the CO_2 produced during the AD remains dissolved in the medium, and the obtained biogas is rich in methane. This methane-rich biogas, also known as biomethane, can then be directly fed into the national gas grid. Secondly, digestion at alkaline conditions enables the cultivation of the microalgae at alkaline conditions. With more CO_2 dissolved in the alkaline culture medium higher biomass yield could be obtained. The combination of the two processes, alkaline anaerobic digestion and alkaline microalgae cultivation, can contribute to the reduction of production costs associated to microalgae cultivation, making the microalgae a more economically viable energy source.

The second part of this thesis is devoted to analyzing the metagenome of the anaerobic microbial communities that produce methane from algal biomass. A better understanding of the composition and function of the microbial community responsible for the degradation of the organic matter and the subsequent production of methane can contribute to an improvement of the process. Therefore, metagenomics was used to characterize the microbial community in terms of taxonomy and function of two different anaerobic reactors, one at mesophilic pH conditions and a second one at alkaline conditions.

The three main manuscripts discussed in this work are:

[1] Nolla-Ardèvol V, Strous M, Sorokin DY, Merkel AY, Tegetmeyer HE. 2012. Activity and diversity of haloalkaliphilic methanogens in Central Asian soda lakes. J. Biotechnol. 161:167–173.

[2] Nolla-Ardèvol V, Strous M, Tegetmeyer HE. Anaerobic digestion of the microalga *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+). (Submitted).

[3] Nolla-Ardèvol V, Peces M, Strous M, Tegetmeyer HE. Metagenome analysis and biogas production from the anaerobic digestion of the protein rich microalga *Spirulina.* (Submitted).

Further discussed work includes the following unpublished results:

[4] Nolla-Ardèvol V. Anaerobic digestion of *Spirulina* at alkaline conditions (pH ~10; 2.0 M Na^+) from fresh soda lake sediments.

[5] Nolla-Ardèvol V. Biomethane potential of different substrates at alkaline conditions.

[6] Nolla-Ardèvol V. Metagenome analysis of the microbial population from an alkaline anaerobic digester fed with the microalga *Spirulina*.

4.1. Anaerobic digestion of *Spirulina* at mesophilic pH [3]

The use of *Spirulina* as substrate for the production of biogas was first studied in the 1980ies by Samson and LeDuy, 1982, 1983, 1986 and Varel et al., 1988. In recent years, due to climate change and the concern with regard to the depletion of fossil fuels, the anaerobic digestion of *Spirulina* has attracted fresh interest (El-Mashad, 2013; Mussgnug et al., 2010).

The results obtained with the mesophilic anaerobic reactor were in accordance to the previous studies where *Spirulina* was used as substrate (El-Mashad, 2013; Samson and LeDuy, 1982; Samson and LeDuy, 1983; Samson and LeDuy, 1986). The optimal organic loading rate (OLR) was identified to be 4.0 g *Spirulina* L^{-1} day^{-1} (dry biomass) and yielded biogas with 68% methane content and a specific biogas production of 393 mL g VS^{-1} (Table 1; [3]). These values were similar to the ones obtained by others. For example, De Schamphelaire and Verstraete, 2009, obtained between 380 to 490 mL biogas g VS^{-1} from the digestion of *Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata* while Tartakovsky et al., 2013 obtained 370 ml of biogas g VS^{-1} from the digestion of *Scenedesmus* (Table 4.1). The biogas production only decreased when the OLR was set to 5.0 g *Spirulina* L^{-1} day^{-1} due to accumulation of toxic compounds (Figure 1; Table 1; [3]).

One of the main bottlenecks of the AD of microalgae in general is the low biodegradability and the resistance to hydrolysis of most of the studied microalgal strains which results in low methane yields (Ward et al., 2014). In the mesophilic reactor for example, the biodegradability of *Spirulina* reached 42% similar to the biodegradability of *Chlamydomonas vulgaris*, 51% (Ras et al., 2011) and *Scenedesmus* 53% (Tartakovsky et al., 2013).

To overcome this problem and to increase methane yields, long hydraulic retention times (HRT) need to be applied allowing the substrate to be further hydrolyzed (Ras et al., 2011). However, increasing the HRT can also have a negative effect on the biogas production as accumulation of inhibitory substances such as ammonia can occur (Ras et al., 2011; Salminen and Rintala, 2002)**.** This can also have a negative effect on the anaerobic digestion at alkaline conditions. In the mesophilic reactor, 20 days of HRT were set as a compromise between the different HRT presented in the literature (Samson and LeDuy, 1986; Varel et al., 1988; Ward et al., 2014).

Table 4.1 Biogas production and methane content
Biogas production and methane content of the anaerobic digestion of different microalgae and other substrates with continuous systems at alkaline and mesophilic conditions

Substrate	Conditions	Yield (mL g VS^{-1})	CH_4 (%)	Reference
Spirulina	pH 7-8	393 BG*	68	Nolla-Ardèvol [3]
Spirulina	pH 10; 2.0 M Na^+	84 BG	86	Unpublished Results [4]
Methanethiol	pH 10; 0.8 M Na^+	50 – 200 BG	78	Van Leerdam 2008
Spirulina	pH 7-8	260 CH_4	70-75	Samson 1982
Spirulina	pH 7-8	310 CH_4	56-74	Samson 1983
Spirulina	pH 7-8	350 CH_4	56-74	Samson 1986
Spirulina	pH 7-7.27	470 CH_4[a]	65	Varel 1988
C. reinhardtii *Ps. Subcapitata*	pH 7-8	380-490 BG	40-60	De Schamphelaire 2009
Post- transesterification *Chlorella* residues	pH 7-7.5	240 BG	61-69	Ehimen 2001
N. salina	pH 7-8; 0.2 M Na^+	0.3 – 0.9[b] BG	60-65	Schwede 2013
Scenedesmus	pH 7-7.4	370 BG	64-66	Tartakovsky 2013

* BG: Biogas; a: ml CH_4 ml day^{-1}; b: m^3 m^3 day^{-1}

At this HRT, no accumulation of toxic compounds occurred when a low OLR was used and negative effects could only be seen when an OLR of 5.0 g *Spirulina* L^{-1} day^{-1} (dry biomass) was applied (Figure 1; Table 1; [3]).

The information and experience gained with the anaerobic digestion of *Spirulina* at mesophilic pH conditions was applied to choose the initial parameters for the set-up and operation of the anaerobic reactors at alkaline conditions.

4.2. Selection of alkaline inoculum and substrate [1]

Conventional anaerobic digestion facilities and laboratory reactors perform the process under mesophilic pH conditions, as the mesophilic methanogenic archaea have limited pH and salt tolerance (Chen et al., 2008; Gerardi, 2003; Sialve et al., 2009)**.** Therefore, in order to set up an anaerobic reactor working at alkaline conditions, pH~10 and 2.0 M Na^+, a microbial population adapted to such conditions is needed. Soda or alkaline lakes are natural alkaline environments inhabited by haloalkaline bacteria and archaea which are capable of tolerating high pH and high salt concentrations and where methanogenesis naturally occurs (Grant, 2006; Ollivier et al., 1994; Sorokin et al., 2004). The presence of active methanogens in sediments from such soda lakes has already been demonstrated [1] (Nolla-Ardèvol et al., 2012). Moreover, a recent study already demonstrated the possibility of using sediments from soda lakes as inoculum for an anaerobic reactor (van Leerdam et al., 2008). In

this study however, the main goal was not the production of biogas but the degradation of methanethiol, an impurity present in liquefied petroleum gas.

Methanogens identified in alkaline soda lakes belong to the orders Methanomicrobiales, Methanosarcinales and Methanobacteriales (Grant, 2006; Surakasi et al., 2007). The genus *Methanocalculus*, from the order Methanomicrobiales, includes the, so far, only isolated and characterized alkaliphilic hydrogenotrophic methanogen *Methanocalculus natronophilus* (Zhilina et al., 2013)**.** This methanogen is capable of growing at pH from 8.0 to 10.2, and requires between 0.9 and 3.3 M of Na^+. It has been isolated from the Tanatar soda lake system (Altai, Russia). Sequencing of the marker gene *mcrA*, which encodes the alpha subunit of the methyl coenzyme M reductase, the enzyme that catalyzes the final step in methanogenesis (Luton et al., 2002), confirmed the presence of members of this genus in the soda lake sediments used as inoculum for the anaerobic alkaline reactor [1] (Nolla-Ardèvol et al., 2012). As the selected sediments had been stored at 4°C for over a year, tests to assess the activity of the methanogenic haloalkaline microbial community under different pH and salt conditions were performed. At alkaline conditions, pH~10 and 2.0 M Na^+, the highest methane production was obtained with H_2 as substrate, indicating first, that the methanogens were still active, and second that, consistent with the type of methanogen detected, the hydrogenotrophic methanogenesis seemed to be the dominant pathway in this alkaline sediments [1] (Nolla-Ardèvol et al., 2012). These findings were in accordance to other studies from similar sediments where hydrogenotrophic methanogens were also present (Antony et al., 2012; Surakasi et al., 2007; Wani et al., 2006). The presence of methanogens from this and other genera in the studied soda lake sediments and their ability to utilize several substrates for the production of methane [1] (Nolla-Ardèvol et al., 2012) showed that this inoculum was suitable for the realization of AD at alkaline conditions.

Once an adequate inoculum had been selected, the appropriate algal substrate had to be chosen. As one of the advantages of the AD at alkaline conditions would be the possibility of coupling the process directly to an alkaline algae cultivation system, it was decided to use as substrate a microalga capable of growing at high pH conditions. Several algae such as the cyanobacteria *Spirulina* and the microalgae *Dunaliella viridis* and *Chlorella minutissima* are known to grow at such conditions

(Ballot et al., 2004; Baumgarte, 2003; Melack, 1981). Because *Spirulina* can grow at high pH (Vonshak, 1997), and because it has been identified as one of the major organisms present in the soda lakes of the Altai province (Grant, 2006; Jones et al., 1998), it was considered as the optimal substrate for the anaerobic alkaline reactor. As the goal of this study is the anaerobic digestion of the microalga and not its cultivation, freeze dried *Spirulina* was used in substitution of fresh algae. Freeze dried *Spirulina* has several advantages over the use of fresh cultured *Spirulina*; it has a constant and known composition (Cañizares-Villanueva et al., 1995), and it is available to others enabling, reproduction of this work. As shown in [3] freeze dried *Spirulina* also appeared to be a suitable substrate for the anaerobic digestion at mesophilic conditions.

4.3. Anaerobic digestion of *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+) [2, 4, 5]

A mixture of sediments originating from the Altai province (Russia) soda lake system were used to inoculate a 1.5L anaerobic reactor which was operated at alkaline conditions, pH~10, 2.0 M Na^+, and fed with freeze dried *Spirulina*. The anaerobic reactor was used in several experiments to determine the optimal HRT and the optimal OLR of the digestion of *Spirulina* [2]. A second batch of fresh sediments, from the same soda lake system, was used to operate a second anaerobic reactor at the identified optimal conditions [4] and for the determination of biomethane potential (BMP) of several substrates at alkaline conditions [5].

The work presented here shows that anaerobic digestion of the microalgae *Spirulina* at alkaline conditions (pH 10 and 2.0 M Na^+) in a semi-continuous anaerobic reactor is possible and that the obtained biogas was, as expected, rich in methane. The alkaline conditions of pH 10 and 2.0 M Na^+ were chosen to offer the highest CO_2 dissolution capacity, in order to retain the CO_2 in the liquid as much as possible, and thus to obtain a high methane content in the produced biogas.

As the production of biogas at alkaline conditions from biomass has so far not been performed, several experiments had to be conducted in order to determine the optimal hydraulic retention time and the optimal organic loading rate [2]. Once these were found, an anaerobic reactor working with the determined parameters was operated for 60 days [4].

4.3.1. Determination of the optimal hydraulic retention time [2]

As mentioned previously, one of the reported bottlenecks of biogas production from microalgae is the difficulty to completely digest the supplied substrate. A common approach to increase the biodegradability of a substrate is to increase the time of residence in the anaerobic reactor (Ras et al., 2011; Weiland, 2010). By increasing the HRT, the hydrolytic microbial consortium has more time to act on the substrate and therefore, a higher degradability is obtained. This approach has, however, a possible negative effect. Increasing the time of residence implies a reduction in the medium exchange, which in turn can lead to an accumulation of inhibitory substances. Therefore, it is necessary to find a specific residence time for which the maximum biodegradability is achieved without compromising the correct function of the process. In the AD of *Spirulina* at alkaline conditions, this best compromise between highest biodegradability and process performance was determined to be 15 days HRT with an OLR of 1.0 g *Spirulina* L^{-1} day^{-1} [2].

To determine this optimal HRT an alkaline anaerobic reactor (Alk-HRT) was set-up in which several HRT, 20, 5, 10, 30 and 15 days, were tested [2]. The selection of the different residence times, and the actual duration of each period, was adapted to the observed circumstances and to avoid reactor failure at each given time point. This selection was also conditioned by the type of substrate used, a protein rich microalga (Ortega-Calvo et al., 1993). The anaerobic digestion of *Spirulina* generates high amounts of ammonium nitrogen which, at mesophilic conditions, does not affect the biogas production, unless, a high organic loading rate is applied (Figure 1; [3]). However, at high pH, and according to the equation by Anthonisen et al., 1976, the released ammonia is present mainly in the dissociated form (NH_3), and is toxic for methanogens (Sterling Jr et al., 2001; Strik et al., 2006). This characteristic influenced the selection of the different HRT tested and it clearly affected the biogas production [2].

Initially a 20 days HRT was used as it gave a reasonable degradability at mesophilic conditions (Table 4.2), however, at alkaline conditions, an accumulation of NH_3 occurred (Figure 2a; [2]). To avoid eventual reactor failure, it was decided to drastically reduce the HRT from 20 to 5 days. With this, the NH_3 concentration was reduced and the biogas production was expected to increase. Unfortunately, a reduction in the biogas production was observed (Figure 2a; [2]). This reduction in

Table 4.2 Specific methane production and biodegradability of *Spirulina*

Specific methane production and biodegradability of *Spirulina* obtained in each period of the different alkaline and mesophilic anaerobic reactors

Reactor	Period	HRT (days)	OLR (g L^{-1} day^{-1})	mL CH_4 g VS^{-1}	BD_{CH4} (%)*	CH_4 (%)	CO_2 (%)
Alkaline Alk-HRT[a]	I	20	1.0	21 ± 6	3	79	19
	II	5	1.0	14 ± 3	2	89	10
	III	10	1.0	11 ± 4	2	81	12
	IV	30	1.0	12 ± 7	2	86	9
	V	15	1.0	31 ± 7	5	83	14
Alkaline Alk-OLR[a]	I	15	0.25	43 ± 12	7	77	5
	II	15	0.50	38 ± 5	6	80	9
	III	15	1.0	27 ± 6	4	88	3
Alkaline Alk-Sed-2[b]	I	15	0.50	47 ± 10	7	88	5
	II	15	1.0	36 ± 8	6	91	2
	Iva	15	0.50	51 ± 7	8	90	2
	IVb	15	0.50	60 ± 5	10	91	1
Alkaline Alk-Opt[b]	-	15	0.25	71 ± 15	11	86	4
Mesophilic[c]	I	20	1.0	246 ± 37	39	69	30
	IV	20	4.0	262 ± 14	42	68	31

Based on results from: a: [2]; b: Unpublished results [4]; c: [3]

* Percentage of biodegradability calculated as in Raposo et al., 2011 and based on the theoretical methane content of *Spirulina*: 627 ml CH_4 g VS^{-1}

the daily biogas production could be attributed mainly to a washout effect. The washout effect generally occurs when microorganisms are purged in excess from the anaerobic reactor medium which leads to a reduction of the biogas production (Gunnerson and Stuckey, 1986; Tartakovsky et al., 2013; Ward et al., 2014; Zhang and Noike, 1994). Reducing the HRT implies an increase in the amount of medium exchanged, in this case from 75 to 300 mL of medium exchanged daily (Table 2; [2]). To reduce the loss of microbial biomass two methods were applied: a settler was installed, through which the medium was exchanged, which retained biomass inside the reactor, and a timer was set in order to stop the stirring for at least 2 hours before the exchange time point to allow a settling of the microbial biomass [2]. However, despite this, a considerable loss of microbial biomass occurred as seen by the reduction in biogas production at low hydraulic retention times

The main explanation for this excess loss of biomass, regardless of the two mitigating procedures, is the fact that hardly any aggregates were formed in the alkaline reactor (data not shown). The formation of microbial aggregates contributes to (i) the precipitation of the microbial biomass and (ii) to the interaction between the different bacteria (Borja, 2011; Yu et al., 2001). Thus, biomass washout and low formation of aggregates resulted in a decrease of biogas production when compared

to the previous period (Figure 2a; [2]). In response to this reduction of the daily biogas production, the HRT was increased first to 10 days HRT and subsequently to 30 days. As explained previously, a risk of applying a long HRT is the possible accumulation of toxic and inhibitory substances which can lead the reactor to failure. This also occurred during the 30 days HRT period. An excessive accumulation of volatile fatty acids (VFAs), undegraded biomass and NH_3, which reached a maximum of 1,200 mg L^{-1}, caused a reactor failure (Figure 2a and Table 2; [2]). After applying measures to recover the reactor, the HRT was set to 15 days in order to reach a compromise between avoiding accumulation of substances and washout of biomass. At the set HRT, the daily biogas production was the highest achieved during this experiment, 37 ml biogas g VS^{-1}, the NH_3 was stable at around 1,000 mg L^{-1} and the biogas production was constant for 100 days (Figure 2; [2]). From the different hydraulic retention times studied and considering all the observed factors it was concluded that the optimal HRT for the anaerobic digestion at alkaline conditions of *Spirulina* with an organic loading rate of 1.0 g L^{-1} day^{-1} (dry weight), was 15 days (Table 2; [2]). At this HRT, the highest methane production, 31.3 ml CH_4 g VS^{-1}, and the highest biodegradability, 5%, of this experiment were obtained (Table 4.2).

4.3.2. Determination of the optimal organic loading rate [2]

Once the optimal hydraulic retention time was determined, it was necessary to find the optimal organic loading rate in order to obtain the highest specific methane production per gram of substrate. The alkaline anaerobic reactor, Alk-OLR, was operated at the identified optimal HRT, 15 days, and three different OLR were applied, 0.25, 0.50 and 1.0 g *Spirulina* L^{-1} day^{-1} (dry weight) [2]. Considering the results obtained with the previous experiment, accumulation of NH_3 and VFAs, it was decided to start the experiment with a low OLR in order to avoid inhibition of the reactor and to try to achieve the maximum possible bioconversion. Setting the HRT time at 15 days had a positive effect on the presence of NH_3 in the medium, which did not accumulate in any of the three different OLR tested indicating that the medium exchange rate was adequate (Table 3; [2]).

As was expected, whenever the OLR was increased, the daily biogas production also increased (Figure 1a; [2]). This increase in biogas was however, not linearly correlated with the increase in substrate added, indicating that part of the additional supplied substrate was not being converted to methane by the microbial community

and accumulated in the reactor medium. This accumulation of undegraded organic matter (COD_T and COD_S) was highly acute when the OLR was set to 1.0 g *Spirulina* L^{-1} day^{-1} and gradually led to reactor failure because of substrate overload (Figure 3; [2]). Reactor failure from substrate overload generally occurs when the microbial community is unable to completely digest the supplied substrate and toxic compounds accumulate (González-Fernández and García-Encina, 2009; Kwietniewska and Tys, 2014; Salminen and Rintala, 2002).

From the three different OLR tested, 0.25 g L^{-1} day^{-1} was the optimal one. With this OLR the highest specific methane production per gram of VS added was achieved (Table 3; [2]). Moreover, with this OLR the biodegradability of *Spirulina* was also the highest obtained so far at alkaline conditions, 7% (Table 4.2). Even though this percentage of biodegradability is much lower than what was achieved at mesophilic conditions, it is an improvement over the highest biodegradability obtained in the previous experiment (Table 4.2). This increase could be attributed mainly to the low OLR applied in combination with the 15 days HRT used which avoided both, accumulation of toxic compounds and bacterial washout (Table 3; [2]).

Parallel to this anaerobic reactor, a second reactor, Alk-Sed-2, was inoculated with a batch of fresh soda lake sediments [4]. This reactor was operated at 15 days HRT, and the OLR was initially set to 0.5 g *Spirulina* L^{-1} day^{-1} (dry weight). The main goal of this reactor was to determine if the use of fresh sediment which had not been stored for over one year and which had not experienced inhibitory conditions would increase the daily biogas production. This reactor also confirmed that apparently, at alkaline conditions, overload of the reactor occurs rapidly when the OLR is set to 1.0 g L^{-1} day^{-1} (dry weight), a threshold identical to the one observed with the Alk-OLR reactor [2]. In addition to the substrate overload, a slight ammonia inhibition was also observed (Figure 4.1b; [4]). During the operation of this second reactor, several strategies were applied to try to increase the biogas production. Of these, the use of a different micronutrients solution supplemented with vitamins led to more satisfactory results [4]. Sufficient supply of micronutrients is crucial as a lack of a certain element can inhibit both bacteria and archaea (Anderson et al., 2003; Zhang et al., 2012). In this case, changing the composition of the initial micronutrients solution and adding other micronutrients such as cobalt, nickel, and zinc, plus the addition of vitamins (D-Biotin, Folic acid, vitamin B12 and others), resulted in an

increase in the daily biogas production (Figure 4.1; [4]). The amount of substrate degraded also increased from 7 to 10% (Table 4.2). This increase in production and biodegradability could be attributed to a better performance of the microbial community due to the addition of the mentioned micronutrients and vitamins.

Setting the HRT to 15 days in both reactors, contributed to maintaining the levels of ammonia and VFAs controlled. Both reactors produced biogas continuously during 100 days before signs of reactor failure could be seen (Figure 1; [2] and Figure 4.1; [4]). This indicates that when a low OLR is combined with a 15 days HRT, the biogas production is continuous without occurrence of inhibitions.

4.3.3. Biogas production under the optimal process parameters [4]

Once the optimal hydraulic retention time and organic loading rate were identified, 15 days and 1.0 g *Spirulina* L^{-1} day^{-1} (dry weight) [2], an alkaline anaerobic reactor, Alk-Opt, was set to study the anaerobic digestion with these determined parameters [4]. With the selected optimal parameters, the biogas production was constant for a period of over 60 days (Figure 4.2; [4]). In this time, no accumulation of toxic metabolites occurred, NH_3 was kept below the inhibitory threshold, and volatile fatty acids and organic matter also remained controlled (Figure 4.2 and Table 4.3; [4]).

Even though the biogas production was constant and no accumulation of inhibitory substances occurred, the daily biogas production was considerably lower than at mesophilic conditions [3], and lower than in other studies (Table 4.1). This low biogas yield could be attributed to several factors:

(i) Substrate type: As already mentioned, the biodegradability of *Spirulina* is relatively low compared to other microalgae and other organic substrates. Moreover, the high protein content of *Spirulina*, 60 – 70% (Ortega-Calvo et al., 1993), limits the maximum organic loading rate at which to operate the reactor due to excessive release of ammonia which increases with the increase in pH and can result in reactor failure [2, 4].

(ii) Poor granule formation: granule formation is a key element for the functioning of several types of anaerobic reactors, specially the UASB reactor (Borja, 2011; Schmidt and Ahring, 1996). Granulation is the process in which suspended biomass agglutinates and forms granules due to a combination of microbial

morphology, the type of substrate used, and accumulation of inorganic salts (Anderson et al., 2003; Borja, 2011; Yu et al., 2001). The presence of granules contributes to a better reactor performance (Anderson et al., 2003), and they also help to reduce inhibition as the methanogenic populations are protected in the inner layers of the granules (Rozzi and Remigi, 2004). Moreover, studies show that biogas yields are reduced when granules are disrupted or low in abundance (van Lier et al., 2001). Several factors contribute to formation of granules. For example the presence of calcium ions, Ca^{2+}, contribute to enhance the three steps of sludge granulation, adsorption, adhesion and multiplication (van Langerak et al., 1998; Yu et al., 2001). pH is also an important factor as it influences the formation and maintenance of granules. Sandberg and Ahring, 1992 showed that increasing the pH above 8.3 resulted in a serious disintegration of granules which lead to process failure. In all the set up alkaline reactors, granules were present in low quantities or they were practically absent (data not shown). This sparse formation of granules could mainly be attributed to the absence of Ca^{2+} ions in the reactor medium and possibly to the high pH. Soda lakes from the Altai province are known to be almost completely calcium free environments (Baumgarte, 2003; Grant, 2006; Ulukanli and Rak, 2002). According to several studies, the addition of calcium to the reactor medium, in the range of 150 to 500 mg L^{-1}, could enhance granules formation (Yu et al., 2001). However, at alkaline conditions, Ca^{2+} reacts with carbonates, CO_3^{2-}, to produce $CaCO_3$ which precipitates. A similar phenomenon would occur with the addition of Mg^{2+} which at alkaline conditions precipitates in the form of $MgCO_3$ (Grant et al., 1990). Adding Ca^{2+} or Mg^{2+} could thus have a negative effect, as precipitated carbonate most likely would disturb the correct functioning of the reactor due to scaling of the reactor and scaling of biomass and reduce the activity of methanogens (Chen et al., 2008). Also, as seen in the biomethane potential of pretreated wheat straw [5], the presence of Ca^{2+} in the alkaline medium appeared to cause some unknown inhibition affecting bacteria and/or archaea, as no biogas production was observed (Figure 5.2; [5]). A possible measure for increasing granule formation would be the addition of synthetic and inert zeolites which could provide a surface for the attachment of the bacterial community.

Other results that support the low presence of granules in the alkaline reactors is the observation that the bacteria and archaea known to form and colonize the granules were practically absent in our reactor. For example, two of the main methanogens

responsible for granule formation, *Methanosarcina* and *Methanosaeta* (Díaz et al., 2006; Saiki et al., 2002; Schmidt and Ahring, 1996) could only be identified when the metagenome analysis was performed with the MG-Rast metagenome analyzer (Meyer et al., 2008), and in both cases, the number of assigned reads was extremely low. Among the bacteria that colonize the granules, the results were similar. *Syntrophobacter* and *Pelobacter*, both bacteria present in granules (Schmidt and Ahring, 1996) were almost completely absent.

(iii) Syntrophic conversions: Another factor causing the low biogas yield could be the low activity of the syntrophic bacteria associated with methanogens such as syntrophic acetate oxidizers (SAO) and syntrophic propionate oxidizers (SPO). SAO and SPO are secondary fermenters that ferment acetate, propionate and other VFAs to H_2 and CO_2 (Hattori, 2008; Lee et al., 2009; Westerholm, 2012). At mesophilic pH conditions, SAO and SPO are mainly active when acetoclastic methanogenesis is inhibited by high levels of NH_3 and high concentrations of acetate (Angelidaki and Ahring, 1993; Salminen and Rintala, 2002; Westerholm, 2012). At alkaline conditions, however, the acetoclastic pathway is practically absent (Sorokin et al., 2014) and the hydrogenotrophic pathway seems to be the most active pathway [1] (Nolla-Ardèvol et al., 2012). Therefore, at alkaline conditions an active syntrophic fermentative community is essential for the oxidation of acetate and propionic acid and the supply of H_2 and CO_2 for the hydrogenotrophic methanogens. However, relatively high levels of acetate, propionic acid and other VFAs, were detected in all alkaline reactors (Tables 2, 3 [2] and Table 4.2 [4]). Even at the optimal process conditions, the levels of acetic acid were relatively high ~ 1.5 g L^{-1} (Figure 4.2; [4] and Table 4.3; [4]).

Interestingly, in the metagenome analysis of the alkaline reactor only one hit was identified belonging to the enzyme formyltetrahydrofolate synthethase, which is a key enzyme used by syntrophic acetate oxidizing bacteria to oxidize acetate to H_2 and CO_2 through the Wood–Ljungdahl pathway (Müller et al., 2013). The absence of this key enzyme correlates with the extremely low abundance of reads assigned to SAO bacteria. For example, *Clostridium ultunense,* a syntrophic acetate oxidizing bacterium (Schnürer et al., 1996) and other SAO such as *Tepidanaerobacter* and *Syntrophaceticus* (Westerholm et al., 2010; Westerholm et al., 2011), both members of the order Thermoanaerobacterales, were not detected. Moreover, the order

Thermoanaerobacterales recruited less than 800 reads. Other possible SAO such as *Geobacter* and *Anaeromyxobacter* (Westerholm, 2012) recruited less than 500 reads. This lack of SAO correlates perfectly with the relatively high concentration of acetic acid present in the alkaline anaerobic reactor [2, 4].

An additional factor affecting the activity of the syntrophic bacteria is the low abundance of granules, as these provide the microenvironment were the syntrophic relationships between SAO / SPO and methanogens take place (Nelson et al., 2012). Thus, the inhibition or low activity of the syntrophic fermenters causes a reduction in the availability of substrates for the hydrogenotrophic methanogens resulting in a low biogas production despite the abundant substrate.

In summary, the type of substrate used, the degradation of which causes the release of NH_3, the scarce presence of aggregates, which favor biomass washout, the possible inhibition of syntrophic fermenters, deduced from the relatively high levels of acetate and of undegraded organic matter (Table 4.3, [4]), most likely caused a so called “inhibited-steady state” of the reactor, characterized by a stable but overall low biogas production (Angelidaki and Ahring, 1994; Astals et al., 2012).

Despite the low biogas production, it is worth noting that the percentage of bioconversion of *Spirulina* increased with each process improvement, from the initial 3% in the Alk-OLR reactor, to 11% in the Alk-Opt reactor (Table 4.2). Likewise, the methane yield also improved reaching 71 ml CH_4 g VS^{-1} in the Alk-Opt, a value similar to that obtained with the alkaline batch tests (Table 4.3). This increase in bioconversion and yield suggest that, with the adequate substrate and reactor set-up, enhanced biogas production could be achieved.

4.3.4. Alkaline digestion of other substrates [5]

Biomethane potential tests (BMP), are a fast and inexpensive approach to determine the methane content of a particular substrate and can also provide details about the biodegradability of a given substrate (Angelidaki et al., 2009; Raposo et al., 2011). Biomethane potential tests have been widely used to assess the potential methane production of algae, *Spirulina*, and other substrates at mesophilic pH conditions (Bourque et al., 2008; El-Mashad, 2013; Mussgnug et al., 2010; Pobeheim et al., 2010; Zamalloa et al., 2012), but have not been used to study the potential methane production of complex substrates at alkaline conditions. Here, BMPs were used to

assess the potential methane production of *Spirulina*, fresh algal biomass and wheat straw at alkaline conditions.

BMP performed with fresh algal biomass have shown that selecting the appropriate type of microalgae, for example with lower protein content, is a crucial step in order to obtain high methane yields. Biogas production from fresh microalgae reached 222 mL biogas g VS^{-1} compared to the 85 mL biogas g VS^{-1} obtained with *Spirulina* as substrate (Table 5.2; [5]). This indicates that running an anaerobic alkaline digester with a more suitable microalgae as substrate, could avoid some of the problems associated with the use of *Spirulina*, mainly the accumulation of NH_3, favoring the biogas production and making this approach a more competitive option.

Wheat straw wastes represent a potential energy resource if they can be properly converted into energy through anaerobic digestion. However, due to its composition, a mixture of lignocellulosic matrix, the anaerobic biodegradability of wheat straw is limited (Motte et al., 2014). To increase the biodegradability several approaches have been proposed, among them the chemical alkaline pretreatment (Song et al., 2014; Zahoor and Tu, 2014). Performing the anaerobic digestion of wheat straw at alkaline conditions could thus increase the biogas production as the alkaline medium would act as a pretreatment.

BMP tests with wheat straw showed that performing anaerobic digestion of this substrate at alkaline conditions was possible and the methane yield obtained, as well as the biodegradability of the substrate were higher than those obtained at mesophilic conditions. Biogas production and biodegradability of wheat straw reached 282 mL biogas g VS^{-1} and 63% of conversion respectively, values comparable to those obtained with microcrystalline cellulose (Table 4.3). The results confirm that the alkaline medium acts as a pretreatment, increasing the biodegradability of the wheat straw. This opens the possibility of performing direct anaerobic digestion of wheat straw, and similar lignocellulosic substrates, in a single reactor, without the need of having to perform pretreatment and digestion in two steps. Generally, the use of pretreatment steps requires energy and/or chemicals which increase the overall production costs and limit the economic feasibility. For substrates which currently require alkaline pretreatments, the possibility of performing the digestion in one single step can thus contribute to a drastic reduction of the processing costs.

Table 4.3 Biomethane potential tests
Biogas production, methane content and biodegradability of different substrates at alkaline and mesophilic conditions performed in batch test

Substrate	Conditions	Yield (mL g VS^{-1})	CH_4 (%)	BD_{CH4} (%)[a]	Reference
Spirulina	Alkaline	85 BG*	92	12	Unpublished results [5]
Fresh algal biomass	Alkaline	223 BG	94	33	Unpublished results [5]
Microcrystalline cellulose (Avicel)	Alkaline	288 BG	90	62	Unpublished results [5]
Wheat straw	Alkaline	282 BG	93	63	Unpublished results [5]
Spirulina	Mesophilic	481 BG	61	83	Mussgnug et al., 2010
D. salina	Mesophilic	505 BG	64	93	Mussgung et al., 2010
C. vulgaris	Mesophilic	369 BG	53	-	Prajapati et al., 2014
Wheat straw	Mesophilic	104 CH_4	-	24¥	Demirbas, 2006
Wheat (Nodes)	Mesophilic	141 CH_4	-	33¥	Motte et al., 2014
Wheat (rachis)	Mesophilic	180 CH_4	-	42¥	Motte et al., 2014
Wheat (Internodes)	Mesophilic	148 CH_4	-	35¥	Motte et al., 2014
Wheat (leaves)	Mesophilic	242 CH_4	-	57¥	Motte et al., 2014

a: Percentage of biodegradability calculated as in Raposo et al., 2011
* BG: Biogas
¥ Calculated using wheat straw theoretical methane content: 426 ml g VS^{-1}

4.3.5. Biogas rich in methane [2, 4, 5]

The main objective of this PhD project was to evaluate the possibility to produce biogas at alkaline conditions as this can have several advantages over the traditional mesophilic systems. The main advantage of the alkaline conditions is that the alkaline medium retains the produced CO_2 as bicarbonate, leading to biogas rich in methane. In all performed experiments, and with all the different substrates tested, this was confirmed experimentally, and the obtained biogas was extremely rich in methane, between 77 and 91% (Table 4.2) [2, 4, 5]. At specific time points, the methane content reached 96% and the CO_2 content was practically zero (Figure 1a; [2] and Figure 4.2; [4]). In addition, the alkaline medium also prevented the release of H_2S which was not detected in the biogas of any of the different alkaline reactors [2, 4, 5]. The absence of H_2S is significant as it is one of the major impurities present in currently produced biogas which causes odors and corrosion (Pavlostathis, 2011).

Biomethane is the term used for the biogas which has been upgraded to increase the percentage of methane by removing CO_2 and impurities, and can be used in the national gas grid or as fuel for vehicles (Persson et al., 2006; Weiland, 2010). With the biogas obtained at alkaline conditions, this upgrade would be unnecessary, or minimized, as the gas meets the minimum methane content required in most of the national gas grid systems. For example, in Germany the minimum required content of

methane in biomethane is 96%, in Norway 95% and in The Netherlands 88% (Persson et al., 2006). Thus, the absence of H_2S and the low percentage of CO_2, would allow the reduction of downstream processing costs, making the production of biogas at these conditions an alternative to traditional biogas producing systems.

4.4. Metagenomics of the anaerobic digestion process [3, 6]

Metagenomics has become a common way to study taxonomy, gene composition and gene function in microbial communities (Simon and Daniel, 2011). A good understanding of the taxonomic composition and the functional interactions between the involved microbial populations, can contribute to the optimization of the anaerobic digestion of the desired substrate.

Two main approaches were used for the taxonomic analysis of the microbial communities present in the mesophilic and in the alkaline anaerobic reactors. In the first approach, sequenced reads were assembled into contigs and subsequently binned, in order to obtain provisional whole genome sequences of abundant community members (Strous et al., 2012). Since tetranucleotide frequencies are relatively conserved between taxa, it is possible to use them for classification purposes. This characteristic, makes binning based classifiers better suited to deal with sequences originating from unknown species (Logares et al., 2012)**,** in contrast to similarity based classification of reads which yields poor results for populations without a nearby reference genome in the databases. Moreover, provisional whole genome sequences allow the inference of an ecological function for each major community member (e.g. biomass hydrolysis, fermentation, methanogenesis etc.). The binning analysis was done with both, the mesophilic pH metagenome [3] and the alkaline metagenome [6].

The second strategy, which was only applied for the mesophilic metagenome, comprised the classification of single reads using the M5NR tool of the MG-Rast metagenome analyzer (Meyer et al., 2008). This approach was used for the comparison between the mesophilic pH metagenome and a publicly available metagenome from a biogas plant (Jaenicke et al., 2011) [3]. In this case it was decided to use the MG-Rast approach because the taxonomic analysis in the original work from Jaenicke et al, 2011 was done based on the classification of reads and not on the classification of contigs.

On the other hand, the functional analysis of the two metagenomes was done with the classification of single reads using several tools of the MG-Rast metagenome analyzer combined with a search for specific protein domains (Pfams).

4.4.1. Characteristic taxonomic structure of anaerobic digester microbial communities [3, 6]

So far, a number of metagenomic studies from several biogas producing plants and lab scale anaerobic reactors treating a variety of different substrates have been performed (Jaenicke et al., 2011; Krause et al., 2008; Li et al., 2013; Schlüter et al., 2008; Sundberg et al., 2013; Wirth et al., 2012). This is, however, the first study of a metagenome derived from the anaerobic digestion of *Spirulina* at mesophilic pH conditions [3].

On the other hand, to date, only one known work involving a metagenomic approach to study the anaerobic microbial community of a biogas reactor at high pH was published by Wong et al., 2013. In this study however, the high pH was only applied as a pretreatment and the anaerobic reactor was operated at neutral pH during 30 days before sampling for DNA, therefore it cannot be considered as an example of the analysis of a truly haloalkaline microbial community. So far the microbiology of this process at alkaline conditions has only been addressed by van Leerdam et al., 2008b who used DDGE to characterize the archaeal community. On the other hand, several studies have already analyzed the microbial composition of soda lakes and related habitats (Kanekar et al., 2008; Lanzén et al., 2013; Ochsenreiter et al., 2002; Rees et al., 2004). The work presented here is therefore the first metagenome analysis of a haloalkaline microbial community that has been adapted to produce biogas in a closed and controlled process [6].

The taxonomic analysis of both metagenomes showed that despite the origin of the inoculum and despite the environmental and process conditions, the microbial community structure of the anaerobic digestion process is remarkably similar at higher taxonomic levels, with Bacteria clearly dominating over Archaea [3, 6]. Furthermore, in both cases the same abundant phyla were detected, Firmicutes, Bacteroides, Thermotogae and Euryarchaeota. At lower taxonomic levels, different representatives of these phyla were dominant, with a clear presence of extremophilic genera detected in the alkaline bioreactor.

At mesophilic conditions, the microbial composition is similar to that obtained in other anaerobic reactors (Jaenicke et al., 2011; Li et al., 2013; Schlüter et al., 2008; Wirth et al., 2012) with the dominance of Firmicutes, Bacteroides and Thermotogae. Moreover, and as in a previous study of anaerobic digestion of a proteinaceous substrate (Kovács et al., 2013), Clostridiales were the most abundant bacteria (Table 3; [3]). Among the archaea, Methanomicrobiales and Methanosarcinales were detected as the dominant methanogens (Suppl. Table 1; [3]), as previously observed in other anaerobic reactors (Li et al., 2013; Liu et al., 2009; Ziganshin et al., 2013).

At alkaline conditions, as expected, and due to the origin of the inoculum, a relatively high percentage (~40%) of the microbial population was unknown and, most of the identified bacteria and archaea were closely related to known halotolerant and/or alkaliphilic microorganisms. The high abundance of unidentified taxa in the population was probably due to the low number of sequenced genomes and 16S rDNA genes belonging to halophiles and alkaliphiles present in the nucleotide databases.

The composition of the microbial community of the alkaline process does not differ much from the already known composition of other anaerobic mesophilic digesters; Bacteria clearly dominate over Archaea (Table 6.3; [6]). At a glance, the major difference between the alkaline anaerobic community and that found in a mesophilic pH reactor was the fact that Bacteroidetes and Firmicutes appeard to be the co-dominant hydrolytic bacteria in contrast to the mesophilic environments where Firmicutes clearly dominate [3]. At a closer look, more differences could be observed with respect to the mesophilic microbial population. In the alkaline reactor, members of the Cytophaga-Flavobacteria-Bacteroidetes group (CFB), classified as “ML635J-40 aquatic group” clearly dominate, representing over 27% of the total abundance (Table 6.3; [6]). This group has been previously identified in other soda lakes (Baumgarte, 2003; Grant, 2006; Humayoun et al., 2003) which indicates that it is probably one of the most abundant and wide spread haloalkaline bacterial groups. Unfortunately, no full characterization of members of the ML635J-40 aquatic group exists. The closest relatives are other members of the CFB and only a few members of this group have been fully characterized (Denger et al., 2002; de la Haba et al., 2011; Zhilina et al., 2004). Halanaerobiales and Natranaerobiales, which are moderate halophilic Clostridia able to ferment multiple substrates, from glucose to

amino acids, and have been detected in several soda lakes (Brown et al., 2011; Ivanova et al., 2011; Mesbah and Wiegel, 2009; Zhao et al., 2011), were also among the most abundant bacteria present in the alkaline metagenome.

In the alkaline reactor the production of methane was carried out mainly by members of the Methanomicrobiales order and especially the genus *Methanocalculus* (Table 6.3 and Figure 6.4; [6]). This genus contains the, to date, only known strict alkaliphilic methanogen, *Methanocalculus natronophilus,* isolated from a soda lake from the same lake system as our inoculum (Zhilina et al., 2013). *Methanocalculus* was one of the methanogens detected, by means of the marker gene *mcrA*, in the sediments that served as inoculum for one of the alkaline reactors [1] (Nolla-Ardèvol et al., 2012). This might indicate that *Methanocalculus* was able to adapt to the process conditions, pH 10 and 2.0 M Na^+, and outcompete the other methanogens present in the sediment [1] (Nolla-Ardèvol et al., 2012). *Methanocalculus* is a hydrogenotrophic methanogen which is unable to use acetate as substrate for methanogenesis (Ollivier et al., 1997; Zhilina et al., 2013). Therefore, the detection of *Methanocalculus* as the main methanogen and the low abundance or probably even absence of syntrophic acetate oxidizers, would explain the relatively high levels of acetate present in the alkaline reactors [2, 4].

These results presented here suggest that, the microbiology of the anaerobic process is constant throughout the different environments and conditions. In the alkaline metagenome, the observed differences are mainly attributed to the capacity of the identified bacteria to thrive in such an extreme environment.

4.4.2. Identification of specific functional characteristics in the alkaline metagenome [6]

The classification of single short reads through the Subsystems hierarchical classifier of the MG-Rast metagenome analyzer showed that, as expected, and as already seen in other functional analyses of anaerobic microbial communities (Li et al., 2013; Schlüter et al., 2008; Wirth et al., 2012), the functions that received the highest number of hits, were those related to basic metabolic processes, energy production and housekeeping genes which are indicative of the presence of an active microbial community (Figure 6.5; [6]).

It is not a surprise that, as most of the identified bacteria in the alkaline anaerobic reactor are known halophiles and alkaliphiles, multiple enzymes involved in the adaptation strategies of the halotolerant and haloalkaline bacteria present at these extreme conditions were identified among the sequenced reads [6]. In this group of genes it is important to highlight the detection of several Na^+, K^+ and Cl^- porters and antiporters which are commonly used to allow flow of these ions through the bacterial membrane in order to maintain the intracellular pH at mesophilic conditions, one of the two strategies of halophiles and alkaliphiles to cope with the osmotic pressure (Kivistö and Karp, 2011; Mesbah et al., 2007b; Ulukanli and Rak, 2002)..The accumulation of organic compounds and compatible solutes which function as osmoprotectants is another strategy applied by halotolerant bacteria to cope with high salt concentrations (Ma et al., 2010; Mesbah and Wiegel, 2012; Ventosa, 2006). Enzymes related to the use of osmoprotectants like ectoins, betaines and choline were also detected in the metagenome.

The functional analysis with the Subsystems classification of reads in combination with the identification of specific protein domains (Pfam) showed that the hydrogenotrophic methanogenic pathway recruited more reads than the acetotrophic pathway [6]. These results suggest that the methanogens present in the alkaline reactor produce CH_4 preferably via the reduction of CO_2 in the presence of H_2, which is in accordance with the relatively higher abundance of hydrogenotrophic methanogens among the detected archaea [6]. These findings corroborate previous reports that indicate that in alkaline lakes one of predominant methanogenic pathways is the hydrogenotrophic pathway and that the acetoclastic pathway is practically absent (Sorokin et al., 2014)

4.4.3. Comparison of metagenomes derived from anaerobic digesters fed with either protein rich or cellulose rich substrates [3]

The availability of public metagenomes can be of interest as it allows researchers to directly compare two or more habitats or environments. In this project, a publicly available metagenome from a biogas plant treating agroindustrial waste (Jaenicke et al., 2011) was downloaded and compared, using the MG-Rast metagenome analyzer (Meyer et al., 2008), with the metagenome of the mesophilic reactor digesting *Spirulina*. With this, it was possible to compare two different anaerobic microbial communities, one responsible for the degradation of mainly cellulosic material and

the second one responsible for the decomposition of a protein rich substrate [3]. This direct comparison also allows us to determine the effect of substrate on the composition and function of the anaerobic microbial community.

Comparing both metagenomes revealed minor differences with regards to the general microbial composition as bacteria clearly dominate over archaea [3]. Differences could be observed among the bacteria abundance at low taxonomic ranks, genus and species, and could be related to the type of substrate degraded. For example, most of the abundant bacteria in the *Spirulina* reactor were the least abundant in the cellulose rich reactor and vice-versa. In the reactor fed with *Spirulina*, a protein rich substrate, *Alkaliphilus,* which is known to be able to use or require amino acids for growth and some of its members are unable to degrade complex sugars such as cellobiose and xylose (Farrow et al., 1995; Lien et al., 1998) recruited over 6% of the reads while it only recruited 2% in the cellulose rich metagenome (Suppl. Table 3; [3]). Furthermore, taxa recruiting most reads in the metagenome from the biogas plant, *Candidatus Cloacamonas* and *Clostridium,* which are known to be able to degrade cellulosic material (Kovács et al., 2013; Tachaapaikoon et al., 2012) recruited less reads in the *Spirulina* metagenome (Suppl. Table 3; [3]). In accordance to these findings, both metagenomes contained genes which encode enzymes for the degradation of the specific type of substrate. In this sense, in the metagenome from the *Spirulina* reactor, a higher number of genes related to the degradation of amino acids, peptides and proteins could be detected compared to those genes related to the degradation of cellulose. The opposite was seen for the metagenome from the biogas plant (Figure 4; Table 4; [3]). A similar result was obtained with the alkaline metagenome. A higher number of genes and Pfams were detected that could be related to the degradation of proteinaceous substrates in contrast to the number of genes related to the degradation of cellulose rich substrates (Table 6.5; [6]).

Major differences between the two studied metagenomes could be seen among the Archaea. *Methanoculleus* and *Methanosarcina* dominated in the *Spirulina* reactor in contrast to the clear dominance of *Methanoculleus* in the biogas plant (Figure 3; [3]). *Methanoculleus* is a hydrogenotrophic methanogen while *Methanosarcina* is one of the methanogens with a broader range of substrates, from acetate, H_2-CO_2 to methyl compounds (Garcia et al., 2000). As methanogens are responsible for the last step in

the anaerobic digestion process, the formation of methane, they are not dependent on the initial type of substrate degraded by the hydrolytic bacteria, but they are affected by the environmental factors inside the reactor. These factors are mainly presence or absence of particular inhibitory substances such as ammonia, or excess acetate (Chen et al., 2008). Possibly, acetoclastic methanogens are more sensitive to the presence of high concentrations of ammonia and VFAs (Angelidaki and Ahring, 1993; Calli et al., 2005). Thus, the dominance of a hydrogenotrophic methanogen, *Methanoculleus*, and a methanogen capable of using multiple substrates, *Methansarcina*, in the *Spirulina* reactor was attributed to a relatively high level of NH_3 and VFAs resulting from the hydrolysis of *Spirulina* biomass (Figure 1; [3]).

In literature, the explanations of the observed differences in the archaeal composition are partially contradictory. One the on hand the environmental conditions in the reactor such as relatively high levels of VFAs and ammonia, favor the dominance of a certain type of methanogens (Angelidaki and Ahring, 1993; Calli et al., 2005). On the other hand, the work by Ziganshin et al., 2013 showed that in some cases, the methanogenic community seemed to be more influenced by the origin of the inoculum than by the environmental conditions in the reactors. In addition, the experiments carried out by Pholchan et al., 2013 showed that the environmental conditions in a reactor are linked to an increase in the diversity of the microbial community. In this case, a combination of both factors, origin of the inoculum and environmental conditions seem to be the most plausible explanation for the presence of two abundant methanogens.

The microbial diversity of both metagenomes was also addressed in order to determine if the digestion of a mono-substrate (*Spirulina*) resulted in a less diverse microbial community when compared to the digestion of a mixture of substrates (maize, green rye and chicken manure) [3]. In accordance to what was observed in the taxonomic analysis, no significant differences could be seen in the bacterial diversity, despite the type of substrate used. This is similar to the results obtained by Pholchan et al., 2013. They demonstrated that the use of a complex substrate does not always imply an increase in microbial richness. In contrast, the diversity of the methanogens was increased in the *Spirulina* reactor in comparison to the biogas plant. This increase in diversity could be attributed to the co-dominance of two

methanogens in the *Spirulina* reactor vs the dominance of a single methanogen in the biogas plant [3].

4.4.4. Can metagenomics facilitate the optimization of the anaerobic digestion process? [3, 6]

Metagenomics is a useful way to study complex microbial communities. With metagenomics, it is possible to answer the question Who is there? and to some extend estimate the relative abundance of the different members of the studied microbial community. Moreover, metagenomics can also provide a first insight into the different functions that are present in the microbial community (Chistoserdova, 2014; Kunin et al., 2008; Thomas et al., 2012).

In the different fields of metagenomics, such as environmental metagenomics (Tringe and Rubin, 2005), medical metagenomics (Virgin and Todd, 2011) and metagenomics applied to industrial processes (Lorenz and Eck, 2005) the same issues are addressed, namely, the identification of the different organisms present, their possible functions and their relationships and interactions. Especially in the medical and industrial process, the general idea is that the information obtained with metagenomics could be used to understand the interactions between the different organism in order to develop new therapeutics or to enhance the studied process (Lorenz and Eck, 2005; Virgin and Todd, 2011). However, to date, one has the impression that so far, the information given by the analysis of a metagenome is more related to the biological aspects. In this sense, despite all the information that can be obtained with a metagenomics approach, in most of the cases, it seems that this information only contributes to increase basic knowledge related to general microbiology and ecology such as which organisms are present and what are their possible functions (Chistoserdova, 2014). The information obtained after analyzing a metagenome has yet to be used to improve an industrial process.

In this sense, the results presented here with regard to the taxonomic analysis are interesting in the perspective of general microbiology as they point to the direction that the microbial communities responsible for the anaerobic digestion of organic matter are relatively stable and similar in their general composition, independent of the substrate used, protein rich or cellulose rich, and independent of the type of inoculum used, mesophilic or alkaline [3, 6]. However, as the information obtained is

only from a specific time point, it is difficult to say if the taxonomic information gained could be used to improve the anaerobic digestion of *Spirulina*. In this direction, and in relation to the production of biogas at alkaline conditions, it would be interesting for future projects to explore options to actively increase the number of syntrophic acetate oxidizing bacteria as their current low abundance seems to be linked to an overall low biogas production [2, 4].

The functional information obtained with metagenomics is just a snapshot which indicates the functional potential of the microbial community, but does not reflect the functional activity at the time of sampling (Moran, 2009; Urich et al., 2008). The functional analysis of both, the mesophilic and the alkaline metagenomes pointed to the direction of a higher abundance of genes related to the degradation of proteins in contrast to the relatively low abundance of genes involved in the degradation of cellulosic material [3, 6]. However, as metagenomics does not give information about the activity of the different functions (Moran, 2009), these results have to be interpreted with caution. More experiments should be carried out in order to deeply analyze the impact of a certain substrate in the composition of the microbial community and their functions. In this sense, it would be interesting to perform studies where DNA samples are obtained from several time points from two or more reactors treating distinct substrates.

A solution to understand which functions are actually performed at a given time point would be the application of metatranscriptomics, a technique similar to metagenomics but in which the mRNAs are sequenced and analyzed (Moran, 2009; Urich et al., 2008). As mRNAs from a specific gene are only produced when it is expressed, the identification and quantification of mRNAs gives information about which functional genes are active and which are not active (Gilbert et al., 2008; Urich et al., 2008).

In this sense, the combination of the binning approach, where provisional whole genome sequences of the abundant community members are generated, with the mapping of transcriptome reads to these provisional genomes, would give firsthand information about the actual function of each of the most abundant organisms present in the reactors (Chistoserdova, 2014). In this direction, a recent work by Yu and Zhang, 2012 already addressed this possibility by analyzing the metagenome and the metatranscriptome of the microbial community present in an active sludge.

The combination of metagenomics and metatranscriptomics applied to the anaerobic digestion process could provide a more comprehensive understanding of the functions and interactions of the different anaerobic bacteria and archaea, and the obtained information could be used to enhance the process.

4.5. Conclusions

In this work, the anaerobic digestion of *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+) has been demonstrated to be feasible and the obtained biogas was rich in methane, with up to 96%. 15 days HRT and 0.25 g *Spirulina* L^{-1} day^{-1}were identified as the optimal process conditions for which the highest biogas yield was obtained, 84 mL biogas g VS^{-1}. Several bottlenecks have been identified which were responsible for the relatively low biogas production: (i) the type of substrate used, rich in proteins, which resulted in excessive release of ammonia, (ii) poor granule formation due to possible lack of calcium, and (iii) low activity of the syntrophic acetate oxidizers which resulted in accumulation of volatile fatty acids.

The anaerobic digestion of wheat straw at alkaline conditions was satisfactory and opens the possibility of performing the biometanization of lignocellulose rich substrates in a single step.

The taxonomic analysis of both metagenomes showed that despite the origin of the inoculum and despite the environmental and process conditions, the microbial community structure of the anaerobic digestion process is remarkably similar at higher taxonomic levels, with bacteria clearly dominating over archaea. Furthermore, in both cases the same abundant phyla were detected, Firmicutes, Bacteroides, Thermotogae and Euryarchaeota. Differences in the microbial composition between the mesophilic and the alkaline reactors were related to the ability of the bacteria identified in the alkaline system to withstand the haloalkaline conditions. The methane production in the alkaline reactor was mainly performed by *Methanocalculus*, a hydrogenotrophic methanogen, while evidence for acetoclastic methanogenesis was practically absent.

5. Perspective

In this work the anaerobic digestion of *Spirulina* biomass and other substrates has been shown to be feasible at alkaline conditions (pH 10; 2.0 M Na^+) [2, 4, 5]. Running the process at the optimal organic loading rate and hydraulic retention time has resulted in the constant production of biogas rich in methane. However, the biogas production was low when compared to a mesophilic pH approach. Several factors have been identified to which the low biogas production could be attributed to, mainly the type of substrate used and the excessive washout of the active biomass. Several actions could be taken in the future in order to overcome these limitations. As has been shown, the use of substrates with low nitrogen content such as microcrystalline cellulose and wheat straw resulted in higher biogas productions. Likewise, the use of fresh algal biomass produced relatively high amounts of biogas. This suggests that with the appropriate substrate the production of biogas could be drastically increased. To avoid washout of the active biomass, a modified reactor configuration could be applied. The use of anaerobic membrane bioreactors could be suitable as it would solve two problems; reducing washout by applying long solid retention times and reducing the accumulation of inhibitory compounds by applying short liquid retention times (Watanabe et al., 2014; Zamalloa et al., 2011). Another approach to reduce the washout could be the addition of zeolites or microcarriers. These would facilitate the attachment and development of syntrophic interaction of the active biomass and would avoid the washout effect (Chauhan and Ogram, 2005; Milán et al., 2001). Other options to increase the biomass retention time would involve the addition of particles, metal ions or similar, to enhance the granule formation (Phalakornkule and Khemkhao, 2012; Yu et al., 1998). With this potential of optimization in mind, anaerobic digestion at alkaline conditions can be a promising alternative process for the production of biomethane for commercial use.

The presented results also open the possibility of producing biogas from wheat straw and similar lignocellulosic substrates in a single pot reaction. As shown, the alkaline medium acts as a substituted for a pre-treatment step which contributes to a more efficient hydrolysis of cellulosic substrates. The possibility of using lignocellulosic material as substrate for biogas without the need of applying a pre-treatment step could drastically reduce the production costs and increase the economic viability of the anaerobic digestion process.

In addition, the anaerobic digestion at alkaline conditions could also be beneficial for the treatment of wastewaters that today are difficult to process. For example, waste waters from the fishery industry which contain high levels of Na^+ could possibly be used as substrate (Chowdhury et al., 2010; Sandberg and Ahring, 1992). Alkaline wastewaters such as waste streams from brewery industries, concentrated sugar wastewaters and leather tannery wastewaters among many others, could also be an optional substrate (Lofrano et al., 2013; Rosenwinkel et al., 2005).

For all the above mentioned possible applications, the anaerobic digestion at alkaline conditions could soon be an interesting technology for the revalorization of biomass or wastewaters.

6. Published and submitted results

[1] Nolla-Ardèvol V, Strous M, Sorokin DY, Merkel AY, Tegetmeyer HE. 2012. Activity and diversity of haloalkaliphilic methanogens in Central Asian soda lakes. J. Biotechnol. 161:167–173. (doi:10.1016/j.jbiotec.2012.04.003)

[2] Nolla-Ardèvol V, Strous M, Tegetmeyer HE. Anaerobic digestion of the microalga *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+). (Submitted).

[3] Nolla-Ardèvol V, Peces M, Strous M, Tegetmeyer HE. Metagenome analysis and biogas production from the anaerobic digestion of the protein rich microalga *Spirulina.* (Submitted).

[1] Activity and diversity of haloalkaliphilic methanogens in Central Asian soda lakes

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Abstract

Methanogens are of biotechnological interest because of their importance in biogas production. Here we investigate the suitability of sediments from Central Asian soda lakes as inoculum for high pH methane-producing bioreactors. Methane production in these sediments was modest (up to 2.5 µmol/mL sediment), with methanol and hydrogen as the preferred substrates. The responsible methanogenic community was characterized based on *mcrA* gene sequences. *McrA* gene sequences so far specific to these habitats indicated the presence of two clusters within the orders *Methanobacteriales* and *Methanomicrobiales*, one apparently including representatives of the genus *Methanocalculus* and another distantly related to the genus *Methanobacterium*.

Introduction

Methanogenic microorganisms are of great interest for environmental biotechnology because of their importance in the anaerobic treatment of industrial wastewaters and the digestion of domestic (bio)waste and wastewater sludge (EC reports on renewable energy, Nayono et al., 2010, Sabra et al., 2010, Tabatabei et al., 2010, van Leerdam et al., 2006). The possibility to produce methane from waste streams is all the more interesting because the produced "biogas" provides a renewable replacement for natural gas. Biogas is already produced commercially at large scale but can never completely replace natural gas because of the relatively small carbon flows in the waste streams compared to the amount of gas necessary for current consumption. For this reason, the supply of renewable energy by the combination of biomass production and its subsequent digestion into methane is being actively explored, for example via the cultivation of microalgae (Mussgnug et al., 2010). Algal biotechnology does not depend on arable land and offers the possibility to effectively recycle nutrients (nitrogen, phosphorous), but it suffers from high process costs.

The supply of sufficient carbon dioxide to the algae is an important bottleneck for the process and contributes to these costs (Norsker et al., 2011, Wijffels, 2008). If it was possible to grow algae at high alkalinity and also digest them into methane under these conditions, this bottleneck may be overcome. Not only would carbon dioxide absorption be more effective, but the carbon dioxide produced during digestion would also mainly stay in solution and not become part of the biogas. This way, carbon dioxide could easily be recycled to the phototrophs in a closed process. For this

process, an inoculum for an anaerobic reactor that contains a methanogenic microbial community adapted to high pH values and high salt concentrations is required. Since pH tolerance of methanogenic species used in conventional biogas plants is restricted (Weiland, 2010), alkaline soda lakes may provide such an inoculum. These extreme environments are characterized by high carbonate alkalinity and high pH and have considerable biotechnological potential (e.g.: Horikoshi, 1999, Margesin and Schinner, 2001, Kanekar et al., 2008, Sorokin et al., 2011a). For example, a high pH bioreactor was previously developed that converts methanethiol into methane (van Leerdam et al., 2008).

Soda lakes are habitats of microbial communities with high primary productivity (Jones et al., 1998, Sorokin et al., 2004, Zhilina and Zavarzin, 1994), especially during the wet season when massive algal/cyanobacterial blooms occur (Grant, 2006, Jones et al., 1998, Melack and Kilham, 1974, Zhilina and Zavarzin, 1994). Methanogenesis occurs as one of the terminal processes during decomposition of organic matter in the anoxic sediments of soda lakes. Several (halo)alkaliphilic methanogenic archaea have been isolated or identified *in situ* based on their 16S rRNA genes (Boone 2002, Liu et al., 1990, Mathrani et al., 1988, Mesbah et al., 2007, Surakasi et al., 2007, Thakker and Ranade, 2002, Wani et al., 2006, Worakit et al., 1986, Zhilina and Zavarzin, 1994). The species which have been isolated belong to the genera *Methanobacterium*, *Methanocalculus*, *Methanoculleus*, *Methanolobus*, *Methanosalsum* and *Methanosarcina*. As is often the case, only few of the 16S rRNA sequences detected *in situ* correspond to any of the species isolated or enriched so far (Antony et al., 2012).

The present study investigates the potential of sediments from the Central Asian hypersaline soda lakes to serve as inoculum for alkaliphilic methanogenic bioreactors for the anaerobic digestion of micro-algae. We combine direct measurements of methane production from different substrates with the molecular characterization of the methanogenic populations via the marker gene *mcrA*, encoding the alpha subunit of the methyl coenzyme M reductase. This enzyme catalyzes the final step in methanogenesis (Friedrich, 2005). Topologies of *mcrA* and 16S rRNA phylogenetic trees are largely congruent (Hallam et al., 2003, Luton et al., 2002, Springer et al., 1995). Although *mcrA* targeted PCR has been used for the detection of methanogens in a number of environmental studies (see, for example, Narihiro and Sekiguchi,

2011, and references therein), *mcrA* sequences have only been once amplified directly from an alkaline lake to date (Antony et al., 2012), and thus not much of the *mcrA in situ* diversity in alkaline habitats is known.

Materials and Methods

Sampling: Sediments were collected from several hypersaline soda lakes of the Kulunda steppe in the Altai province of Russia (Sorokin et al., 2010). After collection they were pooled and stored at 4°C in the dark in an air tight glass container with argon in the gas phase.

Methane production: Incubations were performed in 6 mL vials as follows: First 3 mL of anoxic soda buffer (pH 9.5-10, salt concentration 0.6-2 M, see Table 1), 60 µL of substrate (methanol or acetate, final concentration 5 mM) and 60 µL Na_2S (final concentration 0.5 mM) were transferred into each vial. Then, 0.8-1.5 g sediment (wet weight) was added (density of the sediments: 1.148 g mL^{-1}). Finally, the vials were completely filled with the corresponding anoxic buffer solution and 1.5 mL of the buffer were replaced with 1.5 mL He. For vials with H_2 as substrate the procedure was the same but without adding methanol or acetate, and 1.5 mL of buffer were replaced with 1.5 mL H_2. Vials were incubated for 10 days in the dark at room temperature. Methane concentration in the headspace was measured with an HP 5890 gas chromatograph with a Porapaq Q column and an FID detector. Two replicates were performed for each condition.

Detection and analysis of the mcrA gene in total DNA extracted from soda lake sediments, and from methanogenic pure cultures: Extraction of total DNA from approx. 3 mL sediment was performed as described by Zhou et al. (1996) with minor modifications. Extracted DNA was purified via ion exchange chromatography (NucleoBond AXG 20, Macherey Nagel, Germany) and used as template for PCR with Phusion High fidelity PCR master mix (Finnzymes). Three different DMSO concentrations were tested in the PCR reactions, 0%, 3% and 5%. Primers for amplification of *mcrA* were ME1 and ME2 (Hales et al., 1996). The PCR protocol was 30 sec initial denaturation at 98°C, followed by 30 cycles of 10 sec denaturation (98°C), 30 sec annealing (57°C) and 30 sec extension (72°C), and 10 min final extension (72°C). The obtained PCR products were cloned into cloning vector pSC-B (StrataGene blunt end PCR clonig kit, Stratagene, Canada).

Table 1 Composition of the five buffers used for the sediment incubations

Salt, g/l	0.6 M Na^+		1.0 M Na^+	2 M Na^+	
pH	**9.5**	**10**	**10**	**9.5**	**10**
Medium No.	**1**	**2**	**3**	**4**	**5**
Na_2CO_3 (g/L)	15	23	39.6	64	95
$NaHCO_3$ (g/L)	20	7	14.4	40	15
NaCl (g/L)	3	6	6	18	16
K_2HPO_4 (g/L)	1	1	1	1	1

For sequencing of the clones, pSC-B derivatives with ME1/2 amplicon inserts were subjected to PCR with M13 primers. M13 PCR products were analyzed by Sanger sequencing with primer T7 as sequencing primer.

Partial *mcrA* sequences were also amplified from two *Methanocalculus* isolates (strains AMF-Pr1 and AMF2) obtained from sediment of the same location (Sorokin, unpublished data). Genomic DNA from culture pellets was obtained by three freeze-thaw cycles (liquid nitrogen, 65°C water bath) in a lysis solution (0.5% SDS, 125 mM NaCl, 50 mM Na_2EDTA [pH 8.0], 250 mM Tris-HCl [pH 8.0]), followed by treatment with proteinase K, phenol-chloroform extraction and ethanol precipitation. Primers mlas and mcrA-rev (Steinberg and Regan, 2008) were used to amplify partial *mcrA* from the extracted DNA, using Taq polymerase (Evrogen, Moscow, Russia) in the following PCR protocol: 4 min initial denaturation at 94°C, 30 cycles of 20 sec denaturation (94°C), 20 sec annealing (59°C), 30 sec extension (72°C), and 30 min final extension (72°C). The PCR products were purified using agarose electrophoresis and Wizard SV Gel and PCR Clean-Up System (Promega, USA) and were then sequenced using the Big Dye Terminator kit (version 3.1, Applied Biosystems) on an automatic ABI 3730 sequencer (Applied Biosystems, Inc.).

Analysis of the obtained sequences was performed at the amino acid level. The cloned sequences obtained with the ME primer set were homologous to the region spanning Phe238 to Pro476 of the translated *mcrA* gene of *Methanopyrus kandleri* (GI:1354840), and were compared to homologous sequence parts of *mcrA* sequences available in Genbank (NCBI). The two sequences amplified from the soda lake isolates with primer set mlas/mcrA-rev were homologous to Leu346-Pro476 of

Table 2 *mcrA* sequences used for comparison with *mcrA* clones in Figure 2. Listed are gene ID numbers for protein sequences and originating species.

Gene ID	Species	Reference
284413651	*Methanobacterium oryzae*	Mori et al., 2011
284413655	*Methanobacterium subterraneum*	as above
304315257	*Methanothermobacter marburgensis* str. Marburg	Liesegang et al., 2010
340624872	*Methanococcus maripaludis*	direct submission (Wang et al., 2009)
333911106	*Methanotorris igneus*	direct submission (Lucas et al., 2011)
256810887	*Methanocaldococcus fervens*	as above
312136958	*Methanothermus fervidus*	Anderson et al., 2010
15679140	*Methanothermobacter thermautotrophicus*	Smith et al., 1997
282163186	*Methanocella paludicola*	direct submission (Sakai et al., 2009)
226897268	*Methanolinea* sp. TNR	as above
330506955	*Methanosaeta concilii*	Barber et al., 2011
288561180	*Methanobrevibacter ruminantium*	Lehay et al., 2010
20094093	*Methanopyrus kandleri*	Slesarev et al., 2002
91713341	*Methanococcoides burtonii*	direct submission (Copeland et al., 2006)
143328284	*Methanobacterium formicicum*	direct submission (Ma,K. and Dong,X., 2007)
284413641	*Methanobacterium bryantii*	direct submission (Mori,K. and Harayama,S., 2010)
284413639	*Methanobacterium alcaliphilum*	as above
284413653	*Methanobacterium palustre*	as above
261599977	*Methanospirillum lacunae*	Iino et al., 2010
13259179	*Methanospirillum hungatei* JF-1	Lueders et al., 2001
13259177	*Methanoculleus thermophilus*	as above
145370912	*Methanoculleus bourgensis*	Watanabe et al., 2009
145370896	*Methanosarcina mazei*	as above
145370889	*Methanothermobacter wolfeii*	as above
145370875	*Methanobrevibacter arboriphilus*	as above
154240556	*Methanothermococcus thermolithotrophicus*	Nunoura et al., 2008
154240552	*Methanosalsum zhilinae*	as above
154240550	*Methanohalophilus mahii*	as above
30230517	*Methanocorpusculum parvum*	Simankova et al., 2003

M. kandleri mcrA. Database sequences were identified via Blast searches (Altschul et al., 1990) and aligned by ClustalW or Muscle (Edgar, 2004, Larkin et al., 2007) (Tables 2 and 3).

Phylogenetic trees were generated using FastTree (Price et al., 2010). For bootstrap analysis, 500 random alignment combinations were generated with seqboot (from PHYLIP version 3.67; Felsenstein, 2005). The CompareToBootstrap.pl script (Price M. N.) was used to implement the bootstrap values into the main tree. Trees were drawn using Dendroscope (Huson et al., 2007). The obtained representative clone sequences were submitted to GenBank (accession numbers JN712773 to JN712780). Accession numbers for *Methanocalculus* strains AMF-Pr1 and AMF2 sequences are JQ511367, HM053969 (16S rRNA), and JQ511368, JQ511369 (*mcrA*).

Results

Potential methane production in the soda lake sediments was determined as a function of salt concentration (0.6-2M Na^+) and pH (9.5-10) for three different substrates: methanol, acetate and hydrogen. The total amounts of methane produced after 10 days of incubation are shown in Figure 1. The highest production (2.53 µmol CH_4 mL^{-1}) was measured for the substrate methanol at pH 10 and salt concentration of 1 M. Methane production from hydrogen was within the same order of magnitude and at pH 10 increased with salt concentration. Acetate did not stimulate methane production independent of pH and salt. These results suggest that the sediment contained at least two different populations of methanogens, with different substrate spectra and pH/salt optima.

To further characterize these populations taxonomically, the functional marker gene *mcrA* was used. Amplification of *mcrA* genes from sediment DNA with primer set ME yielded a product of the expected size (760 bp). The product was cloned into *E. coli* and 33 cloned *mcrA* sequences were analyzed by Sanger sequencing. One of the 33 sequences did not encode *mcrA*. For the remaining 32 clones, sequence comparison yielded eight different sequences at nucleotide level. Two of these eight sequences were identical on amino acid level, therefore seven different amino acid sequences were obtained (Figure 2). The *mcrA* genes represented two different taxonomic clades, one most similar to database *mcrA* sequences of the genera *Methanospirillum* and *Methanocorpusculum* and one most similar to *mcrA* sequences of the genera *Methanobacterium* and *Methanothermobacter* (Figure 2).

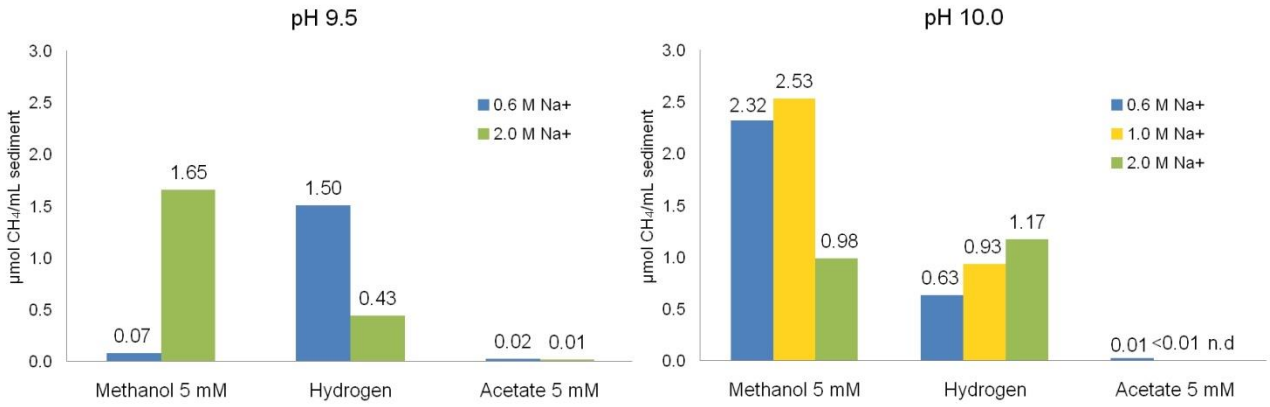

Figure 1. Total methane production for three different substrates as a function of pH and salt concentration. n.d.: not detectable

Most of the obtained sequences were all clearly different from so far published *mcrA* sequences and also from other sequences previously obtained from soda lake sediments or other environments (percent identity at the amino acid level below 93%, see Table 3). However, comparison with partial *mcrA* sequences of two *Methanocalculus* isolates from the same location (strains AMF2 and AMF-Pr1, Sorokin, unpublished data) showed either high amino acid similarity (98%) or even identity to clone 3-21 and 91% amino acid similarity to clone 1-1 (Figure 2). Most similar to clone 1-1 was a partial *mcrA* sequence, “clone MCR25“, amplified from Lonar lake sediment in India (Antony et al., 2012), which was not detected via BlastP search. Similarity between clone MCR25 and clone 1-1 was 97% on DNA and on protein level.

Discussion

Cultivation and digestion of micro-algae at high pH and alkalinity might be a new and interesting option for the biological production of renewable energy sources. Such a process depends on a well adapted haloalkaliphlic anaerobic microbial community that ultimately produces methane. In the present study we investigated the suitability of sediments from Central Asian soda lakes as an inoculum for a high pH methanogenic bioreactor.

Methanogens were shown to be active in the sampled soda lake sediments of the Kulunda steppe in Russia. The highest methane production was obtained with methanol as the substrate at pH 10 (2.5 µmol CH_4 mL^1), much higher than the total

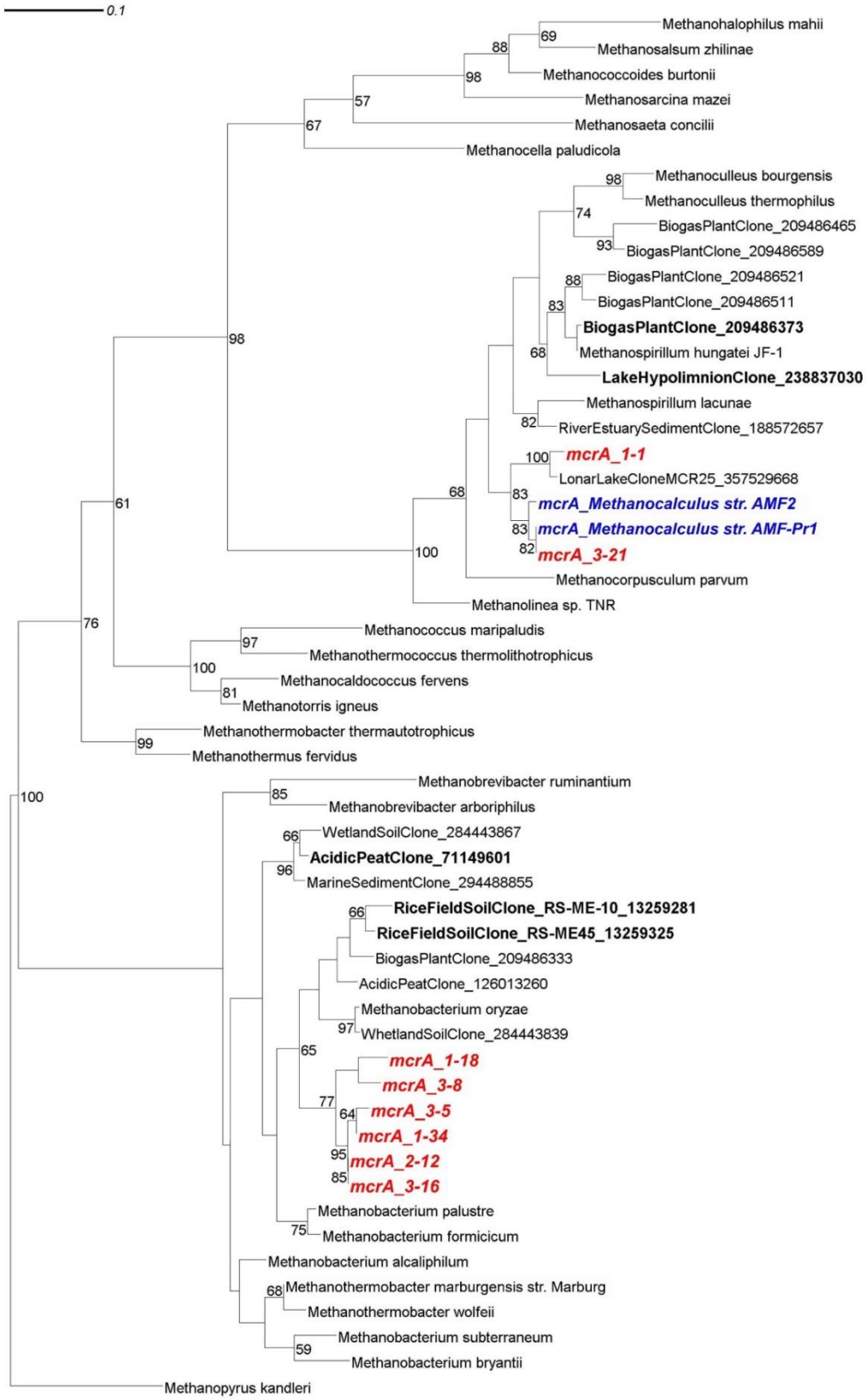

Figure 2. Phylogenetic tree of *mcrA* sequences (amino acid level). Italic type: sequences obtained in this study – red: sequences from sediment clones, blue: sequences from isolate cultures. Bold type: sequences returned as top hits in BlastP searches. Sequences of uncultured clones from GenBank are listed with their GI numbers and were among the top ten BlastP hits of the *mcrA* clones obtained in this study, except for Lonar lake clone MCR25 sequence which was not detected in the performed BlastP searches. Bootstrap values at nodes are obtained from 500 replicates and are presented as percentage values (shown only for branches with at least 50% support). The scale bar represents 0.1 amino acid substitutions per site.

production obtained by Oremland et al. (1982) after 16 days of incubation (5.8 and 2.6 nmol mL^{-1} at pH 9.7 and 11 respectively). In agreement with previous studies, methanol yielded the highest methane production (Oremland et al., 1982, Oren 1988, Sorokin et al., 2004). Winfrey and Ward (1983) studied methane production in salt-marsh sediments from the French coast supplemented with acetate and H_2. In their study however, they did not see any CH_4 production from H_2 and acetate while we do report moderate activity (0.43 – 1.17 µmol CH_4 ml^{-1}) from H_2. CH_4 production from acetate was very low in our study (0.7 - 22 nmol CH_4 ml^{-1}). King (1988) studied methane production from sediments from a hypersaline algal mat which was mainly composed of *Spirulina.* In his study, sediments were supplemented with methanol, acetate and H_2. King reported a total production of 2.5 µmol CH_4 mL^{-1} sediment supplemented with methanol after 6.5 days of incubation. In both studies, King's and ours, compared to methanol, adding H_2 as substrate results in lower methane production, 0.2 µmol CH_4 mL^{-1} and 1.5 µmol CH_4 mL^{-1} respectively. Interestingly, in King's study, acetate is a good substrate for methanogens and after 6.5 days incubation with acetate he obtained around 0.2 µmol CH_4 mL^{-1} sediment while in our study, acetate does not seem to be metabolized into methane in relevant amounts. The fact that King observed considerable methane production from a hypersaline pond where the main biomass came from *Spirulina* gives an example that it should be possible to obtain methane from the anaerobic digestion of *Spirulina* in a bioreactor.

It should also be noted that in the overall digestion processes, methanogenesis may not be the rate limiting step; the results only show that under alkaline conditions, methanol may be an important intermediate during biomass digestion. Methane production from hydrogen was not much lower and at high salt concentrations even exceeded the amount produced from methanol. In agreement with previous studies, acetate was not an important intermediate in anaerobic metabolism *in situ* (Giani et al., 1984, Oremland et al., 1982, Oren 1988, Sorokin et al., 2004, Zhilina and Zavarzin, 1994). Alternatively, the lower methane production obtained with acetate and, to a lesser extent, hydrogen, may be explained by competition with sulfate or sulfur reducers that are known to use hydrogen and acetate (Oren 1988). There is direct evidence of anaerobic acetate oxidation at sulfate-reducing conditions by soda lake syntrophic cultures (Zhilina et al, 2005; Sorokin, unpublished data).

Table 3 Source and references of *mcrA* sequences originating from relevant uncultured organisms used in Figure 2. All sequences listed are among the top ten BlastP hits for the obtained *mcrA* clones. All BlastP top hits are shown. For hits on positions two to ten, sequences were reduced to a set with not more than 97% identity (in ClustalW alignment).

Gene ID	Sequence origin	Top blastp hit of *mcrA* clone[a]	Percentage identity between *mcrA* clone and top blastp hit	Reference
188572657	Shallow sediments of the Pearl River Estuary, Southern China			Jiang et al. (2011)
209486373[a], 209486511, 209486521, 209486589, 209486465, 209486333	Leach-bed reactor of a two-phase biogas plant supplied with cattle manure and triticale silage	1-1	87	direct submission (Nettmann et al., 2008)
238837030[a]	Lake Kivu water column, hypolimnion	3-21	91	direct submission (Buergmann et al., 2009)
13259281[a]	Rice field soil and enrichment cultures	1-18	92	Lueders et al. (2001)
13259325[a], 13259331	Rice field soil and enrichment cultures	3-8	92	Lueders et al. (2001)
126013260	Acidic Peat Bog			direct submission (Metje and Frenzel, 2006)
284443839, 284443873, 284443876, 110592156	Wetland soil			direct submission (Lee et al., 2009)
294488855	Marine sediments from the Cascadia Margin			direct submission (Maruyama et al., 2009)
71149601[a]	Acidic peat from a northern wetland	2-12, 3-5, 3-16, 1-34	90 / 1-34: 89	Metje et al. (2005)

[a]Gene IDs in bold print represent BlastP top hit sequences of the *mcrA* clones that are listed in column three, respectively. Identities between *mcrA* clone and the corresponding top hit sequence are listed as percentage values.

The incubations were performed with sediments that were stored anaerobically in the dark at 4°C for more than one year. Apparently, the microbial community can be preserved with ease, favoring its use as an inoculum for high pH methanogenic bioreactors.

It is interesting that the methane production for each substrate was dependent on both pH and salt concentration. This indicates that different methanogenic communities with different substrate spectra, pH and salt optima may live side by side in these soda lake sediments. This conclusion was further supported by the molecular work.

Sequences of the methanogenesis marker gene, *mcrA*, were successfully amplified from direct sediment DNA extracts, and eight different *mcrA* clones of so far unknown sequences were identified. The topology of the tree in Figure 2 indicates that most of the obtained *mcrA* sequences belong to so far unknown species of the genus *Methanobacterium* or of the genus *Methanothermobacter* (six out of the eight non-redundant sequences of this study). These six clones form a distinct cluster within known *Methanobacterium/Methanothermobacter mcrA* sequences, among which is the *mcrA* sequence of *Methanobacterium alkaliphilum*, an organism enriched from alkaline lake sediments of the Wadi-el Natrun, Egypt (Worakit et al., 1986). With regard to the mesophilic habitat they come from, it is likely that these clone sequences belong to the genus of *Methanobacterium* rather than to *Methanothermobacter,* of which all so far described species are thermophilic. The remaining two clones (1-1 and 3-21) belong to a different cluster that contains *mcrA* sequences of the genera *Methanospirillum*, *Methanocorpusculum, Methanoculleus* and *Methanocalculus*. They can therefore be assigned to the *Methanomicrobiales* order. A more specific affiliation with *Methanocalculus* is possible by the comparison with *mcrA* sequences amplified from two haloalkaliphilic methanogenic isolates from sediment of the same location as the sediment used in this study (Sorokin, unpublished data). Based on their 16S rRNA sequences, the two isolates were assigned to *Methanocalculus* (strains AMF2 and AMF-Pr1). The partial *mcrA* sequences amplified from these strains are very similar (95% on DNA level) or identical to clone mcrA_3-21. The sequence most similar to clone 1-1 (97% on DNA level) was clone MCR25, a partial *mcrA* sequence recently published by Antony et al., (2012) that was amplified from sediment of Lonar lake, a moderately saline alkaline lake in India (Surakasi et al., 2007). In their study, Anthony et al. found only moderate similarity to MCR25 among the accessible database sequences, the most similar sequence being *mcrA* of *Methanolinea* sp. TNR (84% on DNA level). It is possible that clone 1-1 and MCR25 also originate from *Methanocalculus*. The similarities with strain AMF-Pr1 *mcrA* on DNA level are 88% and 89%, and *Methanocalculus* strains were already isolated from Lonar lake (Surakasi et al, 2007). Of these strains, however, only 16S rRNA sequences are available to date. Generally, of methanogens, much fewer *mcrA* sequences are in the databases than are 16S rRNA sequences. This is especially the case for methanogens detected in soda lakes or other alkaline habitats.

It is highly likely that even more methanogens are present in these sediments that were not amplified by the ME primers (Hales et al., 1996) we used or were missed because of the relatively small amount of clones that were sequenced in this study. For example, the methanol utilizing methanogens in the sediment could not be detected by *mcrA* cloning. Amplification of *mcrA* was not always successful with the ME primers (e.g. Scholten et al., 2005), and, especially concerning the amplification of *mcrA* from Methanosarcinae, an alternative primer set, MLf/r, developed by Luton et al. (2002) appears to target a greater sequence variety (Jerman et al., 2009, Jouttonen et al., 2006). Also Hallam et al. (2003) found a bias of ME derived amplicons towards particular *mcrA* groups. However, the ME primers target a longer gene fragment (approx. 760 bases), than the ML primers (approx. 660 bases). Thus the ME amplicons contain more phylogenetic information, which is the main reason why this set was chosen in this study. It is possible that, using the ML primers, a greater proportion of the existing diversity in the sediments could have been detected. Another reason for missing a fraction of the variety of methanogens could be incomplete DNA extraction. A method yielding high molecular weight DNA was used which had been shown to yield relatively high amounts of DNA from environmental samples (Mitchell and Tacaks-Vesbach, 2008, Zhou et al., 1996), yet without bead beating an enzymatic/chemical lysis resistant fraction of the archaeal diversity might have remained undetected (Salonen et al., 2010).

Taxonomic investigation of the anaerobic microorganisms in the Central Asian soda lakes has taken place for two decades, yet hardly focused on methanogens and more on sulfate reducers and acetogens (Sorokin et al., 2010 and 2011b, Sorokin and Kuenen, 2005, Zavarzin and Zhilina, 2000, Zhilina et al., 2011). Taxonomic information on alkaliphilic methanogens at these sites is still lacking, since they have been studied in detail only in other regions. The present study gives a first glance into the taxonomy of the alkaliphilic methanogens active in the Kulunda steppe soda lakes. The results indicate that these remarkable natural systems harbor specific methanogenic communities that are well adapted to high pH and which may be essential for the successful startup of high pH methanogenic bioreactors. The fact that the biotechnological conversion of methanethiol into methane at high pH was already successful (van Leerdam et al., 2006) with these sediments supports this conclusion. The remaining challenge is to realize a complete digestion of biomass to methane at high pH and alkalinity.

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[2] Anaerobic digestion of the microalga *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+)

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Abstract

The anaerobic digestion of the microalga *Spirulina* was successfully achieved under alkaline conditions (pH 10, 2.0 M Na^+). Soda lake sediments were used to inoculate two 1.5 L semi-continuous stirred tank reactors for the production of biogas. Continuous biogas production was observed and the obtained biogas was rich in methane, up to 96 %. Alkaline medium acted as a CO_2 scrubber which resulted in low amounts of CO_2 and no traces of H_2S in the produced biogas. 37 and 56 ml of biogas g VS^{-1} were obtained under the optimal hydraulic retention time, 15 days, and at the optimal organic loading rate, 0.25 g *Spirulina* L^{-1} day^{-1}, respectively. Key parameters affecting the reactor's performance such as ammonia and volatile fatty acids were also identified. Anaerobic digestion at alkaline conditions can be a promising alternative process for the production of biomethane for commercial use.

Keywords

High pH; Biogas; Methane rich; Microalgae; Alkaline Lake; Biomethane

1. Introduction

Microalgae are microorganisms that can transform sunlight and CO_2 into biomass and their suitability as substrate for the production of biogas has already been shown experimentally [1–3]. However, one of the remaining bottlenecks in algae cultivation is the low absorption rate of CO_2 which results in high production costs [4]. The absorption rate of CO_2 could be improved by growing algae at high pH [4,5], as at high pH, CO_2 is more soluble in water and therefore better accessible for algae. Not only would the cultivation of algae at high pH be advantageous for the production of biomass [4–7], but also anaerobic digestion at high pH would have several advantages over traditional systems: (i) the anaerobic reactor would function as a CO_2 scrubber, leading to biogas rich in methane which, with minor upgrade, could directly be used as biomethane [8,9], and (ii) the dissolved CO_2 along with phosphorous and other nutrients could be directly recycled to the photobioreactor and used for algae growth [10,11]. Therefore, the combination of algal biotechnology and anaerobic digestion might be economically favorable at high pH and salt

concentrations, and such a combined system could be a possible source for renewable energy.

High pH alkaline lakes, also known as soda lakes, are natural ecosystems with pH values of up to 12 and high salt concentrations [12]. Some studies have already demonstrated the presence of methanogenic archaea as well as the production of methane in soda lakes and in soda lake sediments [13,14]. *Spirulina* is a microalga known to grow in such soda lakes [15] and has already been used as substrate for biogas production at mesophilic pH conditions [1,16,17]. To date, the successful anaerobic digestion of biomass at high pH has not been reported. Two recent studies have, however, demonstrated the possibility to obtain methane from methanethiol in a bioreactor inoculated with soda lake sediments and operating at pH 10 [18,19]. Here we present, to the best of our knowledge, the first study of biogas production from the microalgae *Spirulina* at alkaline conditions (pH ~10; 2.0 M Na^+) in a semi-continuous stirred tank reactor inoculated with sediments from soda lakes.

2. Methods

2.1. Bioreactor set-up

A 2.0 L semi-continuous stirred tank reactor (S-CSTR) with a working volume of 1.5 L operating at 35 °C and at high pH ~ 10 and high salt concentration (2.0 M Na^+) was set up. The same reactor was used in three different experiments: start-up (Alk-Start), study of the Hydraulic Retention Time (Alk-HRT) and study of the Organic Loading Rate (Alk-OLR). Inoculum for the start-up bioreactor (Alk-Start) was the same mixture of soda-lake sediments used in Nolla-Ardèvol et al., [14]. The substrate, freeze dried *Spirulina,* was acquired from Sonnenmacht GmbH (Germany). To maintain alkalinity, the medium (in which also the substrate for feeding was dissolved) was as follows, in g L^{-1}: Na_2CO_3, 95.0; $NaHCO_3$, 15.0; NaCl, 16.0 and K_2HPO_4, 1.0; micronutrients were as in Vidal et al., [20]. The medium was prepared in lots of 1.0 L, the pH was adjusted to 10.0 at 35 °C, and the medium was stored at 37 °C. Feed was prepared fresh every day with the appropriate amount of *Spirulina* in order to obtain the desired organic loading rate. The daily purge and feed were performed manually with a syringe and through a settler. To avoid excessive loss of microorganisms, the biomass was settled before purging by stopping the stirring for at least 2 hours. Periodically the purged sludge was sampled for analysis; in that case the stirring was

not stopped. pH and redox potential in the reactors were monitored with a Mettler Toledo pH probe (HA405-DPA-SC-S8/225) and a Mettler Toledo Redox probe (Pt4805- DPA-SC-S8/225) respectively (Mettler Toledo GmbH, Germany). Mesophilic temperature conditions were maintained with a Pt-1000 temperature sensor and a heater.

2.2. Analytical methods

In addition to continuous measurements of pH and redox potential, alkalinity and total and volatile solids (TS and VS) in the digesters were periodically analyzed. Biogas production was determined by measuring the pressure build up with a pressure-meter (WAL-BMP-Test system 3150, WAL, Germany) and normalizing to standard conditions (0 °C; 1.0 atm). Biogas composition was analyzed once a week by means of a Shimadzu GC-2010 plus Gas Chromatograph (Shimadzu Corp, Japan) equipped with an Agilent GS-Gaspro capillary column (part # 113-4362) (Agilent Technologies, USA). Samples for biogas composition were obtained using a gas-tight syringe and were kept in 3.0 ml gas-tight vials (Labco Limited, UK) until analysis. Analyses to characterize the digester effluent were carried out periodically directly with the raw sample and with the soluble fraction by centrifuging the samples at 4,600 rpm for 5 minutes and filtering the supernatant through a Rotilabo CME 0.45 µm nylon filter (Carl Roth GmbH, Germany). Once a week, TS and VS were analyzed following the American Public Health Association [21] standard methods. Alkalinity, organic matter (OM), measured as total chemical oxygen demand (COD_T), and ammonium nitrogen (NH_4^+-N) were analyzed using colorimetric methods (Hach Lange GmbH, Germany). Soluble COD (COD_S) and total nitrogen (TN) were analyzed once every two weeks also with Hach Lange colorimetric methods. Free ammonia nitrogen (NH_3-N) concentration was calculated as in Astals et al., [22]. Samples for measuring specific volatile fatty acids (acetate, propionate, iso-butyrate, n-butyrate, iso-valerate and n-valerate) were prepared according to the APHA, 2005 procedure and analyzed using a Shimadzu GC-2010 plus Gas Chromatograph coupled to an FID detector and quipped with a Macherey-Nagel Optima FFA plus capillary column (Macherey-Nagel GmbH & Co. Germany).

2.3. Start-up reactor

Alk-Start reactor was used as a start-up reactor and was inoculated with approximately 150 g of soda-lake sediments and 750 ml of alkaline medium (pH 10,

2.0 M Na^+, total working volume of 850 ml). The initial substrate consisted of a mixture of: *Spirulina*, 2.0 g (dry weight) L^{-1}; glucose, 0.5 g L^{-1}; acetate, 5 mM; and methanol, 5 mM. The headspace of the reactor was flushed with N_2 gas in order to create anoxic conditions. 37.5 ml were purged and fed every two days and the organic loading rate (OLR) was set to 2.0 g *Spirulina* L^{-1} day^{-1}. Purged alkaline sludge was stored in order to be used as re-inoculum in the following days. On day 43, a mixture of 75 ml of fresh alkaline medium plus 75 ml of purged alkaline sludge were added to increase the working volume to 1,000 ml and the OLR was reduced to 1.0 g *Spirulina* L^{-1} day^{-1}. On day 70 the working volume was again increased by adding 100 ml of alkaline medium plus 100 ml of alkaline sludge to a final volume of 1,200 ml. The reactor operated at 1,200 ml until day 175 when it was stopped. At this point, the 1,200 ml of alkaline sludge were left to settle for 14 days after which a "wash" of the alkaline sludge was performed by removing 700 ml of the supernatant and replacing it with fresh pH 10 medium. This procedure was repeated once more and the resulting "washed" alkaline sludge was used to inoculate the following reactor.

2.4. Determination of the optimal hydraulic retention time (HRT)

The same bioreactor setup was used to determine the optimal hydraulic retention time (Alk-HRT). Reactor Alk-HRT was inoculated with 1,200 ml of "washed" alkaline sludge obtained from the start-up reactor plus 300 ml of fresh alkaline medium resulting in a total volume of 1,500 ml. The reactor was operated with an OLR of 1.0 g *Spirulina* L^{-1} day^{-1} (dry weight) and at five different HRT, 20, 5, 10, 30 and 15 days. An initial 25 days adaptation period was performed where the purge and feeding of the reactor was performed daily at a HRT of 20 days but feeding was done only every two days at 1.0 g *Spirulina* L^{-1} day^{-1} (dry weight). Subsequently the HRT was set to 20 days and the experiment started. Table 1 shows the duration and the amount of medium exchanged daily in each different HRT tested. A five day non-feeding period where 50 ml of sludge were exchanged with fresh alkaline medium was applied from day 117 to day 122 in order to recover reactor activity. After 215 days of continuous biogas production the experiment was concluded and the reactor stopped. The same "washing" procedure of the sludge as in Alk-Start was applied before inoculating the next reactor.

Table 1 Operational parameters and duration of each different period for the two reactors used, Alk-HRT and Alk-OLR reactors.

Hydraulic Retention Time experiment (Alk-HRT reactor)						
	Units	**Period I**	**Period II**	**Period III**	**Period IV**	**Period V**
Duration	Days	20	25	40	38	92
From – To	Days	1 -20	21 – 44	45 – 84	85 – 123	124 - 215
HRT	Days	20	5	10	30	15
Purge/Feed	ml day^{-1}	75	300	150	50	100
OLR	g *Spirulina* $(L_R\ day)^{-1}$*	1.0	1.0	1.0	1.0	1.0

Organic Loading Rate experiment (Alk-OLR reactor)					
	Units	**Period I**	**Period II**	**Period III**	**Recovery Period**
Duration	Days	52	46	41	49
From – To	Days	1 – 53	54 – 99	100 – 141	142 – 189
HRT	Days	15	15	15	15†
Purge/Feed	ml day^{-1}	100	100	100	100†
OLR	g *Spirulina* $(L_R\ day)^{-1}$*	0.25	0.50	1.0	1.0†

* Dry weight.
†Values not constant. See section 2.5 for details.

2.5. Determination of the optimal organic loading rate (OLR)

1,200 ml of "washed" sludge from the Alk-HRT reactor plus 300 ml of alkaline medium were used to inoculate the same S-CSTR, now Alk-OLR. The Alk-OLR reactor was operated at 15 days HRT and at different OLR, 0.25, 0.5 and 1.0 g *Spirulina* L^{-1} day^{-1} (dry weight) (Table 1). Before the experiment was started, the reactor was fed every two days and operated at a loading rate of 0.25 g *Spirulina* L^{-1} day^{-1} for a period of 15 days. On day 141 and on day 162, two strategies to recover biogas production were applied. First (day 141), on every other day the feeding was replaced by the addition of alkaline medium without *Spirulina*. In a second approach (day 162), once every seven days the biomass in the reactor was let to settle for 6 hours, subsequently 400 ml of supernatant were removed, centrifuged and the pellet re-inoculated with 400 ml of fresh alkaline medium. This procedure was repeated 4 times. The overall experiment lasted for 189 days after which the reactor was stopped.

3. Results

The anaerobic digestion of the microalgae *Spirulina* at alkaline conditions, pH ~ 10, 2.0 M Na^+, and high alkalinity (60 to 95 g $CaCO_3$ L^{-1}), was studied in several experiments.

3.1. Expansion and start-up

Approximately 150 g of a mixture of soda lake sediments were used to inoculate the start-up anaerobic reactor. During the start-up period, the biogas production was low,

10 to 30 ml of biogas per day and not continuous. Measurement of the biogas composition was nevertheless possible. N_2 in the headspace gradually decreased while the percentage of methane increased. However, the methane content did not increase above 45 to 50% and the carbon dioxide remained high, between 15 and 20%. At the organic loading rate applied (2.0 g of *Spirulina* L^{-1} day^{-1}) both, free ammonia nitrogen (NH_3-N), and total organic matter (OM), determined via total chemical oxygen demand, COD_T, accumulated. To reduce these accumulations, on day 125 of the start-up period the feeding was stopped for 14 days and every two days 60 ml of sludge were exchanged for fresh medium free of substrate. After these 14 days, the OLR was reduced to 1.0 g *Spirulina* L^{-1} day^{-1}, maintaining the every two day feeding regime. These actions reduced both the accumulation of NH_3-N and the COD_T values which led to a slightly increased and more constant biogas production of 30 – 35 ml biogas per day. However, the biogas production was still not continuous and after 175 days of operation the reactor was stopped.

3.2. Determination of the optimal Hydraulic Retention Time (HRT)

In Alk-HRT reactor, five different HRT were tested, 20, 5, 10, 30 and 15 days, periods P-I to P-V respectively. In the course of the experiment the HRT was adjusted to improve the biogas production rate: it was reduced when accumulation of potentially harmful ammonia and volatile acids was observed, and it was increased when the concentration of these compounds was low and biomass washout was more likely to be the cause of reduced biogas production. The pH was constant throughout the experiment and the alkalinity was kept high (Table 2). Biogas production in Alk-HRT reactor was continuous and the produced biogas was, as expected, rich in methane (Fig. 1A). Changes in the hydraulic retention time had a clear effect on the daily biogas production (Fig. 1A). Changing the HRT from 20 to 5 days (P-I to P-II) resulted in a decrease in the daily biogas production while doubling the HRT to 10 days (P-III) did not result in a marked increase in the biogas production (Fig. 1A).

Increasing the HRT to 30 days (P-IV) led to an increase in the biogas production during the first days, however, on day 99 (day 14 of period P-IV), a sudden drop from 27 to 11 ml of gas per day was observed. In the subsequent days, the daily biogas production gradually recovered until day 115 when it dropped to 1.9 ml (Fig. 1A).

Table 2. Effect of the Hydraulic Retention Time on the biogas and methane production, sludge characteristics and specific biogas and methane productions of the Alk-HRT reactor. Mean values and standard deviation of the measurements from each different period.

	Units	Period I	Period II	Period III	Period IV	Period V
OLR	g *Spirulina* (L_R day)$^{-1}$*	1.0	1.0	1.0	1.0	1.0
HRT	Days	20	5	10	30	15
Purge/Feed	ml day^{-1}	75	300	150	50	100
Biogas production and composition						
Daily biogas production	ml biogas day^{-1}	35 ± 9	21 ± 5	18 ± 6	17 ± 10	50 ± 8
Daily methane production	ml CH_4 day^{-1}	29 ± 13	16 ± 9	16 ± 7	14 ± 10	42 ± 15
CH_4	%	79 ± 6	89 ± 3	81 ± 7	86 ± 13	83 ± 9
CO_2	%	19 ± 5	10 ± 2	12 ± 7	9 ± 8	14 ± 6
N_2;O_2	%	2 ± 2	1 ± 1	7 ± 4	5 ± 2	3 ± 5
H_2S	%	n.d	n.d	n.d	n.d	n.d
Sludge characteristics						
pH		10.1 ± 0.04	10.2 ± 0.10	10.1 ± 0.03	10.0 ± 0.04	10.1 ± 0.07
Alkalinity	g $CaCO_3$ L^{-1}	64.9 ± 3.7	68.8 ± 5.5	72.7 ± 2.2	72.4 ± 3.4	82.3 ± 6.9
Total Solids	g Kg^{-1}	111.1 ± 5.9	112.5 ± 7.9	109.5 ± 2.6	111.1 ± 5.3	131.7 ± 4.1
Volatile Solids	g Kg^{-1}	16.8 ± 6.6	18.6 ± 7.1	11.0 ± 3.2	13.0 ± 3.0	23.3 ± 9.4
Fixed Solids	g Kg^{-1}	94.3 ± 0.8	93.9 ± 2.1	98.5 ± 0.9	98.1 ± 2.9	108.4 ± 5.6
Volatile Fatty Acids (VFA)						
Acetic acid	mg L^{-1}	2,168 ± 58	1,161 ± 319	1,965 ± 631	3,721 ± 467	3,105 ± 124
Propionic acid	mg L^{-1}	223 ± 117	118 ± 65	289 ± 122	784 ± 158	688 ± 31
iso-butyric acid	mg L^{-1}	127 ± 10	69 ± 20	109 ± 35	221 ± 32	181 ± 14
Butyric acid	mg L^{-1}	57 ± 10	n.d	63 ± 28	180 ± 28	128 ± 10
iso-valeric acid	mg L^{-1}	300 ± 10	158 ± 50	251 ± 82	471 ± 54	384 ± 40
n-valeric	mg L^{-1}	n.d	n.d	n.d	219 ± 10	41 ± 10
Total VFAs	mg L^{-1}	2,875	1,506	2,677	5,596	4,527
Specific productions						
SBP-VS added	ml_{biogas} (day g VS)$^{-1}$	26 ± 7	15 ± 4	14 ± 4	13 ± 8	37 ± 6
SBP-*Spirulina* added	ml_{biogas} (day g *Spirulina*)$^{-1}$	23 ± 6	14 ± 4	12 ± 4	11 ± 7	33 ± 5
SMP-VS added	$ml_{methane}$ (day g VS)$^{-1}$	21 ± 6	14 ± 3	11 ± 4	12 ± 7	31 ± 7
SMP-*Spirulina* added	$ml_{methane}$ (day g *Spirulina*)$^{-1}$	19 ± 5	12 ± 3	10 ± 3	10 ± 6	29 ± 6

* Dry weight.
n.d: non detected.
SBP: Specific biogas production.
SMP: Specific methane production.

After two days of almost zero biogas production, accumulated potentially inhibitory substances were removed by arresting *Spirulina* feeding and by replacing 50 ml of sludge each day with fresh alkaline medium. After 5 days the feeding was resumed at 1.0 g *Spirulina* L^{-1} day^{-1} and the HRT was set to 15 days (P-V). The five day exchange of sludge for fresh medium had a positive effect and the daily biogas production increased from 27 ml (day 123) to 60 ml of biogas per day (day 162) during period P-V. From this point forward, the biogas production was stable at around 50 ml of gas per day until the end of the experiment (Fig 1A).

3.3. Determination of the optimal Organic Loading Rate

The optimal organic loading rate was determined with alkaline reactor Alk-OLR which was operated at 15 days HRT. The starting OLR was set to 0.25 g *Spirulina* L^{-1} day^{-1} and was gradually increased until reactor failure. As in Alk-HRT, the pH was constant

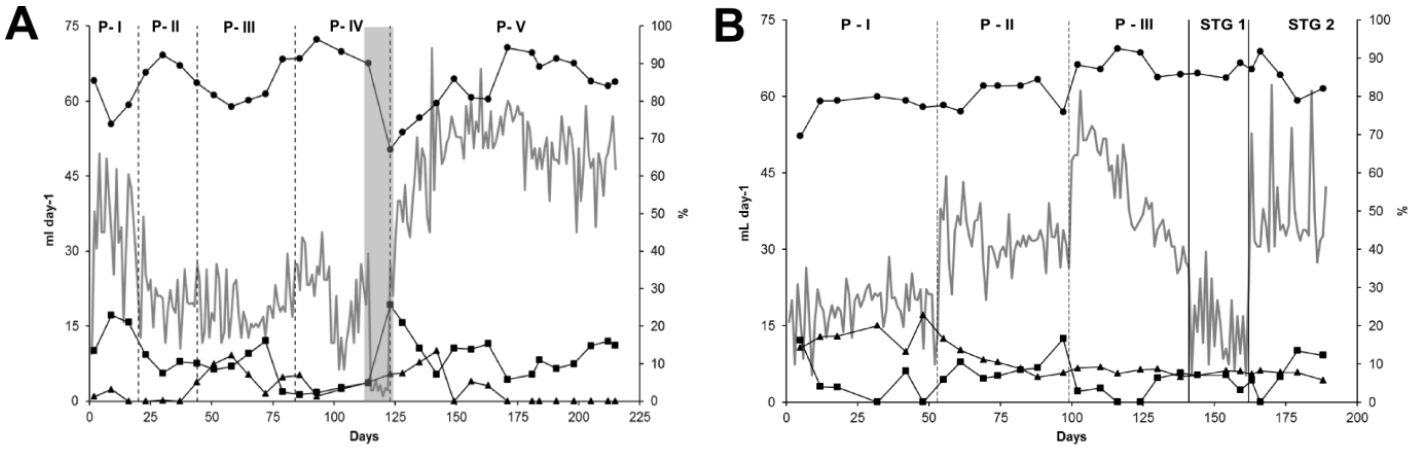

Figure 1 Daily biogas production (gray line) (Left axis) and biogas composition (Right axis): CH_4 (●); CO_2 (■) and N_2 (▲), from the anaerobic digestion of *Spirulina* at alkaline conditions in **(A) Alk-HRT reactor.** Dashed vertical lines indicate a change in the hydraulic retention time: 20, 5, 10, 30 and 15 days; Gray area corresponds to the five day non-feeding period**; (B) Alk-OLR.** Dashed vertical lines indicate a change in the organic loading rate: 0.25, 0.5 and 1.0 g *Spirulina* L^{-1} day^{-1}. Continuous vertical lines indicate the two different strategies to try to recover the biogas production.

at pH 10 and the alkalinity high (Table 3). As expected, an increase in the OLR led to an increase in the biogas production (Fig. 1B). Increasing the OLR from 0.5 to 1.0 g *Spirulina* L^{-1} day^{-1}, however, eventually had a negative effect on the biogas production. After an initial rise in biogas production to 60 ml per day a gradual decrease to 30 ml per day was observed (Fig. 1B). Two strategies for removal of inhibitory substances and recovery of higher biogas production were applied (see section 2.5.). However, none of the applied strategies had the desired effect and the biogas production remained at around 30 ml per day (Fig. 1B). At this point the reactor was stopped.

3.4. Biogas rich in methane

The anaerobic digestion at alkaline conditions produced, as expected, biogas rich in methane (Fig. 1). In the Alk-HRT reactor, the composition of the biogas was not constant and it varied with the changes in the HRT (Fig. 1A). The mean percentage of methane throughout the experiment was around 83% while the carbon dioxide and nitrogen were 12 and 5% respectively (Table 2). The highest methane content was obtained in P-IV, day 93 with 96% and in P-V, day 171 with 94% while the CO_2 content on these two days was 2% and 5% respectively (Fig. 1A). The drop in methane percentage on day 123 (P-IV) was due to a need to open the reactor. From this day on, the methane content rapidly increased from 67 to 86% and then further to 94% on day 171. At the same time, the carbon dioxide in the headspace was gradually reduced. The biogas in the Alk-OLR was likewise rich in methane, with a

Table 3. Effect of the Organic Loading Rate on the biogas and methane production, sludge characteristics and specific biogas and methane productions of the Alk-OLR reactor. Mean values and standard deviation of the measurements from each different period.

	Units	Period I	Period II	Period III
OLR	g *Spirulina* (L_R day)$^{-1}$*	0.25	0.5	1.0
HRT	days	15	15	15
Purge / Feed	ml day^{-1}	100	100	100
Biogas production and composition				
Daily biogas production	ml biogas day^{-1}	18 ± 5	32 ± 5	40 ± 9
Daily methane production	ml CH_4 day^{-1}	14 ± 4	25 ± 8	36 ± 9
CH_4	%	77 ± 4	80 ± 4	88 ± 3
CO_2	%	5 ± 6	9 ± 4	3 ± 3
N_2;O_2	%	17 ± 4	11 ± 3	8 ± 1
H_2S	%	n.d	n.d	n.d
Sludge characteristics				
pH		10.2 ± 0.1	10.1 ± 0.1	10.0 ± 0.1
Alkalinity	g $CaCO_3$ L^{-1}	93.3 ± 2.8	94.8 ± 5.3	94.1 ± 4.0
Total Solids	g Kg^{-1}	111.6 ± 3.0	115.3 ± 3.9	113.6 ± 3.7
Volatile Solids	g Kg^{-1}	5.7 ± 2.9	9.2 ± 3.6	9.7 ± 1.9
Fixed Solids	g Kg^{-1}	105.9 ± 0.5	106.1 ± 0.9	104.0 ± 2.7
Total Nitrogen	g L^{-1}	0.5 ± 0.1	0.7 ± 0.1	1.2 ± 0.3
NH_4^+-N	g L^{-1}	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
NH_3-N	g L^{-1}	0.28 ± 0.04	0.43 ± 0.07	0.73 ± 0.13
Volatile Fatty Acids (VFA)				
Acetic acid	mg L^{-1}	576 ± 95	880 ± 204	1,374 ± 133
Propionic acid	mg L^{-1}	124 ± 19	185 ± 38	378 ± 93
iso-butyric acid	mg L^{-1}	64 ± 16	61 ± 12	101 ± 13
Butyric acid	mg L^{-1}	36 ± 19	21 ± 12	62 ± 19
iso-valeric acid	mg L^{-1}	119 ± 26	135 ± 30	211 ± 20
n-valeric acid	mg L^{-1}	47 ± 15	n.d	n.d
Total VFAs	mg L^{-1}	966	1,282	2,125
Specific productions				
SBP-VS added	ml_{biogas} (day g VS)$^{-1}$	56 ± 15	48 ± 7	31 ± 7
SBP-*Spirulina* added	ml_{biogas} (day g *Spirulina*)$^{-1}$	49 ± 13	41 ± 7	28 ± 6
SMP-VS added	$ml_{methane}$ (day g VS)$^{-1}$	43 ± 12	38 ± 5	27 ± 6
SMP-*Spirulina* added	$ml_{methane}$ (day g *Spirulina*)$^{-1}$	38 ± 10	34 ± 5	24 ± 5

* Dry weight.
n.d: non detected.
SBP: Specific biogas production.
SMP: Specific methane production.

mean value of 82% throughout the three periods (Table 3). In contrast to the Alk-HRT, the composition was more constant and it reached its peak on day 116 with 92% of CH_4 (Fig. 1B). Carbon dioxide was less present in the headspace when compared to the Alk-HRT reactor (6%) while nitrogen percentage was higher (12%) (Table 3; Fig. 1B).

3.5. Parameters affecting the biogas production

Several main parameters such as free ammonia (NH_3), VFAs and organic matter apparently affected the biogas production in both reactors. Changes in the HRT, and therefore in the amount of sludge exchanged daily, had a clear effect on the levels of free ammonia, VFAs and OM present in the Alk-HRT medium (Fig. 2). A gradual accumulation of these compounds from P-I to P-II and especially in P-IV was observed. At the end of P-IV, the NH_3 reached 1,200 mg L^{-1} (Fig. 2A), a

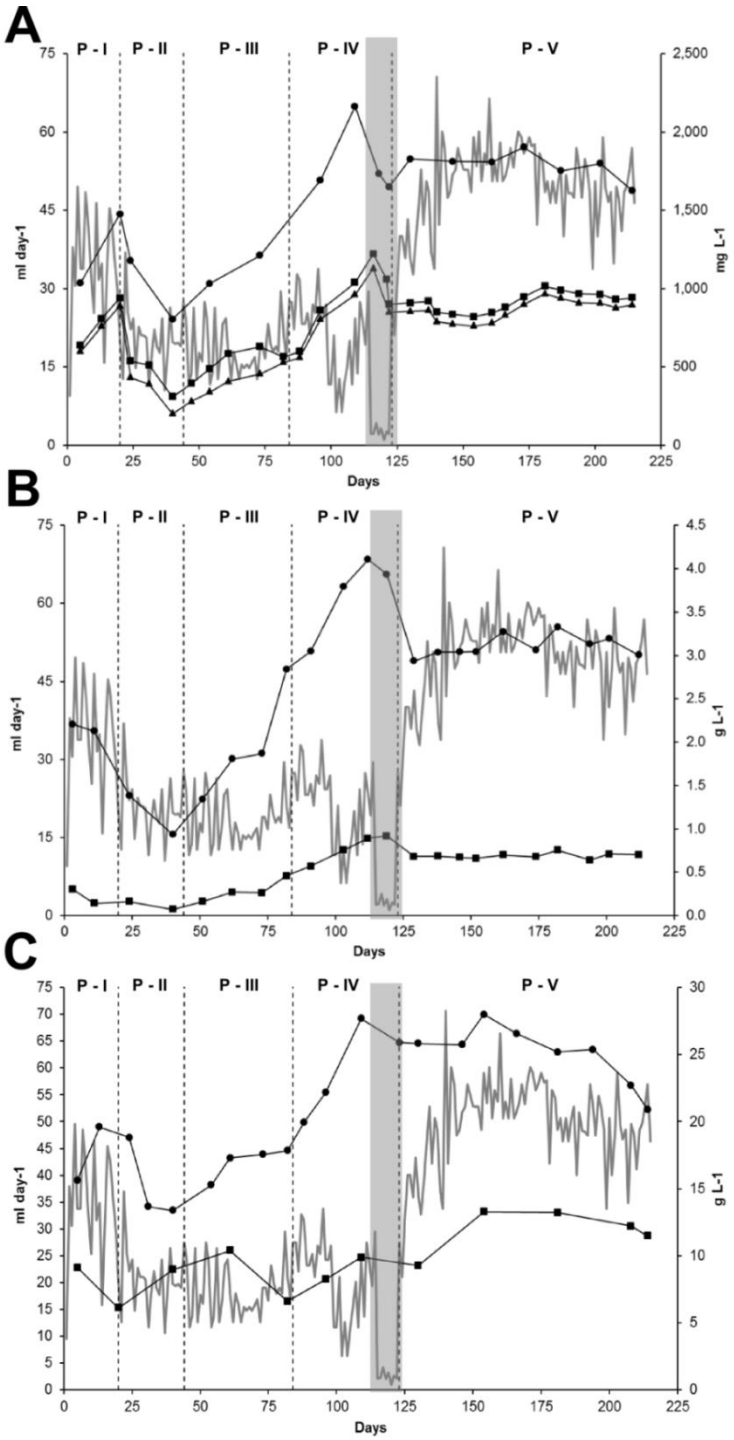

Figure 2 Parameters affecting the anaerobic digestion of *Spirulina* at alkaline conditions in the Alk-HRT reactor. Daily biogas production (gray line); **(A)** Total Nitrogen (●), Total Ammonium Nitrogen (TAN) (■) and Free Ammonia Nitrogen (FAN) (▲) profile; **(B)** Acetic (●) and propionic (■) acid evolution and **(C)** Chemical oxygen demand, Total (●) and Soluble (■). Dashed vertical lines indicate a change in the hydraulic retention time: 20, 5, 10, 30 and 15 days. Gray area corresponds to the five day non-feeding period.

concentration much higher than the previously reported inhibitory thresholds, between 150 and 900 mg L^{-1} [23,24]. At the same time, both the acetic acid concentration and the COD_T reached their maximum, over 4.0 g L^{-1} and 28.0 g O_2 L^{-1} respectively (Fig. 2B,C). Replacing 50 ml of sludge with fresh alkaline medium with

no added substrate five times during five days reduced the free ammonia content to 850 mg L^{-1}, as well as the VFAs and the COD_T, which was low enough for the biogas production to resume (Fig. 2). By setting the HRT to 15 days the accumulation of ammonia, VFAs and COD was prevented and the inhibitory effect reduced, which resulted in a stable biogas production**.**

In the Alk-OLR reactor, the free ammonia did not reach levels as high as in the Alk-HRT reactor (Table 3). The NH_3 concentration in Alk-OLR reached its maximum, 0.73 g L^{-1}, in P-III when 1.0 g *Spirulina* L^{-1} day^{-1} was fed as substrate (Table 3). In contrast to the Alk-HRT reactor, in the Alk-OLR reactor, the concentrations of VFAs remained low throughout the experiment with a slight increase in P-III when 1.0 g *Spirulina* L^{-1} day^{-1} was fed (Table 3). The biogas production in the Alk-OLR was more affected by the accumulation of OM which drastically affected its performance (Fig. 3). When the organic loading rate was increased to 1.0 g *Spirulina* L^{-1} day^{-1}, the COD_T rapidly increased, causing a bioreactor failure due to substrate overload. Strategy one to try to recover the biogas production markedly reduced the total COD, yet once the second strategy was applied it increased to levels similar to those observed P-III (15 g O_2 L^{-1}) (Fig. 3).

4. Discussion

In the work presented here we show that anaerobic digestion of the microalgae *Spirulina* at alkaline conditions (pH 10 and 2.0 M Na^+) is possible and the obtained biogas rich in methane (Fig. 1).

4.1. Biogas rich in methane

As expected, by applying alkaline conditions in our anaerobic digester, methane rich biogas was obtained (Fig. 1). This was due to the fact that the solubility of carbon dioxide in a solution is determined mainly by the pH of the solution and its buffering capacity. Because of the high pH, the (bi)carbonate concentration in the medium can be high while a driving force for carbon dioxide absorption is maintained. Since all absorbed/hydrated carbon dioxide immediately reacts with OH^- to form (bi)carbonate, CO_2 absorption kinetics are faster than at neutral pH. With the high pH and alkalinity used in our experiments, the reactor's medium acted as a CO_2 scrubber and the carbon dioxide remained in solution as carbonates (alkalinity) (Table 2 and 3) which resulted in a low percentage of CO_2 in the headspace (Fig.1). In both reactors this

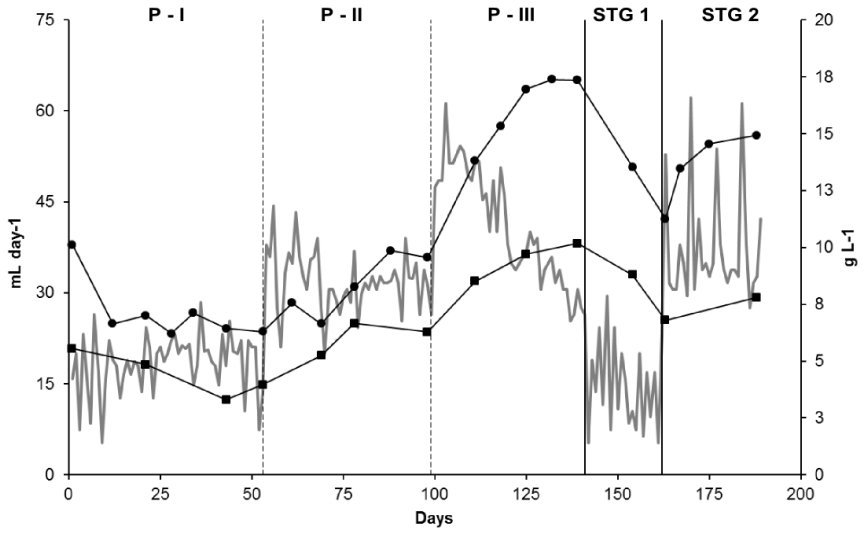

Figure 3 Evolution of the organic matter in the Alk-OLR. Biogas production (gray line) and Total (●) and Soluble (■) Chemical oxygen demand. Dashed vertical lines indicate a change in the organic loading rate: 0.25, 0.5 and 1.0 g *Spirulina* L^{-1} day^{-1}. Continuous vertical lines indicate the two different strategies to try to recover the biogas production.

scrubber effect produced biogas with a high percentage of methane that ranged from 77 to 88% (Table 2 and 3) with peaks up to 96% in the Alk-HRT (Fig.1 A) and 92% in the Alk-OLR (Fig.1 B). These values for methane content are higher than the 78% obtained in the study of van Leerdam et al., [18], which is, to date, the only other study of anaerobic digestion at high pH. A further interesting and important observation was that, as in the case of van Leerdam et al., [18], no H_2S was detected in the biogas during the two experiments. This high methane content and the absence of H_2S make this biogas suitable to be used as biomethane for vehicles and national gas supply grids with none or only a minor upgrade. For example, in Germany the minimum required content of methane in biomethane is 96%, in Norway 95% and in The Netherlands 88% [9].

4.2. Biogas production

Daily biogas production ranged from 35 to 60 ml per day in both reactors Alk-HRT and Alk-OLR (Fig.1) comparable to what was found previously with methanethiol as substrate which to date is the only other known substrate digested at alkaline conditions [18]. This daily biogas production is, however, low if compared to studies performed at mesophilic pH and alkalinity. Samson and Leduy [16,25,26] obtained between 260 and 350 ml of methane from the continuous anaerobic digestion of *Spirulina* while Varel et al., [17] obtained between 300 and 470 ml of methane.

The low daily biogas production could be attributed to a non-complete degradation of the supplied substrate. As can be seen in Fig. 2C and Fig. 3, COD values were high throughout the two experiments which is indicative of a non-complete degradation of the substrate. It is know that *Spirulina* is a difficult substrate to degrade. For example, El-Mashad [27] achieved a 56% of degradation in batch studies and our own studies at mesophilic conditions show similar values, 41% (unpublished data).

4.3. Identification of HRT and OLR as key parameters

Changing the HRT (Alk-HRT experiment) had a clear effect on the biogas production. A reduction of the daily biogas production was observed for short HRTs (P-I to P-III) which could be attributed mainly to a washout of the active biomass [28,29]. This was especially pronounced in P-II (HRT of 5 days) when 300 ml of medium were exchanged daily which caused a considerable drop in the biogas production (Fig 1A). To overcome the washout effect, the HRT was again increased in P-IV. However, this relatively drastic increase in the HRT in P-IV to reduce the washout effect, also negatively affected the daily biogas production, marked by sudden drops on days 99 and 115 (Fig 1A). These drops were attributed mainly to an inhibitory effect due to accumulation of VFAs, organic matter and especially free ammonia (Fig. 2) which can cause an inhibition of the methanogenic community [29,30]. *Spirulina* is a protein rich substrate, (60 to 75% dry weight) [31], and its degradation releases high amounts of nitrogen in the form of ammonium (NH_4^+) which, at high pH, is mostly transformed into NH_3, a highly toxic form for methanogens [32,33]. This accumulation of ammonia, OM and VFAs, led to an almost complete failure of the Alk-HRT reactor (Fig. 2). Reducing the HRT to 15 days in P-V and therefore increasing the amount of exchanged sludge per day had a positive effect in controlling the accumulation of NH_3, VFAs and OM and the biogas production was resumed (Fig. 2**).** Of the five different HRT tested, a HRT of 15 days resulted in the best values for the calculated specific biogas and methane potentials (SBP and SMP), 33 and 29 ml of biogas and methane g *Spirulina*$^{-1}$ respectively (Table 2). For this HRT, both excessive biomass washout and the accumulation of inhibitory substances were avoided which in turn favored the biogas production.

Daily biogas production was also affected by the amount of organic matter fed to the reactor and whenever the OLR was increased the biogas production also increased (Alk-OLR experiment) (Fig 1B). However, the increase in biogas production did not

correlate linearly with the increase in the OLR. In Alk-OLR, from P-I to P-II the OLR was doubled, from 0.25 to 0.5 g *Spirulina* L^{-1} day^{-1}, while the biogas production increased by 77%. From P-II to P-III, the OLR was again doubled, yet the biogas production only increased by 26%. These results indicate that the additional substrate provided was not effectively converted to methane. Instead, it accumulated mainly as total and soluble OM (increasing COD values) and as VFAs (Fig. 3; Table 3). This accumulation eventually caused a substrate overload which resulted in a slow but constant reduction of the daily biogas production during P-III. The two strategies applied to try to recover the biogas production in Alk-OLR helped to reduce the total and soluble COD but did not have the desired effect and the relatively high values for daily biogas production in Alk-OLR from the beginning of P-III were not recovered (Fig. 3). Of the three different OLR tested, 0.25 g *Spirulina* L^{-1} day^{-1} (P-I) was the optimal, yielding the highest SBP and SMP, 49 and 38 ml of biogas and methane per gram of *Spirulina* respectively (Table 3).

Based on the chemical composition of *Spirulina* ($C_4H_7O_1N_{0.8}S_{0.02}$) [31] its theoretical biomethane potential is 627 ml CH_4 g VS^{-1} [34]. Using these values, it was possible to determine the percentage of biodegradability in each experiment. In Alk-HRT, 4.9% of conversion into methane was achieved with the 15 days HRT while in the Alk-OLR the highest biodegradability, 6.7%, was obtained with an OLR of 0.25 g *Spirulina* L^{-1} day^{-1}. These percentages of biodegradability obtained for *Spirulina* in both reactors were much lower than the reported previous work where it reached 55% [16]. However, in both experiments the percentage of conversion increased when the conditions were optimized which indicates that there is still room for improvement.

4.4. Low biogas production

The relatively low biogas production during the periods of best performance for each reactor (HRT=15 days and OLR = 0.25 g *Spirulina* L^{-1} day^{-1}) (Table 2 and 3) might either be explained by inherently low metabolic rates of the haloalkaline microbial community, but could also indicate that the reactors are working under what is known as “inhibited steady state”, a state in which reactors are working stable but with low biogas yields [22,35]. We attribute the low biogas production and conversion in both reactors to the extreme conditions in which the reactors were operated, to the type of substrate used (rich in proteins) and, mainly to the inhibition caused by the accumulation of free ammonia, VFAs and organic matter. It was not within the scope

of this study to exactly identify and specify the factors that led to reactor failure before a constant biogas production was achieved but to demonstrate that continuous production of biogas that is rich in methane from the anaerobic digestion of organic matter at alkaline conditions is possible, and the results presented will be a valuable starting point for optimization in future studies.

5. Conclusions

The anaerobic digestion of the microalga *Spirulina* was possible at alkaline conditions, pH 10, 2.0 M Na^+. Continuous biogas production was observed and the obtained biogas was rich in methane (up to 96%). However, the biogas production was low and affected by several factors such as free ammonia nitrogen and volatile fatty acids accumulation. These drawbacks might be overcome by using alternative substrates and/or reactor configurations where a long biomass retention time and a short hydraulic retention time can be combined (e.g. membrane or granule bioreactor). With this potential of optimization in mind, anaerobic digestion at alkaline conditions can be a promising alternative process for the production of biomethane for commercial use.

Acknowledgments

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Abbreviations

HRT: Hydraulic retention time; OLR: Organic loading rate; OM: Organic Matter; COD_T: Total chemical oxygen demand; COD_S: Soluble chemical oxygen demand; VFAs: Volatile fatty acids; SBP: Specific biogas potential; SMP: Specific methane potential; Alk-Start: Alkaline start-up reactor; Alk-HRT: Alkaline HRT reactor; Alk-OLR: Alkaline OLR reactor.

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[3] Metagenome analysis and biogas production from the anaerobic digestion of the protein rich microalga *Spirulina*

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Abstract

Background

Anaerobic digestion is a biological process during which complex organic compounds are transformed into methane and carbon dioxide by a consortium of microorganisms. A good understanding of the interactions between the populations that form this consortium can contribute to successful anaerobic digestion of the desired substrate.

In this study we combine the analysis of the biogas production by a laboratory anaerobic digester fed with the microalgae *Spirulina*, a protein rich substrate, with the analysis of the metagenome of the consortium responsible for digestion, obtained by high-throughput DNA sequencing. The obtained metagenome was also compared with a metagenome from a full scale biogas plant fed with cellulose rich material.

Results

The optimal organic loading rate for the anaerobic digestion of *Spirulina* was determined to be 4.0 g *Spirulina* L^{-1} day^{-1} with a specific biogas production of 350 mL biogas g *Spirulina*$^{-1}$ with a methane content of 68%.

Firmicutes dominated the microbial consortium at 38% abundance followed by Bacteroidetes, Chloroflexi and Thermotogae. Euryarchaeota represented 3.5% of the total abundance. The most abundant organism (14.9%) was related to *Tissierella*, a bacterium known to use proteinaceous substrates for growth. Methanomicrobiales and Methanosarcinales dominated the archaeal community. Compared to the full scale cellulose-fed digesters, Pfam domains related to protein degradation were more frequently detected and Pfam domains related to cellulose degradation were less frequent in our sample.

Conclusions

The results presented in this study indicate that *Spirulina* could be a suitable substrate for the production of biogas. The proteinaceous substrate appeared to have a selective impact on the bacterial community that performed anaerobic digestion. A direct influence of the substrate on the selection of specific methanogenic populations was not observed.

Keywords

Metagenome; Metagenome comparison; *Spirulina*; Biogas; Binning; Microbial community

Background

The problems associated with climate change, and the limited supply of fossil fuels has led to an increasing interest in renewable energy sources. One of these alternative energy sources is biogas (a mixture of mainly methane and carbon dioxide) which is obtained through the anaerobic digestion of organic matter [1]. In recent years, energy crops, crops used to produce energy in form of biofuels, have contributed over 50% of the total biogas production [2]. However, the use of such crops as substrate for biogas production has several drawbacks: (i) use of arable land; (ii) consumption of large quantities of water and (iii) increased use of fertilizers [3, 4]. An alternative to energy crops could be the use of algal biomass. This would overcome the main problems mentioned above; algae do not compete for arable land and with algae it is possible to close the water and nutrient balances [4].

Anaerobic digestion of the microalgae *Spirulina* was studied in the late 80s by several authors [5–7], however, the circumstances at that time, low oil prices and less environmental concerns, led to a loss of interest. The need to use non-fossil energy sources and the biorefinery concept has brought back the attention to using algal biomass to produce biofuels [4, 8–10]. In this context, the use of the microalga *Spirulina* as substrate for the production of biogas is again an interesting option.

Anaerobic digestion is a biological process in which a wide range of anaerobic bacteria hydrolyze and ferment complex organic compounds first into organic acids, then further to acetate, hydrogen and carbon dioxide, which are subsequently transformed into methane by methanogens [11]. A good understanding of the taxonomic composition and the functional interactions between the involved microbial populations, can contribute to the optimization of the anaerobic digestion of the desired substrate. High-throughput DNA sequencing technologies and their application for metagenome analysis have greatly enhanced the study of microbial communities of environmental samples. Several metagenome studies both of biogas

producing plants and lab scale anaerobic digesters have been performed to date [12–16].

In the study presented here we combine the analysis of the anaerobic digestion process of *Spirulina* with the analysis of the metagenome from the microbial community in the digester. Total DNA was extracted from a lab scale bioreactor digesting *Spirulina* and sequenced using the Ion Torrent (PGM) platform. Sequencing reads where assembled into contigs and these were analyzed with regard to the predicted genes, and by binning to acquire provisional whole genome sequences of abundant community members [17].

In contrast to the cellulose rich substrates commonly used to date in many large scale biogas production plants, *Spirulina* is a protein rich substrate [18]. To determine if the microbial community in the *Spirulina* fed lab-scale digester displays significant adaptation to the substrate, the MG-Rast metagenome analyzer [19] was used to compare the gene content of the obtained metagenome to that of a publicly available metagenome from a fully operative biogas plant fed mainly with cellulose rich material [14].

Results and discussion

Biogas production via the anaerobic digestion of *Spirulina*

The anaerobic digestion of freeze dried *Spirulina* was studied using a 2.0 L semi continuous stirred tank reactor (S-CSTR) operated at pH 7.5 – 8.2, at 37ºC and with a 20 days hydraulic retention time (HRT). After a 71 days start-up period constant daily biogas production (742 ml biogas day^{-1}), and constant process parameters (alkalinity, total solids (TS), volatile solids (VS)) were observed, indicating that the bioreactor had reached a pseudo steady state condition. Starting from this pseudo steady state, five different organic loading rates (OLR), from 1.0 to 5.0 g *Spirulina* L^{-1} day^{-1} (dry weight) were studied to determine the optimal OLR for freeze dried *Spirulina*. The biogas production in each period was constant and, as expected, increased when the OLR was increased (Table 1). The biogas production ranged from 470 mL of biogas day^{-1} (69% of methane) in period I, with an OLR of 1.0 g *Spirulina* L^{-1} day^{-1}, up to 2,210 mL biogas day^{-1} (62% methane) in period V, with an OLR of 5.0 g *Spirulina* L^{-1} day^{-1}.

Table 1 Biogas production and sludge characteristics

Biogas and methane production, biogas characteristics, specific biogas and methane productions and sludge characteristics from the anaerobic digestion of *Spirulina* obtained with the five organic loading rate tested. Shown are the mean values with the standard deviation.

	Units	Period I	Period II	Period III	Period IV	Period V
	Days	116	74	100	21	29
OLR	g *Spirulina* (L_R day)$^{-1}$	1.0	2.0	3.0	4.0	5.0
Daily biogas production	mL biogas day^{-1}	470 ± 69	986 ± 176	1,487 ± 252	2,096 ± 118	2,210 ± 325
Daily methane production	mL CH_4 day^{-1}	327 ± 49	648 ± 117	972 ± 190	1,397 ± 79	1,399 ± 260
CH_4	%	69 ± 5	69 ± 2	70 ± 13	68 ± 1	62 ± 4
CO_2	%	30 ± 5	30 ± 2	25 ± 10	31 ± 1	37 ± 4
N_2;O_2	%	1 ± 1	1 ± 1	5 ± 16	1 ± 1	1 ± 1
H_2S	%	n.d ± n.d	n.d ± n.d	n.d ± n.d	n.d ± n.d	n.d ± n.d
Specific biogas and methane productions						
SBP-VS added	mL_{biogas} (day g VS)$^{-1}$	354 ± 52	369 ± 66	372 ± 63	393 ± 22	334 ± 47
SBP-g *Spirulina* added	mL_{biogas} (day g *Spirulina*)$^{-1}$	313 ± 46	329 ± 58	330 ± 55	349 ± 19	297 ± 42
SMP-VS added	$mL_{methane}$ (day g VS)$^{-1}$	246 ± 37	243 ± 44	243 ± 47	262 ± 14	211 ± 39
SMP-g *Spirulina* added	$mL_{methane}$ (day g *Spirulina*)$^{-1}$	218 ± 33	216 ± 39	216 ± 42	233 ± 13	188 ± 34
Sludge characteristics						
pH		7.5 ± 0.3	7.9 ± 0.1	8.5 ± 0.1	8.6 ± 0.1	8.6 ± 0.1
Alkalinity	g $CaCO_3$ L^{-1}	8.6 ± 0.7	11.4 ± 0.8	16.5 ± 3.1	20.8 ± 1.2	25.4 ± 2.4
Total Solids	g Kg^{-1}	17.6 ± 1.3	21.7 ± 1.2	26.6 ± 2.6	29.3 ± 1.4	38.2 ± 5.0
Volatile Solids	g Kg^{-1}	10.2 ± 1.0	13.7 ± 1.2	18.9 ± 2.5	21.1 ± 1.2	29.1 ± 4.6
COD_T	g O_2 L^{-1}	17.2 ± 1.0	24.1 ± 3.5	33.2 ± 2.5	36.6 ± 3.8	54.8 ± 8.34
CODs	g O_2 L^{-1}	3.9 ± 1.0	5.5 ± 0.5	8.6 ± 2.9	7.2 ± 0.1	19.3 ± 6.5
BOD_5	g O_2 L^{-1}	2.0 ± 0.6	3.3 ± 1.1	4.4 ± 0.1	4.9 ± 0.1	12.9 ± 0.1
Total Nitrogen	g L^{-1}	3.2 ± 0.3	4.5 ± 0.6	5.8 ± 0.3	6.7 ± 0.6	8.0 ± 0.8
NH_4^+-N	g L^{-1}	2.2 ± 0.2	2.9 ± 0.2	2.8 ± 0.2	3.0 ± 0.2	3.7 ± 0.4
NH_3-N	g L^{-1}	0.1 ± 0.1	0.3 ± 0.1	1.1 ± 0.2	1.4 ± 0.2	1.6 ± 0.3
Acetic acid	mg L-1	376 ± 301	646 ± 168	1647 ± 858	1,836 ± 919	3,117 ± 1,526
Propionic acid	mg L-1	35 ± 10	173 ± 66	629 ± 614	713 ± 623	1,582 ± 521

n.d: non detected

The increment in biogas production was not completely proportional to the loading rate (Table 1). Apparently, at higher loading rates digestion of the algal biomass was no longer complete which eventually led to substrate overload causing a reactor failure (Fig. 1). This was apparent from: (i) the drop in biogas production at the end of period V (Fig. 1), (ii) the decreasing methane content of the biogas at high loading rate and (iii) the increase in all the parameters related to organic matter in the sludge, TS, VS, total and soluble chemical oxygen demand (COD) and five day biological oxygen demand (BOD_5) (Table 1). This accumulation of organic matter was especially acute during period V with a 30% increase for TS, 37% for VS, 50% for total organic matter (COD_T), and 163% for BOD_5 compared to period IV. Soluble organic matter, acetic acid, propionic acid and NH_3 also accumulated in period V (Fig. 1, Table 1). *Spirulina* is a protein rich substrate [18], therefore its nitrogen content is high, which led to the accumulation of total nitrogen in the sludge (Table 1). The degradation of proteins leads to the production of ammonia nitrogen (NH_3-N) which increased gradually from 0.1 g L^{-1} in in period I to 1.6 g L^{-1} in period V (Table 1, Fig. 1). This increase can be attributed both to the increase in substrate concentration and, according to the equation of Anthonisen *et al.* [20] (eq 1), to the increase of pH

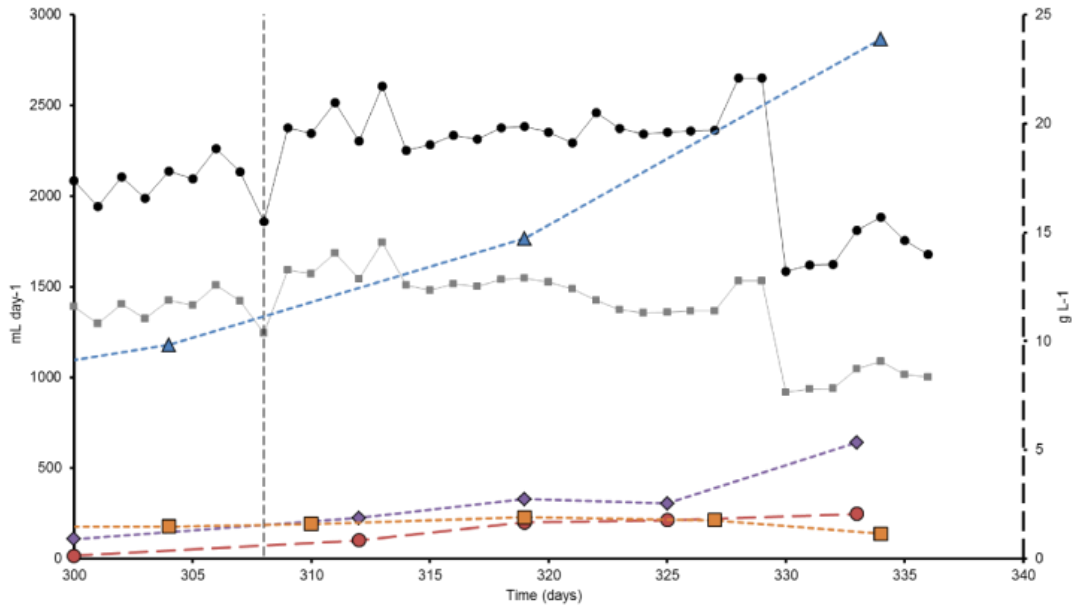

Fig. 1 Biogas, methane production and sludge characteristics
Left axis: daily biogas (●) and methane (Gray-■) production normalized to standard temperature and pressure conditions (20°C; 0 atm); **Right axis:** Soluble COD (Blue- ∆); acetic acid (Purple-◊); propionic acid (Red-o), NH_3-N (Orange-■) obtained from the anaerobic digestion of *Spirulina.* Vertical dashed line indicates a change in the organic loading rate from 4.0 g *Spirulina* L^{-1} day^{-1} to 5.0 g *Spirulina* L^{-1} day^{-1}.

from 7.5 to 8.6. Methanogens are sensitive to ammonia [21] and the accumulation of this compound can lead to digester failure. The reported levels of free ammonia nitrogen in period V are high and comparable to other reported levels that caused reactor inhibition [21–24]. The accumulation of non-degraded biomass and VFAs along with the relatively high total nitrogen (8.0 g L^{-1}) and free ammonia nitrogen (1.6 g L^{-1}) concentrations led to the reduction of the biogas production from 2,651 mL day^{-1} on day 329 to 1,586 mL day^{-1} on day 330 (Fig. 1) which might indicate a substrate overload in the digester.

One of the main bottlenecks of the anaerobic digestion of microalgae is its apparently low biodegradability, which result in low methane yields [25]. To overcome this problem long HRT need to be applied in order to increase the residence time allowing the substrate to be further hydrolyzed [8]. However, this on the long run can have a negative effect on the biogas production as accumulation of inhibitory substances such as ammonia can occur [8, 26]. The optimal organic loading rate for freeze dried *Spirulina* in our experiment was 4.0 g *Spirulina* L^{-1} day^{-1} (period IV). At this OLR, the specific biogas production (SBP) per gram of *Spirulina* was 350 mL biogas with 68% methane content (Table 1). The highest biodegradability was

Table 2 Sequencing statistics

Dataset-1 sequencing data and assemblies statistics.

	Bases	Reads	Mean Read length	GC %
Sequencing data				
PGM raw data	1 GB	5,630,598	155 (bp)	38
Post Trimmomatic data	974 MB	5,240,830	185 (bp)	38

Assembled contigs							
Assemblies	**# Submitted reads**	**Minimum read length**	**# Contigs**	**# Contigs >500bp**	**N50 contig size**	**Mean contig size**	**Largest contig size**
Assembly A*	5,240,830	50 (bp)	54,246	21,998	3,810 (bp)	1,807 (bp)	171,327 (bp)
Assembly B*	5,240,830	50 (bp)	278,958	30,987	1,226 (bp)	1,115 (bp)	18,073 (bp)
Assembly C*	1,984,110	220 (bp)	27,994	14,915	4,380 (bp)	1,899 (bp)	75,139 (bp)

* See Material & Methods for details about assembly settings.

obtained, 42% and no accumulation of inhibitory substances occurred in this period. Both values, SPB and biodegradability are similar to those observed in other studies [5, 7, 10]. Our results show that 20 days HRT seem to be an adequate compromise between an optimal methane yield and accumulation of toxic compounds.

Metagenome analysis of the anaerobic digester community

DNA was extracted from the sludge of the *Spirulina* digester and sequenced on a 318^{TM} Chip with the Ion Torrent Personal Genome Machine (PGM) platform. Obtained sequence reads were quality trimmed (Table 2) (see Material and methods for details) and the remaining reads were either assembled into contigs or analyzed directly with the MG-Rast metagenome pipeline.

Binning and 16S rDNA taxonomy analysis

Three different assemblies were used for the detection of ribosomal 16S genes to taxonomically characterize the microbial community (see Material and Methods for details). Assembly A produced the largest contig, 171,327 bp (Table 2). Due to the stringent settings, assembly B produced the highest number of contigs but shorter, while assembly C (only reads with a minimum read length of 220bp were assembled) produced contigs with the longest mean size (Table 2).

Contigs from assembly A were binned using the Metawatt v1.7 pipeline to investigate the most abundant populations of the microbial consortium in more detail. From the 113 obtained bins, after manual selection and curation, 10 remained which displayed characteristic tetranucleotide frequencies, assembly coverages and consistent phylogenetic signature and together accounted for almost 80% of the total sequence data (Table 3; Additional File-1 Suppl. Fig. 1). As was observed in other anaerobic

Table 3 Characteristics and taxonomical classification of selected microbial bins
Characteristics and 16S rDNA taxonomical classification of the 10 selected bins obtained from the *Spirulina* metagenome.

Bin characteristics									
Bin	**Contigs (#)**	**Size (Mb)**	**N50 contig length (Kb)**	**GC (%)**	**Cov (X)**	**tRNA (#)**	**Conserved genes (#)**	**Abun (%)**	**16SrDNA taxonomical classification**
A	443	1.87	9.8	32.0	66.8	21	113 / 139	14.9	*Un. Tissierella;*
B	489	2.58	17.2	29.8	42.4	19	105 / 139	13.1	*Un. Clostridiales;*
C	443	2.40	13.0	31.9	36.6	21	79 / 139	10.5	*Un. Clostridiales*
D	468	3.37	22.5	42.0	22.3	77	103 / 139	9.0	*Proteiniphilum*
E	384	1.91	16.2	47.3	33.3	49	92 / 139	7.6	*Un. Anaerolineaceae*
F	397	2.44	27.7	31.1	23.4	49	106 / 139	6.9	*Un. Thermotogaceae*
G	225	2.53	39.5	51.6	21.8	71	164 / 139	6.5	*Unknown*
H	879	3.05	7.9	37.8	11.5	44	82 / 139	4.2	*Un. Bacteroidetes*
I	4,267	4.10	1.6	49.2	7.1	70	75 / 139	3.5	*Unknown*
J	8,444	5.11	0.7	55.5	5.6	31	161 / 139	3.5	*Un. Methanomicrobia*

Cov: Coverage
Abun: Abundance
Un: Uncultured

digesters, populations affiliated with Firmicutes were most abundant, and constituted 38% of the total community [13–16, 27], followed by Bacteroidetes (abundance approx. 13%), Chloroflexi (8%) and Thermotogae (7%). Euryarchaeota represented only 3.5% of the total abundance. Two bins of unknown taxonomic origin accounted for 10% of the sequenced data (Table 3).

16S rDNA sequences corresponding to 8 of the 10 bins were identified among the contigs of the three different assemblies and/or recovered independently by iterative read mapping with EMIRGE (Fig. 2a, Additional File-2 Suppl. Table 1). Unfortunately, the three 16S sequences obtained by EMIRGE and affiliated with Firmicutes (EM-1, EM-2 and EM-3) could not be assigned conclusively to any of the three Firmicutes bins (A, B and C). However, based on comparison of sequencing coverage between the recovered EMIRGE 16S sequences and the contigs in the Firmicutes bins it was most likely that the dominant organism, Bin-A, 14.9% abundance, (Table 3) was most closely related to *Tissierella praeacuta* (EM-1, Fig 2a). Bins B and C were assigned to uncultured Clostridiales (Table 3). The 16S rDNA sequence classified as *Atopostipes* (contig01659 of assembly A) was assembled well in all three assemblies, yet it could not be linked to any of the bins. Based on the low sequencing coverage of this 16S sequence, the population was probably of relatively low abundance (Additional File-2 Suppl. Table 2).

As in a previous study of anaerobic digestion in which a proteinaceous substrate was used, Clostridiales were the most abundant bacterial order [27]. The genus

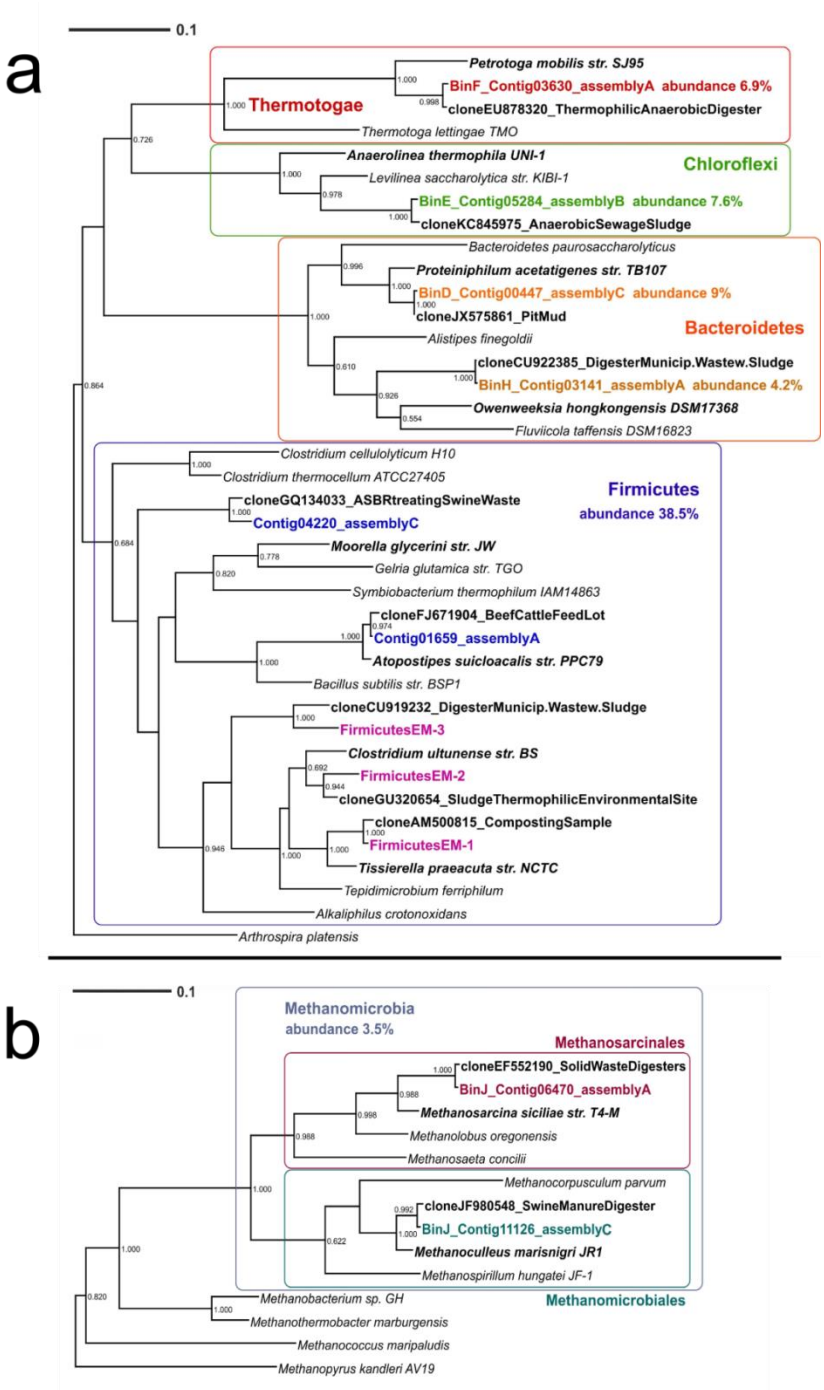

Fig. 2 16S rDNA bacterial and archaeal phylogenetic trees

Phylogenetic trees of **(a)** Bacterial and **(b)** Archaeal 16S rDNA sequences. Although the binning was only performed with contigs of assembly A, most of the binned 16S rDNA sequences were also assembled in assemblies B and C. If so, for the tree the longest assembled sequence was chosen. 16S rDNA sequences not assembled in assembly A, but in B or C, or detected by EMIRGE are also included. Colored: sequences obtained from metagenomic reads. Assignment to Metawatt bins is indicated if applicable. Reference sequences in **bold**: top hits in blast search against NCBI non-redundant nucleotide collection, ***bold+italics***: top hits in blast search against NCBI reference RNA sequences. Additional reference sequences in the Bacteria tree represent genera detected in other anaerobic digesters. 16S rDNA sequences of *Arthrospira platensis* and *Methanopyrus kandleri* were chosen as outgroups, respectively. Bootstrap values at nodes are obtained from 500 replicates and are only shown for branches with at least 50% support (values > 0.499). The scale bar represents 0.1 nucleotide substitutions per site. Accession numbers of reference sequences are available in Additional File-2 Suppl. Table 7.

Tissierella has already been detected in other anaerobic digesters yet in much lower abundance [14, 28, 29]. The specific function of members of this taxon in anaerobic digesters is still not clear. However, members of this genus are known to require the presence of certain amino acids and formate for growth and they seem to be unable to utilize carbohydrates such as glucose, cellobiose or xylose [30] which is in accordance to the type of substrate used in our experiment. *Proteiniphilium*, (Bin-D, Table 3) a member of the Bacteroidales that utilizes peptone and is unable to grow on carbohydrates [31], was previously identified in several biogas studies [12, 14, 28, 32]. Anaerolineales and Thermotogales were also identified in other biogas reactors but in much lower abundance [15, 33, 34]. Their function in anaerobic digestion is not clear yet, however, their relatively high abundance when compared with other anaerobic reactors could be explained by the fact that they are known to utilize proteins as substrate [35, 36], therefore they might play an important role in the degradation of protein rich *Spirulina*.

Among the Archaea, we identified one bin, Bin-J, with 3.5% of abundance, for which the 16S rDNA fragments were classified as uncultured Methanomicrobia (Table 3). Methanomicrobiales were also identified as the most abundant methanogens in an anaerobic reactor fed exclusively with casein [27, 37]. A closer look at the 16S rDNA phylogeny (Fig. 2b and Additional File-2 Suppl. Table 1) suggests the possibility that two methanogenic populations may have been binned together, one related to Methanomicrobiales and one to Methanosarcinales. Members of both these orders are frequently encountered in anaerobic digesters [13, 38] and they use H_2, CO_2, formate and acetate as their C source [14, 39]. Formate and acetate are both fermentation products of Clostridiales and Anaerolineales [30, 35], both populations present in high abundance in our experiment (Table 3).

Effect of substrate at taxonomic and functional level

In parallel to this genome-focused analysis, the effect of substrate on both taxonomic composition and presence of functional genes was also studied at the level of individual sequences, by comparing the unassembled reads from our study to a publicly available metagenome from a biogas plant using the MG-Rast platform. Sequencing dataset *Spirulina*-S1, was obtained from our anaerobic reactor fed with *Spirulina*, a protein rich substrate, while the second sequencing dataset, Maize-Rye

Table 4 Comparison of identified COG/NOG and specific Pfams
Comparison of the COG/NOG categories obtained with the MG-Rast platform (E-value $1e^{-5}$; min 60% identity; 15 bp min length) and the selected Pfams obtained with the Hmmscan (E-value cutoff 1.0).

	***Spirulina*-S1 dataset**		**Maize-Rye dataset**	
Initial # reads	1,019,333		1,019,333	
Initial # ORF	6,115,998		6,115,998	
	Hits	**%**	**Hits**	**%**
COG/NOGs				
Total # hits	215,831		344,104	
Information Storage and Processing	48,761	22.6[a]	86,925	25.3[a]
Cellular processes and signaling	38,183	17.7[a]	60,697	17.6[a]
Metabolism	89,707	41.6[a]	125,357	36.4[a]
Poorly characterized	39,180	18.2[a]	71,125	20.7[a]
AaM / CM Ratio[b]	1.26		0.89	
PFAMs				
Total identified PFAMs	1,132,766		2,205,177	
Cellulose degradation PFAMs	1,881	0.17[c]	6,554	0.30[c]
Amino acid degradation PFAMs	10,628	0.94[c]	16,052	0.73[c]
Protein degradation PFAMs	7,148	0.63[c]	11,498	0.52[c]
Ratio Proteases / Cellulases (P/C)	3.80		1.75	
Ratio Amino acid / Cellulases (A/C)	5.65		2.45	

a % of total identified COG/NOGs
b “Amino acid transport and metabolism” / “Carbohydrate transport and Metabolism”
c % of total identified PFAMs

(M-R), was originating from a biogas plant fed with a mixture of substrates, mainly cellulose rich substrates [13].

Effect on the microbial community composition

The taxonomic composition of the *Spirulina* reactor metagenome obtained from the two analyses, single read analysis with the MG-Rast pipeline and binning of assembled contigs, was not identical but similar. Therefore, a comparison of the *Spirulina*-S1 data with the M-R data set based on single read analysis was feasible.

The general taxonomic composition did not appear to depend on the type of substrate used. Based on the MG-Rast M5NR analysis of the metagenomic reads, bacteria clearly dominated in both datasets while Archaea represented less than 10% in the M-R dataset and merely 3% in the *Spirulina*-S1 dataset (Table 4). Amongst bacteria, Firmicutes, Bacteroidetes and Proteobacteria dominated in both sets, but differences could be seen in the abundances of other phyla. Hits in Thermotogae were more abundant in the protein rich substrate digester data (5.4%) compared to 2% in the cellulose rich substrate digester dataset. This was also true for Chloroflexi, 2.7%

in *Spirulina*-S1 when compared to the M-R dataset, 1.3%. On the other hand, within the Archaea such a variety at phylum level was not seen, and as expected, Euryarchaeota dominated with over 90% of all Archaea in both datasets.

Among the Bacteria, Clostridiales dominated in both datasets with 40% and 30% of the total assigned reads in the *Spirulina*-S1 dataset and the M-R dataset respectively, followed by Bacteriodales, Thermoanaerobacterales and Bacillales (Fig. 3a). In both datasets, M-R and *Spirulina*-S1, the genus to which most of the reads were assigned was Clostridium (18 and 14% of recruited reads respectively) (Additional File-2 Suppl. Table 3). Furthermore, as in the case of Kovács *et al*. [27], who used casein and pig blood as substrate, a relatively high number of reads in the *Spirulina*-S1 dataset (almost 40,000 reads – 6% of total hits) were most similar to members of the genus *Alkaliphilus*, which is in contrast to the M-R dataset, where only about 2% of the reads were assigned to this genus (18,000 hits). Bacteroides, who recruited 7.5% of the hits in the M-R dataset accounted for 4.1% in the *Spirulina*-S1 dataset. Among other substrates, Bacteroides are known to utilize cellobiose and xylose [32], both absent in *Spirulina*, which could explain their lower abundance in our reactor. Interestingly, *Candidatus Cloacamonas*, which accounted for 4,1% of the bacteria M-R reads, 36,032 hits, recruited less than 0.1% of the hits in the *Spirulina*-S1 dataset (280 hits) (Additional File-2 Suppl. Table 3). This bacterium was also present in high abundance at the initial adaptation period of two biogas reactors fed with casein and pig blood and its detection was not possible after 12 weeks of substrate adaptation, which might indicate that it cannot survive without a source of carbohydrates [27]. Major differences regarding bacterial taxa were also seen for Thermotogales and Anaerolineales which were considerably more abundant in the dataset from the protein rich substrate digester, with 5 and 1% respectively when compared to the dataset from the cellulose rich substrate biogas plant, 1.7 and 0.1% respectively (Fig. 3a). These two orders are known to include bacteria which utilize proteins as substrate [34, 35, 37].

Among the Archaea orders, Methanomicrobiales clearly dominated in the M-R dataset recruiting almost 75% of the hits. In the *Spirulina*-S1 dataset, a co-dominance of Methanosarcinales (31% of the hits) and Methanomicrobiales (29% of the hits) was observed (Fig. 3b; Suppl. File-2 Table-3), confirming the analysis of the assembled 16S rDNA sequences (Fig. 2b). Methanosarcinales are known to be able

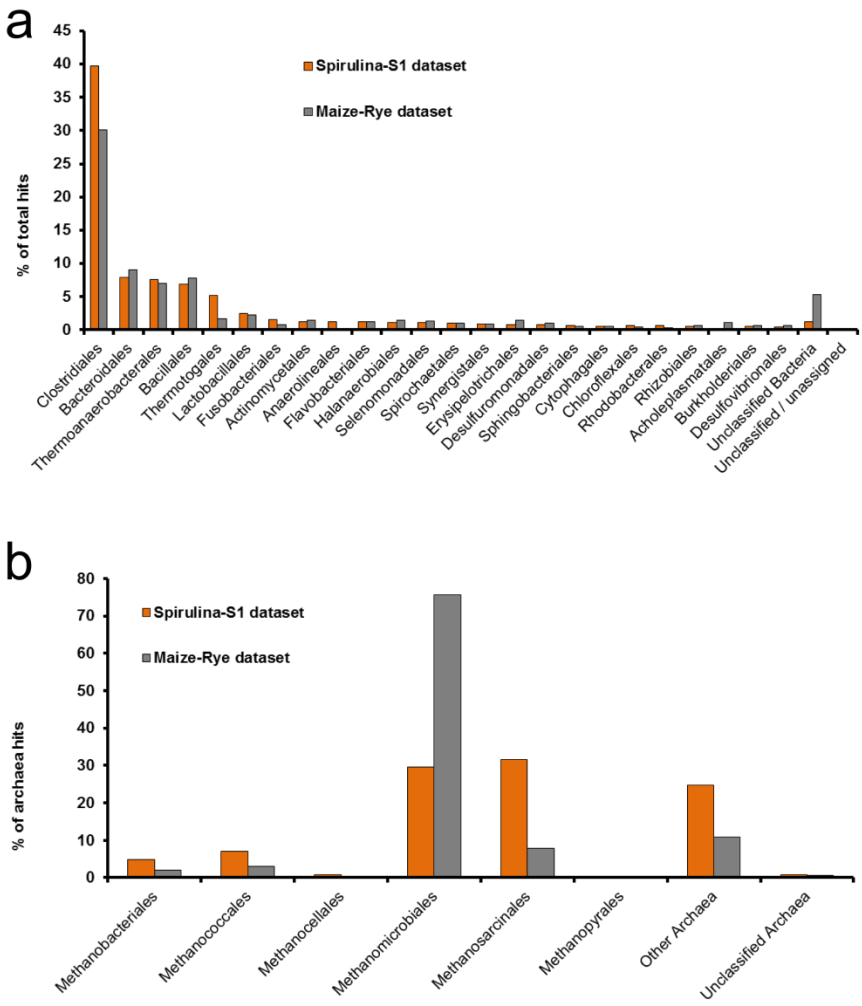

Fig. 3 Comparison of the taxonomic classification of *Spirulina*-S1 and M-R reads at order level
Percentage of the total taxonomic assigned reads of each dataset obtained with MG-Rast M5NR representative hit tool (E-value $1e^{-5}$; min 60% identity; 15 bp min length). **(a)** Bacterial orders with the 20 most abundant assigned read hits and **(b)** all Archaeal orders with assigned hits from the metagenomic reads.

to use acetate, H_2 and CO_2 as substrate [39], which are the main fermentation products of *Tissierella* and other Clostridia [30], and they are usually dominant in reactors where VFAs and NH_3 are present in high concentration as in our case (Fig. 1) [41, 42]. On the other hand, Methanomicrobiales do not use acetate but can grow on H_2, CO_2 and formate [43] the latter a common fermentation product of bacteria belonging to the Chloroflexi phylum [35] which in our dataset represent almost 8% of the abundance (Table 2). This combination of factors, high VFAs and ammonia plus the production of formate, H_2 and CO_2 can explain why the protein rich reactor was not dominated by one single methanogen. The observed co-dominance of two

methanogenic populations, as in our *Spirulina* reactor, was also observed in several other studies [15, 28, 44]. For example Ziganshin *et al* [44] observed co-dominance of *Methanoculleus* and *Methanosaeta* in reactors fed with cattle manure and dried distillers grains, and of *Methanosarcina* and *Methanoculleus* in reactors treating maize straw and cattle manure, while Li *et al* [15], also detected the same co-dominant methanogens in a reactor treating multiple substrates (chicken waste, pig manure and excess sludge). This co-dominance of methanogens detected in such a broad range of substrates might indicate that, rather than the type of used substrate, the characteristics of the sludge (NH_3, VFAs, temperature, pH, etc.) and the initial type of inoculum (wastewater, manure, etc.) determine which Archaea become dominant in anaerobic digesters.

In order to see whether the diversity of the species community was affected by the used substrates, the species diversities in both datasets, were compared with Lorenz curves and Simpson's diversity index (SDI) [45–47]. The mixture of substrates used in the biogas plant (maize silage, 63%; green rye, 35% and chicken manure, ~2%), might lead to a higher bacterial diversity than in the reactor solely fed with *Spirulina*. However, Lorenz curves, describing population evenness, of both datasets were similar (Additional File-1 Suppl. Fig 3a), as well as Simpson's diversity index values (SDI=0.0078 for the *Spirulina*-S1 dataset and SDI=0.0062 for the M-R dataset; where SDI=1 indicates low diversity and SDI=0 indicates high diversity). The similarity in evenness and diversity in both bacterial populations can be explained by the fact that *Spirulina* as such is not a "simple" substrate, as would be glucose, starch or glycerol, and therefore needs a microbial population with a certain complexity to be fully digested. Apparently, the differences between the substrate types (complex mono-substrate or substrate mixture) used in the two compared systems did not affect the diversity of the whole population, yet rather the abundance of certain bacterial taxa (Fig. 3).

For Archaea, on the other hand, Lorenz curves and SDI indicated a difference in their diversity in the two studied datasets. The archaeal population in the *Spirulina* reactor was more even than the archaeal community in the maize-rye biogas plant (Additional File-1 Suppl. Fig 3b). Given the same species richness, a more even population is also more diverse. Indeed, by calculating the SDI a higher diversity for the archaeal community in the *Spirulina* reactor was observed (SDI=0.0761) than in

the M-R biogas plant (SDI=0.3695). The lower SDI for the Archaea in the M-R dataset is best explained by the clear dominance of *Methanoculleus* in the archaeal reads of this dataset (Fig. 3b).

Taken together, the results suggest that the type of substrate used in anaerobic digestion mainly affects the bacterial composition, to some extent, at low taxonomic levels, especially at genus and species level. Proteolytic Bacteria were probably present in all dominant phyla of the *Spirulina*-S1 digester, whereas in the Maize-Rye biogas plant, cellulolytic Bacteria were dominant. Regarding the Archaea, an influence of the substrate on their presence and composition is not as clear as for the Bacteria. This could be explained by the fact that the Archaea perform the final step in the process of anaerobic digestion, and their presence is probably more dependent on the population composition of the primary substrate degraders (Bacteria), of their metabolic products, the presence or absence of inhibitory compounds and the origin of the inoculum, rather than on the substrate itself.

Effect on the abundance of functional genes

Both datasets, *Spirulina*-S1 and M-R were compared at functional level with two approaches, MG-Rast's COG/NOG comparison, and identification of specific protein domains (Pfams) related to the cellulose degradation pathway and to protein and amino acid degradation (Table 4).

Due to the different types of substrates used, it was expected to see differences regarding the COG/NOGs related to amino acids and protein metabolism. However, with respect to the detected functional genes, the differences between both datasets were minor (Fig. 4). The highest difference was observed in category L "Replication, recombination and repair" which represented 8.6% of the hits in the *Spirulina*-S1 dataset and 11.6% in the M-R dataset. This category contains COGs related to groups of genes which participate in the replication process of the microbial community and were more abundant in the M-R dataset. This difference could be explained by the fact that at the time of sampling of the *Spirulina*-S1 dataset, day 336, the reactor was suffering from substrate overload which resulted in an accumulation of toxic compounds (NH_3, VFAs) that hindered the correct function of the bacteria and reduced the daily biogas production (Fig. 1). COG/NOGs related to category E, "Amino acid transport and metabolism" were slightly more abundant in the protein

rich dataset (9.7%), than in the cellulose rich dataset (7.3%) while COGs from "Carbohydrate transport and metabolism" (category G), were less abundant in the *Spirulina*-S1 dataset 7.7% than in the M-R dataset 8.1% (Fig. 4). Amongst the amino acid metabolism related COGs, some differences could be seen. For example, COG4608, an "Oligopeptide transport system", recruited 0.32% of all assigned hits in the *Spirulina*-S1dataset and 0.22% in the M-R dataset. Similar differences were seen in other amino acid COGs such as COG1164 an" Oligopeptidase" and COG2195 a "Di and tri-peptidase" amongst many others. In contrast, important differences could be seen among the Carbohydrate category GOGs, especially in those COGs directly related to the degradation of cellulose, xylanose and other complex sugars. For example, COG3507/COG3664 a "Beta-xylosidase" represented 0.10% in the M-R dataset and only 1/10 of this abundance (0.01%) in the *Spirulina*-S1 dataset. Similarly, COG2160, an "L-arabinose isomerase", was almost absent in the *Spirulina*-S1 dataset (0.005% of all assigned hits) but represented almost 0.05% in the M-R dataset. Similar results were observed with other COGs such as COG3693 a "Beta-1,4-xylanase" and COG0366 a "Glycosidase" which were both significantly less abundant the *Spirulina*-S1 dataset. This difference in the abundance of COGs correlates with the type of substrate used, *Spirulina*, which has low content of complex sugars [18]. These differences in percentage might be subtle for some of the COGs but they still indicate that the selected microbial community might display particular functions in response to the used substrate.

Since the COG/NOG categories only give information about general function, specific protein domains (Pfams) related to cellulose degradation and to protein and amino acid degradation were searched for in both datasets (Additional File-2 Suppl. Tables 4, 5 and 6). Pfams domains associated with cellulose summed up to 0.30% of the total identified Pfams in the M-R dataset compared to 0.17% in the *Spirulina*-S1 dataset (Table 4). The proteases related Pfams were slightly more abundant in the *Spirulina*-S1 dataset with 0.63% of all identified Pfams compared to 0.52% in the M-R dataset (Table 4). Also the amino acid degradation Pfams were more abundant in the *Spirulina*-S1 reads (0.95% of all the Pfams) than in the M-R reads (0.74%).

As the percentages of abundances were relatively low, it is not certain that the substrates significantly affected the microbial community at functional level. Two Pfam ratios, Proteases to Cellulases ratio (P/C ratio), and Amino acid to Cellulase

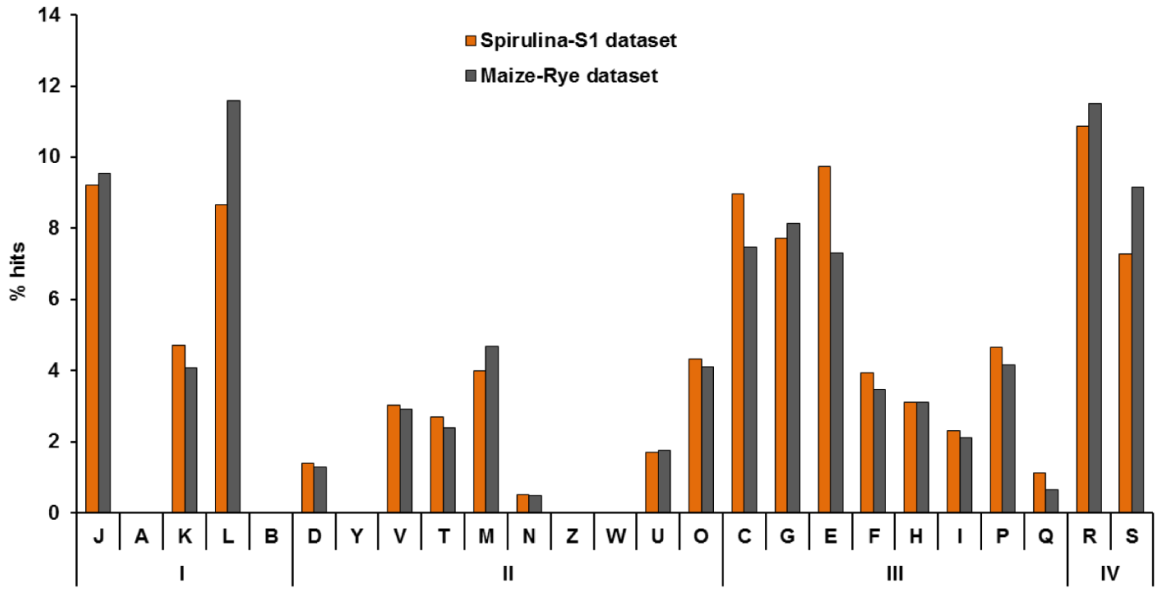

Fig. 4 COG/NOG functional hierarchical classifications
Comparison of the COG/NOG classified reads of the *Spirulina*-S1 and the M-R metagenomes obtained with the MG-Rast metagenome analyzer (E-value $1e^{-5}$; min 60% identity; 15 bp min length). **X axis: (I) Information storage and processing**: **A,** RNA processing and modification; **B**, chromatin structure and dynamics; **J**, translation, ribosomal structure and biogenesis; **K**, transcription; **L**, replication, recombination and repair; **(II) Cellular processes and signaling: D**, cell cycle control, cell division, chromosome partitioning; **M**, cell wall/membrane/envelope biogenesis; **N**, cell motility; **O**, posttranslational modification, protein turnover, chaperones; **T**, signal transduction mechanisms; **U**, intracellular trafficking, secretion and vesicular transport; **V**, defense mechanisms; **W**, extracellular structures; **Z**, cytoskeleton; **(III) Metabolism: C**, energy production and conversion; **E**, amino acid transport and metabolism; **F**, nucleotide transport and metabolism; **G**, carbohydrate transport and metabolism; **H**, coenzyme transport and metabolism; **I**, lipid transport and metabolism; **P**, inorganic ion transport and metabolism; **Q**, secondary metabolites biosynthesis, transport and catabolism; **(IV) Poorly characterized: R**, general function prediction only; **S**, function unknown.

ratio (Aa/C) were also calculated to determine the relative abundance of each group. The P/C ratio in the *Spirulina*-S1 dataset, 3.80, was double the P/C ratio in the M-R dataset, 1.75, and a comparable result was obtained with the Aa/C ratios, 5.65 for the *Spirulina*-S1 and 2.45 for the M-R dataset (Table 4). When a COG/NOG "Amino acid transport and metabolism" to "Carbohydrate transport and metabolism" ratio was calculated (AaM/CM), the result was similar, 1.26 for the *Spirulina*-S1 dataset and 0.89 for the M-R dataset (Table 4). The differences in the obtained P/C, Aa/C and AaM/CM ratios suggest that the microbial community in both reactors did adapt to the type of substrate degraded, protein or cellulose rich. Since both substrates were not either pure carbohydrates or proteins, but consisted of both in different proportions, further studies are necessary to determine whether the observed differences in the presence of functional genes would become more distinct for longer run times of the

Spirulina digester, or if the microbial community was already at functional equilibrium when it was sampled for the metagenome analysis presented here.

Binning of contigs vs classification of single reads

In this work, the same metagenome has been taxonomically analyzed by two different approaches, assembly of reads into contigs followed by a binning strategy combined with a16S rDNA analysis, and blasting of single reads against a general nucleotide database. Despite the differences in methodology, the results obtained can be extrapolated; the microbial picture is very similar in both cases (Table 3; Fig. 3). The advantage of using the binning approach is that the obtained bins form a consensus genome of the most abundant organisms which better represent the microbial community and its abundances (Table 3), and the 16S rDNA taxonomical classification of contigs is more reliable than the classification of short reads. In contrast, MG-Rast classification of short reads only gives information about the taxa but does not provide any extra information on the populations abundances. In this sense it can be argued that 80% of the genes identified by MG-Rast belong to the 8 bins which together account for 80% of the abundance even though they have, based on single read assignment, been assigned to many different taxa.

Conclusions

The results presented in this study indicate that *Spirulina* could be a suitable substrate for the production of biogas with a mean production of 350 ml of biogas per gram of substrate. As in previous studies, a dominance of Clostridia and Bacteroidetes was observed in bacteria while Methanomicrobiales and Methanosarcinales dominated amongst the archaea. The microbial community present in the anaerobic digester adapted to the type of substrate used, both in taxonomy and function. Binning of contigs and 16S rDNA analysis gives reliable information about the identification and abundances of the different bacterial populations, in contrast to the traditional classification of reads.

Material and methods

Bioreactor set-up

One 2.0 L semi-continuous stirred tank reactor (S-CSTR) with a working volume of 1.5 L, operated at 37°C with 20 days hydraulic retention time (HRT) was set up to study the anaerobic digestion of freeze dried *Spirulina*. The overall experiment lasted 440 days which included a 33 days adaptation to *Spirulina* and a 71 days start-up period. The remaining 336 days were divided into 5 periods (P-I to P-V) in which the organic loading rate (OLR) was gradually increased from 1.0 g to 5.0 g *Spirulina* L^{-1} day^{-1} (dry weight). The inoculum was obtained from a local wastewater treatment plant (Heepen Klaerwerk, Bielefeld, Germany) and the substrate, freeze dried *Spirulina,* was acquired from Sonnenmacht GmbH (Germany). Biogas production was measured with an on-line Milligascounter MGC-1 equipped with the Rigamo software v3.0 (Ritter Engineering, Germany) and normalized to standard conditions (0 °C; 1.0 atm). pH and redox potential were monitored, but not controlled, with Mettler Toledo pH (HA405-DPA-SC-S8/225) and redox (Pt4805- DPA-SC-S8/225) probes (Mettler Toledo GmbH, Germany). Mesophilic conditions were obtained with a Pt-1000 temperature sensor and a heater. In order to avoid rupture of the bacterial granules, constant stirring was performed with a floating magnet (Fisher Scientific GmbH, Germany). Daily purge and feed were performed manually with a syringe. Before purging, the biomass was settled by stopping the stirring for at least 30 minutes. Periodically the purged sludge was sampled for analysis; in that case the stirring was not stopped. The medium used to dissolve the freeze dried *Spirulina* for dosing at the desired OLR was modified after Vidal *et al.* [48] excluding the NH_4Cl. The *Spirulina* mixture was prepared freshly once per day.

Analytical methods

The performance of the laboratory digester was continuously monitored by the on-site pH probe, the biogas measurement device and by periodical analysis of alkalinity. Carbon dioxide content of the biogas was determined daily by bubbling the produced biogas through an alkaline solution (KOH 50 g L^{-1}). Biogas composition was determined once a week by means of a Shimadzu GC-2010 plus Gas Chromatograph (Shimadzu Corp, Japan) equipped with an Agilent GS-Gaspro capillary column (part # 113-4362) (Agilent Technologies, USA). Samples for biogas

quality and composition were obtained using an airtight syringe. If biogas composition was not analyzed immediately, samples were kept in gas-tight vacutaniers (BD-Plymouth, UK). Analyses to characterize the liquid effluent were carried out periodically. Total solids (TS) and volatile solids (VS) were analyzed once a week following the APHA standard methods [49]. Five day biological oxygen demand (BOD_5) was analyzed with a WTW Oxitop® according to the APHA 2005 5210D procedure. Alkalinity, total and soluble chemical oxygen demand (COD_T and COD_S), total nitrogen (TN) and ammonium nitrogen (NH_4^+-N) were analyzed by colorimetric methods (Hach Lange GmbH, Germany). The free ammonia (NH_3-N) concentration was calculated by as in Astals et al [50]. Analyses were performed directly to the raw sample or to the soluble fraction by centrifuging the samples at 4,600 rpm for 5 minutes and filtering the supernatant through a Rotilabo CME 0.45 µm nylon filter (Carl Roth GmbH, Germany). Specific volatile fatty acids (acetate, propionate, iso-butyrate, n-butyrate, iso-valerate and n-valerate) were analyzed using a Shimadzu GC-2010 plus Gas Chromatograph coupled to an FID detector and equipped with a Macherey-Nagel Optima FFA plus capillary column (Macherey-Nagel GmbH & Co. Germany).

Bioreactor adaptation and start-up

The start-up of the bioreactor consisted of an adaptation period for the microbial community to the use of *Spirulina* as the main substrate. To do so, initially the reactor was fed with plain glucose (1.66g L^{-1} day^{-1}) which was gradually substituted for freeze dried *Spirulina*, following the substitution strategy from Vergara-Fernandez et al. [51] until only *Spirulina* was fed at 1.66 g L^{-1} day^{-1}. Once the microbial community was adapted to *Spirulina*, the bioreactor was fed with 2.0 g *Spirulina* L^{-1} day^{-1} until the biogas production and the process parameters (alkalinity, TS and VS) were constant. In order to start the experiment with the lowest possible residual *Spirulina* biomass in the bioreactor's sludge the feeding was stopped until the biogas production was below 100 mL day^{-1}. After the starvation period, the study of the anaerobic digestion of freeze dried *Spirulina* began.

Metagenome analysis

DNA sample preparation, sequencing and quality trimming

15.0 mL of sludge obtained from the bioreactor digesting freeze dried *Spirulina* were used for DNA extraction (sampling day 336, biogas production, 1,676 mL biogas day^{-1}, methane content 60%). DNA was extracted according to Zhou *et al.* [52] with minor modifications. 2.5 µg of extracted DNA were used to prepare a 200bp insert size sequencing library for the Ion Torrent Personal Genome Machine (PGM) platform (Life Technologies, USA). The instructions according to the Ion Xpress™ - Plus gDNA Fragment Library Preparation manual were followed, except for the initial DNA fragmentation, which was done using a GS FLX Standard Nebulizer Kit (Roche Applied Science, Germany), nebulization for 3 min at 32 psi. Sequencing template preparation was performed using the OneTouch Instrument and the OneTouch ES module. Enriched ISP particles were sequenced with the Ion PGM™ 200 Sequencing Kit (Life Technologies, USA) on a 318™ Chip with 520 flows following the manufacturer's instructions. Automated analysis was performed with the Torrent Suite™ Software v3.2 using default settings. Additional quality filtering was done using the Trimmomatic tool v3 [53], with settings for removal of trailing bases of a q-value lower than 20, and removal of reads shorter than 50 bases.

Assembly of quality trimmed reads

Quality trimmed reads longer than 50bp were assembled into contigs by means of the Genome Sequencer De Novo Assembler Software v2.6 (Newbler assembler, Roche Applied Science, Germany). In total, three read assemblies were performed, one with default settings for genomic DNA (assembly A), one with more stringent settings for better assembly of 16S rDNA sequences (assembly B), according to Fan *et al*., 2012 [54] and a third one (assembly C) with default settings but using only reads with a minimum length of 220 bp in order to better assemble Clostridial sequences. Additionally, EMIRGE [55] was used to reconstruct 16S rDNA fragments that did not assemble with our procedures.

In-depth taxonomy analysis

Contigs from assembly A were binned into provisional whole genome sequences of abundant populations in order to taxonomically analyze the microbial population.

Contigs were binned, based on tetranucleotide pattern combined with interpolated Markov models (IMMs), and submitted to a blast search [56] against a database containing all bacterial genomes downloaded from NCBI on May 2013 (ftp://ftp.ncbi.nlm.nih.gov/genomes/bacteria/all.gbk.tar.gz) using the Metawatt v1.7 pipeline (http://sourceforge.net/projects/metawatt) (for further details concerning the binning pipeline see Strous *et al.* [17]). Binning options were set as follows: read length 200 nt; minimum bin size 100 kb and minimum contig size 500 bp. Generated bins were manually revised and assigned to a taxon by blasting all contigs from the selected bins against the 16S rRNA SILVA database [57]. Coverage and bin size of each particular bin were used to estimate the abundance of each population. Furthermore, transfer-RNAs of each bin were identified with ARAGORN [58] and the genome completeness for each population was estimated by the identification of 139 conserved Pfams as described by Campbell *et al.* [59].

Phylogeny of assembled 16S rDNA sequences

To identify 16S rDNA sequences among the assembled contigs, all contigs from the three assemblies were submitted to a blastn search against the RDP database (v10-32) [60]. Sequence parts with a hit were extracted and aligned parts with a minimum length of 1000 (Bacteria) or 500 bases (Archaea) were further analyzed. Together with the 16S rDNA fragments detected using EMIRGE, the assembled 16S rDNA sequences were submitted both to the RDP classifier [61] and the SINA classifier [62] with the confidence threshold or minimum sequence similarity set to 80% respectively. The sequences were also submitted to a blastn search against the current (Feb. 2014) NCBI nucleotide collection (nr/nt), and reference RNA sequences (refseq_rna). For both blastn searches the top blast hit for each query sequence was obtained. All sequences (contig parts, blast search hits, further representative 16S rDNA sequences) were aligned with muscle [63]. Phylogenetic trees were generated with FastTree [64] with the GTR+CAT model, bootstrapping (500 reps.) was done using seqboot (v3.67, http://evolution.genetics.washington.edu/phylip.html [65]), and the CompareToBootstrap.pl script (Price M. N., http://www.microbesonline.org/fasttree/treecmp.html) was used to implement the bootstrap values into the main tree. Trees were drawn using dendroscope [66].

Metagenome comparison

To determine if the substrate had any effect on the microbial community both in composition and function, a publicly available metagenome from a fully operational biogas plant treating mainly cellulose rich material: maize silage, 63%; green rye, 35% and low amounts of chicken manure, around 2%, was downloaded from the NCBI database (SRR034130.1) [14] and compared to our lab scale metagenome.

To compare both metagenomes the same normalization procedure as in Jaenicke *et al.* [14] was applied to the 50bp quality trimmed reads dataset which resulted in a dataset with 1,019,333 reads (*Spirulina*-S1). See Additional File-3 Supplementary Material and Methods for further normalization details.

Taxonomic and functional comparison

Spirulina-S1 dataset and the biogas plant dataset, Maize-Rye dataset (M-R), were uploaded to the MG-Rast pipeline. Taxonomy analysis was done with the M5NR representative hit classification while functional analysis was done with the COG/NOG classification both with an e-value of $1e^{-5}$, 60% minimum identity and 15 bp minimum length. Furthermore, specific protein domains (Pfam) related to cellulose degradation, and protein and amino acid degradation were identified in both datasets. In short, both datasets were first translated into amino acids and searched for open reading frames (ORFs) and subsequently blasted against the Pfam-A protein database [67]. See Additional File-3 Supplementary Material and Methods for details.

Microbial diversity

To assess the microbial diversity of both populations, *Spirulina*-S1 and M-R, two approaches were used: (i) the determination of the evenness by Lorenz curves [45, 68] and (ii) the determination of the diversity by calculating Simpson's diversity index (SDI) [46, 47]. These approaches were applied at species level (i) on the bacterial population and (ii) on the archaeal population.

Accession numbers

Metagenomic reads and assembled contigs are accessible via NCBI under the Bioproject PRJNA239997. The sequenced reads were submitted to the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/) with the sample accession

number SRS565943. Contigs of the tree assemblies (A, B and C) were submitted to GenBank, under the accession numbers JMBV00000000, JMBW00000000 and JMBX00000000. The versions described in this paper are versions JMBV01000000, JMBW01000000 and JMBX01000000. The sample numbers for the three assemblies are SAMN02727904, SAMN02727905, SAMN02727906; they are grouped in sample group SAMN02671764. The 11 16S rDNA sequences used for the generation of the phylogenetic trees in Fig. 2 were submitted to GenBank under the sample number SAMN03078811, with accession numbers KM851210-KM851220.

All metagenomes analyzed with the MG-Rast metagenome analyzer are publicly available with the following IDs: *Spirulina-S1* metagenome (4545162.3); Maize-Rye metagenome (4545349.3).

Abbreviations

Spirulina-S1: metagenomic sub-dataset 1 from the *Spirulina* reactor; M-R: Maize-Rye metagenome dataset. SDI: Simpson's diversity index; S-CSTR: semi-continuous stirred tank reactor; HRT: Hydraulic retention time; OLR: Organic loading rate; TS: Total solids; VS: Volatile solids; BOD_5: Five day biological oxygen demand; COD_T: Total chemical oxygen demand; COD_S: Soluble chemical oxygen demand; TN: Total nitrogen; NH_3-N: Ammonia nitrogen; NH_4^+-N: Ammonium nitrogen; VFAs: Volatile fatty acids; SBP: Specific biogas potential; SMP: Specific methane potential.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VNA designed and performed the experiments presented herein, evaluated the data and drafted the manuscript. MP performed part of the analysis, evaluated the data and drafted the manuscript. MS conceived the study, assisted in experimental design and in drafting the manuscript. HET supervised the work, assisted in evaluation of the data and drafted the manuscript. All authors participated in the experimental design, evaluation of the data, read and approved the final manuscript.

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Additional files

Additional File-1 “Supplementary Figures”

File contains:

- Suppl. Fig. 1 Contig assembly depth, GC content and taxonomic classification of selected bins.
- Suppl. Fig. 2 Phylogenetic tree of assembled Firmicutes 16S rDNA sequences from all three assemblies.
- Suppl. Fig. 3 Population evenness by means of Lorenz curves

Additional File-2 “Supplementary Tables”

File contains:

- Suppl. Table 1 RDP and SINA classification results for assembled and EMIRGE detected 16S rDNA sequences.
- Suppl. Table 2 Assembly depth values and classification results of selected contigs encoding 16S rDNA sequences.
- Suppl. Table 3 Taxonomic classification of metagenomic reads.
- Suppl. Table 4 Identified Pfams related to cellulose degradation.
- Suppl. Table 5 Identified Pfams related to protein degradation.
- Suppl. Table 6 Identified Pfams related to amino acid degradation.
- Suppl. Table 7 Accession numbers of reference 16S rDNA sequences in phylogenetic trees.

Additional File-3 “Supplementary Material and Methods”

File contains additional information regarding the normalization of metagenomic datasets and the detection of specific protein domains.

7. Unpublished results

[4] Nolla-Ardèvol V. Anaerobic digestion of *Spirulina* at alkaline conditions (pH ~10; 2.0 M Na^+) from fresh soda lake sediments.

[5] Nolla-Ardèvol V. Biomethane potential of different substrates at alkaline conditions.

[6] Nolla-Ardèvol V. Metagenome analysis of the microbial population from an alkaline anaerobic digester fed with the microalga *Spirulina*.

[4] Anaerobic digestion of *Spirulina* at alkaline conditions (pH ~10; 2.0 M Na^+) from fresh soda lake sediments

V. Nolla-Ardèvol

1. Introduction

In a previous work [2] the study of the anaerobic digestion of the protein rich microalga *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+ and high alkalinity ~100 g L^{-1} $CaCO_3$) was initiated. In this previous work, the optimal process conditions, 15 days HRT and 0.25 g *Spirulina* L^{-1} day^{-1} (dry weight) OLR were identified. The obtained results showed that it was possible to digest *Spirulina* at alkaline conditions and that as expected the obtained biogas was rich in methane, 88%, with peaks up to 96% (Figure 1; Table 2; [2]). However, the biogas production was low throughout the process. This low production could be attributed to two main factors: (i) the inoculum utilized, Sediment-1, was an alkaline sediment which was kept at 4ºC for over a year and (ii) during the process of adaptation and parameter optimization, the microbial community responsible for the degradation of *Spirulina* was exposed to high concentrations of ammonia nitrogen [2].

To corroborate these findings, a second alkaline reactor was inoculated with fresh alkaline sediment (Sediment-2) originating from the same group of soda lakes. The purpose of this second experiment was to determine if with a fresh and non-inhibited microbial population, the biogas production could be increased.

Eventually, a third alkaline reactor was inoculated and operated at the previous determined optimal process conditions, 15 days HRT and 0.25 g *Spirulina* L^{-1} day^{-1} OLR [2]. This reactor was set to study the biogas production and biogas quality at the optimal conditions.

2. Material and Methods

2.1. Experiment set-up

A 2.0 L semi-continuous stirred tank reactor (S-CSTR) with a working volume of 1.5 L operating at mesophilic conditions (35 °C) and at high pH ~ 10 and high salt

concentration (2.0 M Na^+) was used to study the anaerobic digestion of *Spirulina* at alkaline conditions. The S-CSTR set up and operated was as described in [2] and was used for two experiments:

(i) Experiment one (Alk-Sed-2) was utilized to further study the effect of the organic loading rate (OLR). The inoculum of the reactor consisted of a fresh mixture of soda lake sediments sampled from central Asian soda lakes and obtained in July 2012 (Sediment-2). Substrate and medium were the same as in [2]. Two different micronutrients solutions were used throughout the experiment (Table 4.1). Solution 1 was used from day 1 to day 159 and solution 2 from day 160 to the end of the experiment. Vitamin solution RPMI-1640 (Sigma-Aldrich product # R-7256) was added to the micronutrient solution 2 from day 182 to the end of the experiment. The reactor was operated at the optimal hydraulic retention time identified in the previous study, 15 days [2].

250 g of soda lake sediment-2 plus 300 mL of alkaline pH 10 medium were used as inoculum leading to a total working volume of 500 mL. The start-up substrate consisted of a mixture of *Spirulina*, 0.5 g L^{-1} (dry weight); glucose, 0.5 g L^{-1}; methanol, 10 mM and sodium acetate, 10 mM. The mixture, inoculum plus substrates was transferred into a 1.0 L glass bottle, the headspace was flushed with Helium gas for 5 minutes and the bottle was incubated anaerobically at 37 ºC and constantly stirred.

The start-up of the 1.0 L reactor consisted of a 15 days adaptation period in this 1.0 L reactor. During this period the reactor was fed every two days with 0.5 g *Spirulina* L^{-1} and the purged alkaline medium was kept in a sealed bottle for further use. Subsequently, the content of the 1.0 L bottle was transferred into the 2.0 L S-CSTR. 200 mL of the alkaline sludge purged during the start-up period plus 800 mL of fresh alkaline medium were added to achieve a final working volume of 1,500 mL. The headspace of the reactor was again flushed with Helium for 5 minutes. The reactor was fed every two days at an OLR of 0.5 g *Spirulina* L^{-1} (dry weight) for another 15 days. After this second adaptation period the feeding regime was switched to a daily feed of 0.5 g *Spirulina* L^{-1} and the experiment was started. After 100 days the OLR was doubled to 1.0 g *Spirulina* L^{-1} day^{-1} (dry weight). EDTA and vitamins were added to the micronutrients at day 112 and 182 respectively (See Results and discussion for details).

Table 4.1 Micronutrient solution composition
Solution was prepared in 1 L batch and added to the macronutrient solution at a concentration of 10 mL per litter

	Solution-1*	Solution-2**
From day - to day	0 – 159	160 – end
Compound	mg L^{-1}	mg L^{-1}
$FeSO_4 \cdot 7H_2O$	-	2,000
$FeCl_2 \cdot 4\ H_2O$	2,000	-
$MnCl_2 \cdot 4H_2O$	500	500
H_3BO_3	50	300
$ZnCl_2$	50	-
$CoCl_2 \cdot 6H_2O$	-	200
$Na_2SeO_3 \cdot 5H_2O$	164	164
$NiCl_2 \cdot 6H_2O$	-	92
$ZnSO_4 \cdot 7H_2O$	-	100
$AlCl_3 \cdot 8H_2O$	-	90
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	50	50
$CuCl_2 \cdot 6H_2O$	38	38
Yeast extract	200	200

* Modified from Vidal et al., 1997
** Dr. Dimitry Y Sorokin personal communication

(ii) Experiment two (Alk-Opt) consisted in the anaerobic digestion of *Spirulina* at the optimal conditions identified in the previous work [2]. The HRT was set to 15 days and the OLR to 0.25 g *Spirulina* L^{-1} day^{-1}. The inoculum for this second experiment was the 1.5 L sludge that remained in the Alk-Sed-2 reactor at the end of the experiment. The medium used was identical to experiment one supplemented with the micronutrient solution-2 plus vitamin solution RPMI-1640. Prior to the start of the reactor, the alkaline sludge was exhausted for a period of 10 days to allow the consumption of any possible remaining undegraded biomass. The start-up of Alk-Opt reactor consisted of a 15 days period where the feeding was performed every two days, at the end of which the experiment started.

2.2. Analytical methods

In addition to continuous measurements of pH and redox potential, alkalinity and total and volatile solids in the digesters were periodically analyzed. Biogas production, biogas composition and determination of the sludge characteristics (Total and Volatile solids, Total Nitrogen, Chemical Oxygen Demand, Volatile Fatty Acids etc.), were determined as in [2].

3. Results and discussion

3.1. Alk-Sed-2: Biogas production from fresh soda lake sediment

The anaerobic digestion of *Spirulina* at alkaline conditions was studied with a fresh inoculum of soda lake sediments, Sediment-2. The microbial community adapted to the reactor's configuration within 5 days as seen by the detection of biogas on day 5 after inoculation of the 1.0 L start-up reactor (data not shown). During the next 10 days, biogas production was constant. After transferring the sludge to the 2.0 L reactor and the addition of extra alkaline medium and sludge, biogas production was detected at day 3 after the transfer (data not shown). In this second start-up period, biogas production was constant at 29 mL per day and the composition shifted from 21 to 80% of methane, 11 to 6 % of CO_2 and 68 to 14% of N_2 gas.

Figure 4.1a shows the biogas production and the biogas composition throughout the experiment. In period P-I, with an OLR of 0.5 g *Spirulina* L^{-1} day^{-1}, the daily biogas production was constant with a mean daily biogas production of 36 mL biogas (Table 4.2).This production was in accordance with previous findings where with the same OLR, 32 mL of daily biogas production were achieved (Table 3; [2]). During this period, the biogas obtained was, as expected, rich in methane, ranging from 79 to 92%, slightly higher than in the previous study [2]. This slightly increase in biogas production could be attributed mainly to the fact that, in contrasts to the first experiment [2], the microbial community of this second reactor had not suffered previous inhibitions and therefore bacteria and archaea were fully active. In addition to this, the sediments used for the inoculation of the reactor, Sediment-2, were freshly obtained, in contrast to the previous inoculum which had been stored at 4ºC for over a year.

During period P-I, both, the total nitrogen and the ammonia present in the reactor's medium were relatively low, 830 and 500 mg L^{-1} respectively (Figure 4.1b). Both levels were in the same order as the ones obtained in P-II of reactor Alk-OLR [2]. At this OLR of 0.5 g *Spirulina* L^{-1} day^{-1}, the levels of organic matter measured as total and soluble COD, as well as the levels of VFAs, mainly acetic and propionic acid, remained stable throughout period P-I (Figure 4.1c, d).

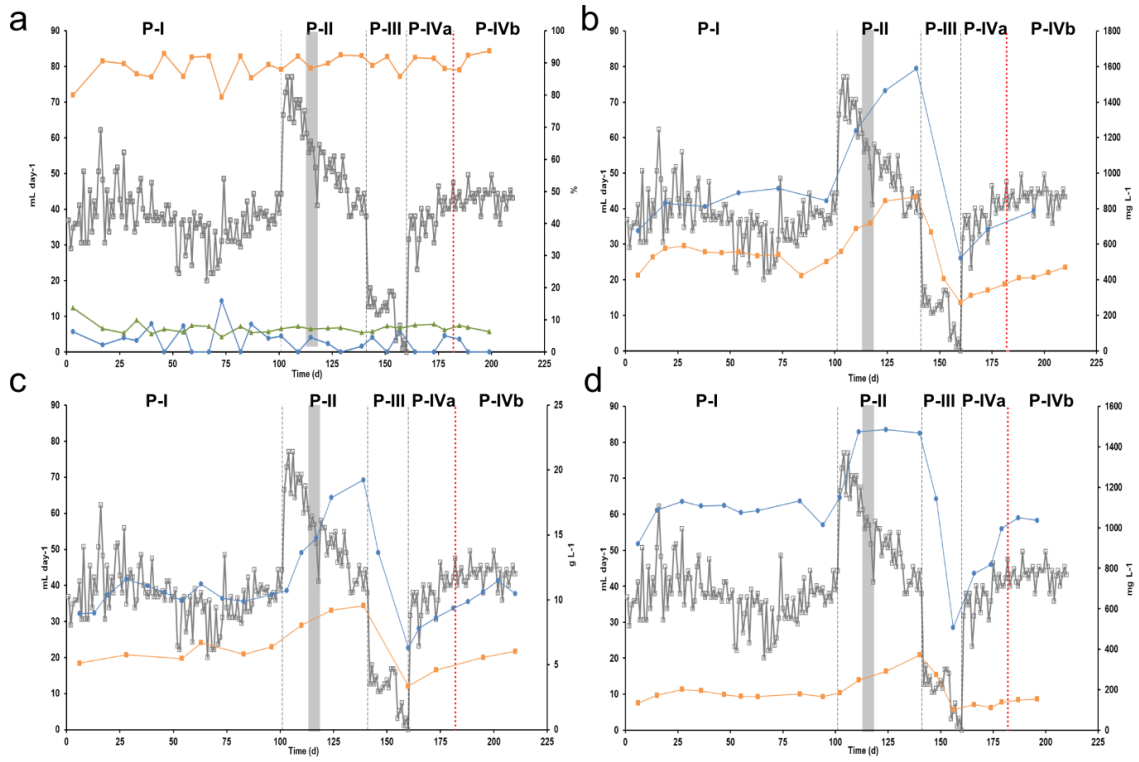

Figure 4.1 Alk-Sed-2 reactor performance
Alk-Sed-2 reactor performance from the anaerobic digestion of *Spirulina* at alkaline conditions. Daily biogas production (gray line – left axis); **a)** biogas composition: CH_4 (■); CO_2 (●) and N_2 (▲); **b)** Total nitrogen (●) and free ammonia (NH_3) (■); **c)** Total (●) and soluble (■) organic matter (COD_T and COD_S); **d)** Acetic (●) and propionic (■) acid. Dashed gray vertical lines indicate a change in the organic loading rate, red vertical dotted line indicates vitamin solution addition time point and gray area corresponds to the non-feeding period (see text for details).

After 100 days of constant biogas production, the organic loading rate was doubled from 0.5 g to 1.0 g *Spirulina* L^{-1} day^{-1} (P-II). The daily biogas production, as expected, almost doubled from 40 to 60 mL day^{-1} and it gradually increased during the next 4 days when it reached its maximum, 77 mL of biogas (Figure 4.1a). From this point onwards, the biogas production experienced a slow but constant reduction. This reduction was similar to what occurred when the organic loading rate was increased from 0.5 to 1.0 g *Spirulina* L^{-1} day^{-1} in the Alk-OLR reactor (Figure 3, Period P-II; [2]).

In both cases, the change in the OLR had an initial positive effect and the biogas production increased, but after a short period of time it started to gradually decrease. As can be seen in Figure 1, all the parameters that were constant in P-I, TN, NH_3, COD_T, COD_S and VFAs, experienced a rapid increase within the firsts days after the change in the OLR. Accumulation of free ammonia inhibits methanogens, especially aceticlastic methanogens (Angelidaki and Ahring, 1993; Koster and Lettinga, 1984)

Table 4.2 Alkaline Alk-Sed-2 reactor biogas production

Biogas production, biogas characteristics, specific biogas and methane productions from the anaerobic digestion of *Spirulina* at alkaline conditions. Mean values and standard deviation of the measurements from each different period

	Units	Period I	Period II	Period IVa	Period Ivb
	Days	100	40	22	28
OLR	g *Spirulina* (L_R day)$^{-1}$	0.5	1.0	0.5	0.5
HRT	Days	15	15	15	15
Micronutrients		Micro-1	Micro-1	Micro-2	Micro-2 + Vit
pH		10.02 ± 0.1	9.97 ± 0.02	10.07 ± 0.03	9.98 ± 0.02
Alkalinity	g $CaCO_3$ L^{-1}	104 ± 9	99 ± 3	99 ± 2	99 ± 1
Biogas production and composition					
Daily production	mL biogas day^{-1}	36 ± 8	53 ± 13	37 ± 5	43. ± 3
Daily production	mL CH_4 day^{-1}	32 ± 7	48 ± 11	33 ± 4	39 ± 3
CH_4	%	88 ± 4	91 ± 2	90 ± 2	91 ± 3
CO_2	%	5 ± 5	2 ± 2	2 ± 3	1 ± 2
N_2;O_2	%	7 ± 2	7 ± 1	8 ± 1	7 ± 1
H_2S	%	n.d ± n.d	n.d ± n.d	n.d ± n.d	n.d ± n.d
Specific biogas and methane productions					
SBP-VS added	mL_{biogas} (day g VS)$^{-1}$	55 ± 12	40 ± 9	57 ± 8	65 ± 4
SBP-g *Spirulina* added	mL_{biogas} (day g *Spirulina*)$^{-1}$	49 ± 11	35 ± 8	50 ± 7	57 ± 4
SMP-VS added	mL_{CH4} (day g VS)$^{-1}$	47 ± 10	36 ± 8	51 ± 7	60 ± 4
SMP-g *Spirulina* added	mL_{CH4} (day g *Spirulina*)$^{-1}$	42 ± 9	32 ± 7	45 ± 6	52 ± 4

n.d: non detected
SBP: specific biogas production
SMP: specific methane production

which in turn increases the levels of acetic acid in the medium. Under such circumstances, high NH_3 and high acetate, syntrophic acetate-oxidizing bacteria (SAO) should take over and oxidize acetate into H_2 and CO_2 (Karakashev et al., 2006). However, in the reactor, acetic acid levels also accumulated which might indicate that the SAO bacteria were also inhibited or not fully active (Hattori, 2008; Karakashev et al., 2006). The accumulation of total organic matter (COD) is generally indicative of substrate overload which occurs when the amount of substrate fed in to the reactor exceeds the degradation capacity of the hydrolytic bacteria (Borja, 2011; Schnürer and Jarvis, 2010). At the same time, the total organic matter increased more rapidly than the soluble organic matter which is a clear indication that the added biomass was not completely degraded by the hydrolytic bacteria. In this particular case, it was observed that there was an accumulation of ammonia nitrogen, acetic acid and total organic matter accompanied by a reduction of the daily biogas production (Figure 4.1). The combination of all these factors led to a drop in the biogas production.

One of the possible reasons for the low hydrolysis of organic matter and the low consumption of volatile fatty acids might be that the bacteria were unable to utilize, due to the particular process conditions (pH~10; 2.0 M Na^+), some of the essential elements present in the micronutrients dissolved in the medium. It is well known that micronutrients play an important role in the function of the different microbes involved in the anaerobic digestion process and the absence of a certain compound can reduce their activity (Zhang et al., 2012a). Therefore, to increase the solubility of the micronutrients, and with it the uptake by the microbial community, EDTA, a chelating agent present in the composition of multiple mediums used for anaerobic digestion (González-Gil et al., 2002; Liu et al., 1999; Strik et al., 2006), was introduced to the micronutrient solution. Initially, it was decided not to add this compound in the micronutrient solution-1 because the initial approach of the project was to design an alkaline medium as simple as possible and with the idea that *Spirulina*, itself would provide the sufficient minerals and vitamins (Cañizares-Villanueva et al., 1995; Ortega-Calvo et al., 1993). As can be seen in Figure 4.1, day 112 (gray area), the addition of 2.0 g L^{-1} of EDTA to the micronutrient solution did not have a positive effect, on the contrary, the reduction in daily biogas production accelerated. The biogas production in this period of EDTA addition fell from 67 to 41 mL day^{-1}, that is, 26 mL in 7 days, while in the previous 10 days the reduction was only of 10 mL, from 77 to 67 mL of biogas per day. This rapid reduction in the biogas production clearly indicated that the addition of EDTA did not stimulate the activity of the microbial community. At this point, day 118, it was decided to return to the previous micronutrient solution-1. Returning to the original micronutrient solution had slight a positive effect and the biogas production recovered to the levels before the addition of EDTA. However, the inhibition persisted and the biogas production continued to drop from 58 to 37 mL of biogas per day (Figure 4.1).

At the end of P-II the parameters related to the degradation of organic matter, TN, NH_3, VFAs and COD_S reached their peak (Figure 4.1b, c, d). Total and soluble organic matter increased in this period from 10.7 to 19.2 and from 6.3 to 9.5 g O_2 L^{-1} respectively. Total nitrogen almost doubled in this period, from 845 mg L^{-1} to 1,500 mg L^{-1} while the ammonia nitrogen also increased, from 558 to 866 mg L^{-1}. After 40 days of feeding at an OLR of 1.0 g *Spirulina* L^{-1} day^{-1} and no indication of reactor recovery the supply of *Spirulina* was stopped.

To remove inhibitory substances and excess of undegraded organic matter, 100 mL of reactor liquid was replaced with alkaline medium without substrate every two days. This procedure was repeated during 19 days until the biogas production was almost zero and it drastically reduced the concentrations of acetic acid, total and soluble organic matter and nitrogenous compounds (Figure 4.1). During this period the methanogenic archaea were still active and the biogas production was constant at around 10 mL of biogas per day with 88% methane content.

After this starvation period, the feeding was resumed at 0.5 g *Spirulina* L^{-1} day^{-1} (dry weight) and a new micronutrient solution, solution-2 (Table 4.1), specifically designed for haloalkaline microbial communities (Dr. Sorokin personal communication) was used. As can be seen in Figure 4.1a period P-IVa, as soon as the feeding was resumed the biogas production was also resumed. The biogas production during this period was similar to P-I, around 37 mL of biogas per day and the biogas obtained was also rich in methane, 91%. At day 182, when the biogas production was fully recovered, a cocktail of vitamins was added to the micronutrient solution-2 in order to determine its effect on the performance of the microbial community. The addition of vitamins (Figure 4.1; Red dotted line) did have a slight positive effect and the biogas production increased steadily until it reached its maximum of 43 mL day^{-1}. The absolute increase in biogas production was not very high, yet the addition of vitamins appeared to have a positive effect on the consumption of acetic acid which, was stabilized at around 1.0 g L^{-1}, levels similar to the ones in P-I (Figure 4.1).

The optimal specific biogas and specific methane productions (SBP and SMP) were obtained with the addition of vitamins to the micronutrient solution-2, period P-IVb. In this period, 57 and 52 mL of biogas and methane per gram of *Spirulina* were obtained respectively. The methane content in this period was also the highest achieved, 91% (Figure 4.1a; Table 4.2). These results are higher than the previous findings with the Alk-OLR (49 mL of SBP and 38 mL of SMP) and much higher than with the Alk-HRT reactor, 33 and 29 mL of biogas and methane per gram of substrate (Tables 2 and 3; [2]).

This experiment confirmed that the biogas production from *Spirulina* at alkaline conditions was possible, yet with several limitations. The main bottleneck appears to be the slow and incomplete degradation of substrate, provably due to sub-optimal conditions for the hydrolytic bacteria and the methanogenic archaea, the latter being

inhibited by the accumulation of volatile fatty acids, ammonia and undegraded organic matter. This bottleneck was also identified in the previous experiments [2]. However, in the current experiment, an overall higher biogas production was obtained which could attribute to the fact that the inoculum used, Sediment-2, was relatively fresh in comparison to Sediment-1 which was used in the previous set of experiments.

3.2. Alk-Opt: Biogas production at optimal operational conditions (15 days HRT; 0.25 g *Spirulina* L^{-1} day^{-1} OLR)

In a previous publication, the optimal process conditions for the successful anaerobic digestion of *Spirulina* at alkaline conditions, 15 days hydraulic retention time and 0.25 g *Spirulina* L^{-1} day^{-1} were identified [2]. Reactor Alk-Opt was operated at these conditions for 67 days (Figure 4.2a). The biogas production during this period oscillated between 20 and 37 mL of biogas per day with a mean daily production of 27 mL per day (Table 4.3). The obtained biogas was rich in methane, 86%, with peaks up to 90% (Figure 4.2a; Table 4.3), which are in the same range as the previous findings [2].

Performing the digestion at the optimal conditions avoided the problems encountered during the previous experiments As can be seen in Figure 4.2b,c, the organic matter (COD) did not accumulate throughout the experiment. Moreover the ammonia levels remained low, 150 mg L^{-1}, and acetic and propionic acid were also controlled and no accumulation occurred after 67 days of continuous biogas production (Figure 4.2d). The no accumulation of organic matter (COD_T), the constant levels of soluble organic matter (COD_S) and the controlled levels of acetic acid indicate that the degradation and consumption rates were in equilibrium. In addition, the low ammonia levels in the reactor's medium avoided the inhibition of methanogens which could transform the available acetate, CO_2 and H_2 into methane. The results presented in Figure 4.2 and Table 4.3 indicate that at this set of conditions (15 days HRT and 0.25 g *Spirulina* L^{-1} day^{-1}) the microbial community was able to successfully digest part of the supplied substrate and was not affected by inhibitory substances.

The obtained SBP, 74 mL of biogas per gram of added substrate, was much higher than the ones obtained with the previous experiments, 33 mL in Alk-HRT, 49 mL with Alk-OLR [2] and 57 mL with the Alk-Sed-2.

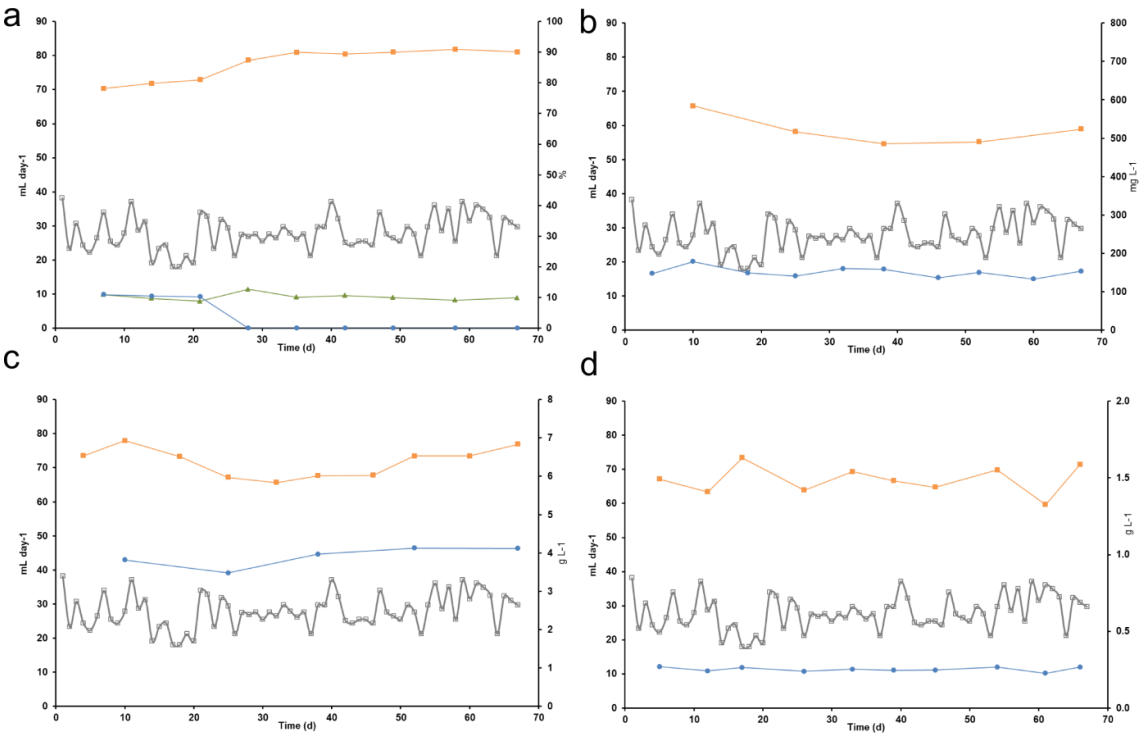

Figure 4.2 Alk-Opt reactor performance
Alk-Opt reactor performance from the anaerobic digestion of *Spirulina* at alkaline conditions with optimal process settings, 15 days hydraulic retention time and 0.25 g substrate L^{-1} day^{-1} organic loading rate. Daily biogas production (gray line – left axis); **a)** biogas composition: CH_4 (■); CO_2 (●) and N_2 (▲); **b)** Total nitrogen (■) and free ammonia (NH_3) (●); **c)** Total (■) and soluble (●) organic matter (COD_T and COD_S); **d)** Acetic (■) and propionic (●) acid.

This increase in the SBP was attributed to several factors:

(i) The selected micronutrients, Solution-2, and the addition of vitamins to the feeding mixture provided an improved environment for the optimal growth of the microbial community involved in the anaerobic process.

(ii) With this low organic loading rate, 0.25 g *Spirulina* L^{-1} day^{-1}, equilibrium was reached between the hydrolysis and the consumption rates which avoided accumulation of undegraded organic matter, of ammonia nitrogen and of volatile fatty acids, compounds that could cause inhibition and therefore reduce the capacity of the anaerobic community.

(iii) The selected HRT, 15 days, avoided an excessive washout of the active biomass and at the same time removed sufficient inhibitory substances.

(iv) The inoculum used for the Alk-Opt experiment was a relatively fresh inoculum which was transferred directly from the Alk-Sed-2 experiment and, contrary

Table 4.3 Alkaline Alk-Opt reactor biogas production

Biogas production, biogas characteristics, specific biogas and methane productions from the anaerobic digestion of *Spirulina* at alkaline conditions with optimal process settings, 15 days hydraulic retention time and 0.25 g substrate L^{-1} day^{-1} organic loading rate. Mean values and standard deviation of the measurements.

	Units	**Steady state**
	Days	67
OLR	g *Spirulina* $(L_R\ day)^{-1}$	0.25
HRT	Days	15
Biogas production and composition		
Daily production	mL biogas day^{-1}	27 ± 4
Daily production	mL CH_4 day^{-1}	23 ± 5
CH_4	%	86 ± 5
CO_2	%	4 ± 3
N_2;O_2	%	10 ± 1
H_2S	%	n.d ± n.d
Sludge characteristics		
pH		10.1 ± 0.1
Alkalinity	g $CaCO_3$ L^{-1}	91 ± 4
Total Solids	g Kg^{-1}	110 ± 1
Volatile Solids	g Kg^{-1}	5 ± 0.3
Fixed Solids	g Kg^{-1}	105 ± 0.5
Total COD	g O_2 L^{-1}	6.4 ± 0.4
Soluble COD	g O_2 L^{-1}	3.9 ± 0.3
Total Nitrogen	g L^{-1}	0.52 ± 0.04
NH_4^+-N	g L^{-1}	0.01 ± 0.01
NH_3-N	g L^{-1}	0.15 ± 0.01
Volatile Fatty Acids (VFA)		
Acetic acid	mg L^{-1}	1,488 ± 92
Propionic acid	mg L^{-1}	252 ± 15
iso-butyric acid	mg L^{-1}	21 ± 5
Butyric acid	mg L^{-1}	20 ± 6
iso-valeric acid	mg L^{-1}	47 ± 25
n-valeric acid	mg L^{-1}	n. d
Total VFAs	mg L^{-1}	1,828
Specific biogas and methane productions		
SBP-VS added	mL_{biogas} $(day\ g\ VS)^{-1}$	84 ± 14
SBP-g *Spirulina* added	mL_{biogas} $(day\ g\ Spirulina)^{-1}$	74 ± 12
SMP-VS added	mL_{CH4} $(day\ g\ VS)^{-1}$	71 ± 15
SMP-g *Spirulina* added	mL_{CH4} $(day\ g\ Spirulina)^{-1}$	62 ± 13

n.d: non detected
SBP: specific biogas production
SMP: specific methane production

to the inoculums used for the Alk-HRT and Alk-OLR, was not exposed to severe inhibitory conditions.

Almost 10% of conversion of *Spirulina* was achieved with the optimal process conditions, which was higher than the bioconversion values of the previous studies, 8.3% in reactors Alk-Sed-2, 4.9% in Alk-HRT and 6.7% in Alk-OLR [2], and similar to the bioconversion obtained in the Alk-BT2 batch test of *Spirulina* (Table 5.2; [5]).

However, this percentage is relatively low compared to the value obtained at mesophilic pH conditions, 41% [3].

In summary, applying the optimal settings determined for the alkaline anaerobic digestion of *Spirulina* prevented accumulation of VFAs, NH_3 and organic matter (COD), and led to an increase in biogas production. The daily biogas production was, however, still far from the production obtained at mesophilic pH conditions, 262 mL CH_4 g VS^{-1} [3]. This low biogas production could be attributed to the fact that, even though the reactor was operating at the optimal identified conditions, it was doing it in the so called “inhibited steady-state”, a condition where the reactor is working correctly but with a low biogas production (Angelidaki and Ahring, 1994). This condition could be attributed to the type of substrate used, to the extreme applied conditions which slow down the microbial conversion of the substrate, or to an inherent low activity of the microbial community.

4. Conclusions

The results presented here contribute to support and validate the results obtained in the previous work [2]. It was confirmed that a rapid inhibition, due to ammonia and VFA accumulation, occurred when a relatively high organic loading rate (1.0 g *Spirulina* L^{-1} day^{-1}) was applied. The results also show that when the reactor was operated at the optimal process conditions, 15 days HRT and with and OLR of 0.25 g L^{-1} day^{-1}, the continuous anaerobic digestion of *Spirulina* was possible with no accumulation of inhibitory substances. The obtained biogas was rich in methane which makes it suitable to be used as biomethane, requiring only minor purification. The results also indicate that further improvement should be possible in order to increase the overall biogas production.

[5] Biomethane potential of different substrates at alkaline conditions

V. Nolla-Ardèvol

1. Introduction

Biomethane potential tests (BMP), are a fast and inexpensive approach to determine the methane content of a particular substrate (Angelidaki et al., 2009; Raposo et al., 2011). Moreover, BMP tests can also give information about the percentage of biodegradability, BD_{CH4} (%), of a substrate (Raposo et al., 2011), which helps to assess its potential as feed for an anaerobic reactor. Biomethane potential tests have been widely used to evaluate the methane production potential of algae and other substrates at mesophilic pH conditions (Bourque et al., 2008; El-Mashad, 2013; Mussgnug et al., 2010; Pobeheim et al., 2010). In this work, BMP tests were performed to study the capacity of certain substrates for methane production at alkaline conditions (pH~10 and 2.0 M Na^+).

The biomethane potential from *Spirulina* at mesophilic pH conditions has already been determined by several authors (El-Mashad, 2013; Zamalloa et al., 2012), however, its BMP at alkaline conditions has never been tested. In this study, BMP of *Spirulina* and microcrystalline cellulose (Avicel) was determined at alkaline conditions. The main advantage of using Avicel is that, its chemical composition ($C_6H_{10}O_5$) and also its theoretical methane potential, 415 mL CH_4 g VS^{-1}, are known, making it suitable to act as a positive control (Raposo et al., 2011). A mixture of fresh algal biomass consisting of *Dunaliella* (Eukaryotic) and *Geitlerinema* (Cyanobacteria) was also tested for its BMP. These two microalgae were obtained from the same soda lakes where the sediments for the alkaline anaerobic digester were originate, the soda lake system of the Kulunda steppe [2, 4]. Biomethane potential of *Dunaliella* has already been extensively performed at mesophilic pH conditions (Dębowski et al., 2013; Mussgnug et al., 2010; Sialve et al., 2009) and more recently at high salt concentrations using halophilic sediments (Mottet et al., 2014). Using a fresh microalgae substrate, which has been cultured under specific conditions, can give more precise information about the real capability of the alkaline microbial consortium to produce methane under controlled conditions. Chitin, a long chain polymer of N-acetylglucosamine derived from glucose, was also used as a substrate. In such

haloalkaline lakes, massive presence of the crustacean Artemia, or Brine shrimp, occurs (Kompantseva et al., 2009). When these crustaceans die their skeletons, which are mainly composed of chitin, precipitate and accumulate in the sediments top layers (Kompantseva et al., 2009 and Dr. Dimitry Sorokin personal communication). In addition, several studies have shown the possibility of digesting this compound under anaerobic conditions (Boyer, 1986; Pel and Gottschal, 1986; Reguera and Leschine, 2001).

The anaerobic degradation of wheat straw to produce biogas has been studied at mesophilic conditions mainly as co-substrate with other more easily digestible substrates such as manure (Zhang et al., 2013), or after being subjected to pretreatments in order to increase its digestibility (Zahoor and Tu, 2014). However, the direct digestion of un-pretreated wheat straw is generally not efficient due to the nature of its components, lignin, cellulose and hemicellulose in different proportions (Taherzadeh and Karimi, 2008). To increase its biodegradability, pretreatment of wheat straw is a common procedure as it contributes to decompose cellulose and hemicellulose into more easily biodegradable substrates (Song et al., 2014) For example, alkaline pretreatments increase the organic solubilization and increase the surface area available for the action of enzymes (Song et al., 2014; Zahoor and Tu, 2014). To date, only one reference has been found in which wheat straw was used as substrate in biomethane potentials were soda lake sediments were used (Porsch et al., 2013). Therefore, in this study, both un-pretreated wheat straw and pretreated wheat straw were tested as substrates in order to determine whether the alkaline medium has a “pretreatment” effect and therefore make the hydrolysis of the cellulolytic material easier.

Finally, glycerol, a by-product of the biodiesel industry was used as co-substrate since it is an easily degradable molecule which is known to greatly increase the biogas production when co-digested in the presence other substrates (Astals et al., 2012; Astals et al., 2013).

2. Material and methods

Alkaline sludge from reactors Alk-HRT [2] and Alk-Sed-2 [4] were used to perform several sets of batch tests:

(i) Batch tests Alk-BT1: Batch tests were performed in 100 mL glass bottles with a gas tight rubber septum. Each batch test was inoculated with 50 mL of alkaline sludge obtained from the Alk-HRT reactor [2] which had previously been substrate-exhausted by incubation at 37ºC for 20 days. Three different substrates were used: Freeze dried *Spirulina*, microcrystalline cellulose (Avicel PH-101; Sigma-Aldrich Art # 11365) and Chitin (Sigma-Aldrich Art # C7170). *Spirulina* was also co-digested with Avicel (50% w/w), Chitin (50% w/w) and Glycerin (5% w/w). A control test was performed without substrate. In addition, 25 mL of fresh alkaline pH 10 medium were added to each batch test to achieve a final working volume of 75 mL and a headspace volume of 25 mL.

(ii) Batch test Alk-BT2: These batch tests were inoculated with alkaline sludge from the Alk-Sed-2 reactor [4] which was previously substrate-exhausted for 20 days at 37ºC. The substrates added in the test were freeze dried *Spirulina*, Avicel, wheat straw, pretreated wheat straw and mixture of fresh algal biomass (*Dunaliella* and *Geitlerinema)*. Wheat straw was kindly provided by Dr. Kleinsteuber, and prior to its use it was grinded and filtered through a 0.5 mm sieve. Pretreatment of wheat straw was as follows. 5 grams of sieved straw were incubated at room temperature for 24 hours in a mixture of 0.5 g $Ca(OH)_2$ (lime) and 115 mL of distilled water. Prior to its addition to the batch tests as much possible of the liquid was removed (Dr. Kleinsteuber personal communication). Fresh algal biomass was kindly cultured and provided by MSc Viktor Klassen. Algal biomass was cultured at alkaline pH ~10 with a continuous supply of air for 14 days, and the harvesting procedure consisted in 5 minutes centrifugation at 3,000 g. Harvested biomass was stored at -20ºC to prevent degradation until used. Batch tests without substrate addition, *Spirulina*, Avicel and pretreated wheat straw were performed in 100 mL bottles and inoculated with 75 mL of alkaline sludge. Batch tests for wheat straw and fresh cyanobacterial biomass were done in 250 mL bottles with 75 mL of alkaline sludge.

Batch tests from both sets were performed in triplicate, substrate concentrations were added according to VDI_4630 guide lines of the Verein Deutscher Ingenieure (VDI, 2004), closed with a gas tight rubber septum and the headspace was flushed with Helium for 1 minute prior to incubation at 37ºC in an incubating chamber. Biogas production was measured by determining the pressure build up inside the headspace with a portable pressure meter (WAL-BMP-Test system 3150, WAL, Germany). As it

took only about 30 seconds to measure the pressure in the headspace for each reactor, a constant temperature inside the headspace was assumed (El-Mashad, 2013). Bottles were manually shaken before and after the pressure measurement. When needed the headspace was evacuated to release pressure. Composition of the produced biogas was measured with a Shimadzu GC-2010 gas chromatograph with settings as in [2]. Gas samples were obtained from the headspace and directly injected into the chromatograph with a gas-tight syringe.

Ammonia nitrogen was measured according to [2] in both exhausted sludge, Alk-HRT and Alk-Sed-2, prior to the addition of the different substrates and again at the end of the experiments. Likewise, pH was also measured at the beginning and end of each experiment. Theoretical methane potential (BMP_{TH}) for both protein containing (*Spirulina*) and protein free substrates (Chitin, Avicel, Wheat straw, Glycerol) and Biodegradability percentage BD_{CH4}(%) were determined according to Raposo et al., 2011. *Spirulina's* BMP_{TH} was calculated with Ortega-Calvo et al., 1993 chemical composition, $C_4H_7ON_{0.8}S_{0.02}$. The same chemical composition was used for the cyanobacterial biomass. For Avicel the chemical composition of cellulose ($C_6H_{10}O_5$) was used, for Chitin, $C_{16}H_{28}O_{11}N_2$, and for glycerol $C_3H_8O_3$. 426 mL CH_4 g VS^{-1} was used as the BMP_{TH} for wheat straw (Kaparaju et al., 2009).

3. Results and discussion

Biomethane potential tests (BMP) are a fast and inexpensive method to assess the potential biogas production of a particular substrate (Angelidaki et al., 2009; Raposo et al., 2011) and were used to determine the suitability of several types of substrates as potential candidates for the anaerobic digestion at alkaline conditions. *Spirulina* and Avicel were used in both sets of batch tests, Alk-BT1 and Alk-BT2, because two different soda lake sediments were tested throughout the PhD project. It was suspected that the microbial community present in the sludge originated from sediment-1 and used as inoculum for Alk-BT1 was strongly inhibited and that part of its components, mainly hydrolytic bacteria, had been lost during the previous processes [2]. This assumption was made after evaluating the results obtained with the Alk-BT1 tests. Therefore, to try to obtain a more accurate methane potential of these two compounds it was decided to repeat the batch test using alkaline sludge originated from fresh sediment-2, Alk-BT2, which did not suffer from inhibition [4].

The first important result obtained was that the anaerobic digestion at alkaline conditions is a relatively slow process compared to standard mesophilic conditions were the average BMP process lasts between 13 and 87 days with a mean duration of 32 (Raposo et al., 2011). Here, the batch anaerobic fermentation tests were incubated at 37ºC for a period of 100 days in the case of Alk-BT1 and 325 days in the case of the Alk-BT2.

3.1. Alk-BT1 batch tests: biogas production under inhibitory conditions

In the first set of batch tests, Alk-BT1, the highest biomethane potential was achieved with microcrystalline cellulose (Avicel) where a total of 33.2 mL of methane g VS^{-1} were obtained. This production however was far from its theoretical value 415 mL CH_4 g VS^{-1} (Table 5.1).

Likewise, the BMP of *Spirulina*, 6.2 mL CH_4 g VS^{-1}, was extremely low compared to its theoretical value, 627 mL CH_4 g VS^{-1}. When chitin was used as substrate no production was observed (Table 5.1) which was unexpected as this compound is abundant in alkaline soda lakes (Kivistö and Karp, 2011; Kompantseva et al., 2009; Kompantseva et al., 2010), its composition is chemically related to cellulose and it has already been degraded at mesophilic pH conditions (Boyer, 1986; Pel and Gottschal, 1986).

The absence of biogas production in the chitin test could be explained by (i) lack or complete inhibition of sulfate reducers and specific chitin degraders which are essential for its degradation (Boyer, 1986) and (ii) the digestion of chitin at such extreme conditions might be a slow process requiring more than the 100 days incubation period in this experiment.

The co-digestion of substrates did not increase the overall production and the biogas production was below 10 mL g VS^{-1} in all studied substrates (Table 5.1). When *Spirulina* was co-digested with Avicel, the BMP obtained was similar to plain *Spirulina* but much lower than plain Avicel. This reduction of production when compared to sole Avicel could indicate that the microbial community in the co-digestion test did not use the supplied Avicel, as was already suffering from inhibition caused by the degradation of *Spirulina.* This supposition can be explained by the

Table 5.1 Alkaline batch tests Alk-BT-1 biogas and methane potentials
Biogas and methane potential of different substrates at alkaline conditions after 100 days of incubation at 37ºC

	Substrates					
	Spirulina	Avicel	Chitin	*Spirulina* + Avicel	*Spirulina* + Chitin	*Spirulina* + Glycerol
Theoretical CH_4 (mL g VS^{-1})*	627	415	423	521	525	619
Experimental Biogas (mL g VS^{-1})	8.6	37.3	0.3	7.6	8.6	5.5
% CH_4	72	89	60	78	81	65
% CO_2	15	6	20	10	11	16
% N_2	13	5	20	12	18	19
Experimental CH_4 (mL g VS-1)**	6.2	33.2	0.2	6.0	7.0	3.5
Biodegradability BD_{CH4} (%)*	1.0	8.0	0.04	1.1	1.3	0.6
Initial pH	10.01	10.01	10.01	10.01	10.01	10.01
Final pH	9.93	9.71	10.23	10.05	10.07	9.97
Initial NH_3 (mg L^{-1})	267	267	267	267	267	267
Final NH_3 (mg L^{-1})	1,121	171	366	858	823	1,164

*Calculated as in Raposo et al 2011
** Calculated with the experimental methane %

observation that the degradation of plain Avicel started after 5 days of incubation while in the same period of time, degradation of plain *Spirulina* already reached its maximum and a plateau followed.

This plateau after only 5 days of incubation was mainly attributed to an accumulation of NH_3 derived from the degradation of *Spirulina* which resulted in the complete inhibition of the methanogenic community. A similar plateau after 5 days of incubation was also observed in the co-digestion of *Spirulina* and Avicel indicating that the methanogenic community was inhibited and therefore could not use the additional microcrystalline cellulose. If the methanogenic community would have not been inhibited, then an increase in the biogas production should appear after day 5 of incubation, as occurred with plain Avicel. Co-digesting *Spirulina* with chitin produced similar results and no increase in the biogas production was observed when compared to digestion of plain *Spirulina*. One of the benefits of co-digesting two or more substrates is that possible inhibitory metabolites released during the digestion of the compounds are diluted (Astals et al., 2012). This dilution effect became visible by a reduction in the levels of ammonia present in the batch tests where *Spirulina* was co-digested with Avicel or chitin at a 1:1 ratio (Table 5.1). However, in both cases, this reduction was still not sufficient and inhibition of the methanogenic bacteria eventually occurred. Adding glycerol, a carbon rich substrate, did not stimulate the biogas production, on the contrary it reduced the production

when compared to digesting plain *Spirulina*. This was unexpected as glycerol generally boosts the biogas production when used as co-substrate (Astals et al., 2012; Astals et al., 2013), but gain could be attributed to an inhibition of the methanogenic community.

In all batch tests, except in Avicel, the obtained biogas was, in some cases, not as rich in methane as expected by the results obtained with the continuous system [2]. The highest methane content, 89%, was obtained with the digestion of plain Avicel while *Spirulina* produced biogas with 72% methane (Table 5.1). Apparently the expected CO_2 scrubber effect of the alkaline medium was not so acute in some of the batch tests, especially in those with extreme low biogas production, chitin and the co-digestion of *Spirulina* plus glycerol.

An additional fact pointing towards a general inhibition of the alkaline sludge used as inoculum could be seen in the biodegradability of each compound (Table 5.1). The highest biodegradability, BD_{CH4} (%), was achieved with Avicel but it accounted for less than 10 percent of the total methane potential. The biodegradability of *Spirulina* at these conditions was extremely low, 1%, while chitin remained almost undegraded, 0.04%. Co-digestion of *Spirulina* with Avicel and chitin slightly increased the biodegradability compared to *Spirulina* only.

The low biogas production in all batch tests could be attributed to (i) ammonia inhibition in the case of tests whit *Spirulina* as substrate and (ii) to a low activity of the hydrolytic bacteria, or to a low number of these bacteria in the cellulose digestion test. Ammonia nitrogen, NH_3, a molecule that is toxic for methanogenic archaea (Calli et al., 2005; Strik et al., 2006), accumulated in the tests that digested *Spirulina* (Table 5.1). When *Spirulina* was used as the sole substrate ammonia reached 1,121 mg L^{-1} at the end of the incubation period, a value which is in accordance to the inhibitory levels obtained in reactor Alk-HRT [2]. On the other hand, when non-proteinaceous substrates were used, the ammonia levels remained low (Table 5.1). In these cases the low biogas production could not be attributed to ammonia inhibition but to a low number or low activity of hydrolytic bacteria.

These results show that the alkaline medium utilized as inoculum was extremely inactive either by ammonia inhibition which affects methanogens or by a low number or lack of activity of the hydrolytic bacteria.

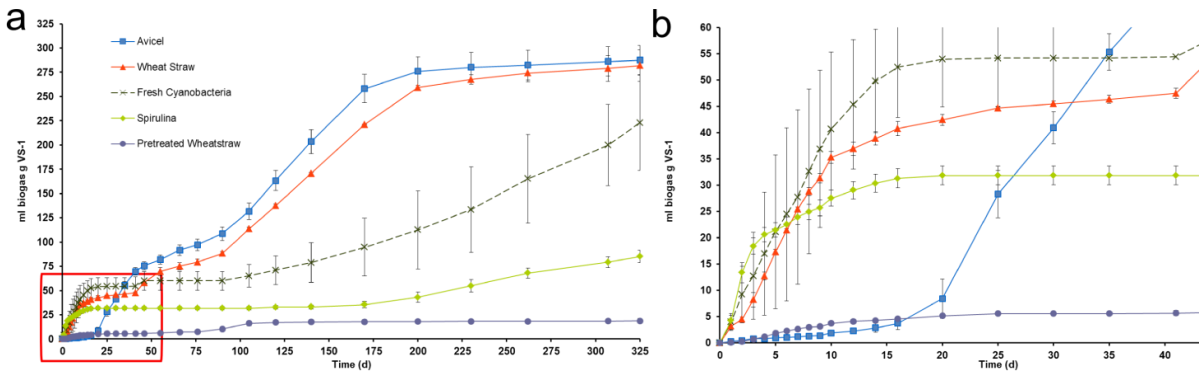

Figure 5.1 Alk-BT2 Biomethane potential
Alk-BT2 cumulative biogas potential alkaline conditions (pH 10; 2.0 M Na^+) and 37°C incubation **a)** After 325 days of incubation; **b)** 40 initial days of incubation (enlarged red box).

3.2. Alk-BT2 batch tests: biogas production under non-inhibitory conditions

As the previous results suggested that the alkaline inoculum utilized was heavily inhibited, a new set of batch tests were prepared with alkaline sludge obtained in the first days of the start-up process of reactor Alk-Sed-2 [4].

Using a non-inhibited inoculum had, as expected, a positive effect on the biogas production and the overall biogas yields were much higher than the ones obtained with the Alk-BT1 tests (Figure 5.1; Table 5.2). Contrary to what was observed in the Alk-BT1 tests, the biogas produced from all substrates was rich in methane. The highest methane content, 94%, was obtained with fresh algal biomass as substrate which is comparable to the results obtained with the continuous alkaline Alk-Opt reactor (Table 3; [4]). In all tests, the alkaline medium acted as a carbon dioxide scrubber and its presence in the headspace was minor and in some cases absent (Table 5.2).

Wheat straw and Avicel were the substrates with the highest biomethane potential, 262 and 259 mL CH_4 g VS^{-1} respectively (Table 5.2). The biodegradability of microcrystalline cellulose also increased using a fresh inoculum, from 6.5% in Alk-BT1 to 62.4% in Alk-BT2. Both the biomethane potential and the biodegradability were, however, still far from the theoretical maximum methane potential for cellulose, 415 mL CH_4 g VS^{-1} and were also lower than results at mesophilic pH conditions

Table 5.2 Alkaline batch tests Alk-BT-2 biogas and methane potentials
Biogas and methane potential of different substrates at alkaline conditions after 325 days of incubation at 37ºC

	Substrates				
	Spirulina	Avicel	Wheat straw	Pretreated Wheat straw	Fresh algal biomass
Theoretical CH_4 (mL g VS^{-1})*	627	415	426	415	627
Experimental Biogas (mL g VS^{-1})	85	287	281	18	222
% CH_4	93	90	93	92	94
% CO_2	0	5	3	0	2
% N_2	7	5	4	8	4
Experimental CH_4 (mL g VS^{-1})**	78	259	262	17	210
Biodegradability BD_{CH4} (%)*	12.5	62.4	63.2	4.2	33.4
Initial pH	10.01	10.01	10.01	10.01	10.01
Final pH	9.70	9.51	9.60	9.84	9.7
Initial NH_3 (mg L^{-1})	u.d.	u.d.	u.d.	u.d.	u.d.
Final NH_3 (mg L^{-1})	1123	93	133	119	401

*Calculated as in Raposo et al 2011
** Calculated with the experimental methane %
u.d.: Hach Lange LCK302 test under detection limit (47 mg L^{-1} NH_4-N)

reported in literature which range from 340 to 390 mL CH_4 g VS^{-1} (Raposo et al., 2011; Turick et al., 1991).

Spirulina was also degraded better in Alk-BT2 tests. Its BMP increased to 85 mL of methane per gram of volatile solids and the biodegradability increased by factor 12 compared to the previous batch test (Table 5.2). This BMP is higher than the specific methane potential obtained with reactor Alk-Opt, 71 mL CH_4 g VS^{-1}, which was operated under optimal process conditions (Table 3; [4]). However, these results are still far from the ones obtained at mesophilic pH and alkalinity, around 250 mL of CH_4 g VS^{-1} (Mussgnug et al., 2010).

Interestingly, when a fresh mixture of algal and cyanobacterial biomass was used as substrate, the methane potential drastically increased, reaching 210 mL of methane g VS^{-1}. Likewise, the biodegradability of the fresh algal biomass was also greater than the biodegradability of freeze dried *Spirulina*, 33.4 and 12.5% respectively (Table 5.2).

This increase in biogas production and biodegradability could be attributed to the type of algal mixture used, *Geitlerinema,* a cyanobacterium and *Dunaliella* a microalga. The latter has a higher BMP at mesophilic pH conditions, 323 mL CH_4 g VS^{-1} when compared to *Spirulina* 293 mL CH_4 g VS^{-1} (Mussgnug et al., 2010). This higher BMP potential of *Dunaliella* over *Spirulina* is mainly due to the fact that

Dunaliella does not have a cell wall (Mussgnug et al., 2010), and is therefore more easily biodegradable compared to *Spirulina,* which has a proteinaceous cell wall. *Dunaliella* also has a lower content of proteins, 57%, compared to *Spirulina*, 70% (Becker, 2007) which leads to less ammonia production when digested. This explains the ammonia concentrations in the fresh algal batch tests which were much lower, 401 mg L^{-1}, than in the *Spirulina* batch tests, 1,123 mg L^{-1} NH_3 (Table 5.2). This lower ammonia content is clearly below the inhibitory levels reported in our continuous anaerobic reactor, 1,200 mg L^{-1} [2] and therefore methanogens in these batch tests were less inhibited than in the *Spirulina* batch tests. Not much is known about *Geitlerinema*'s biomass composition and biodegradability but it is known that it has a filamentous morphology with no spirals which possibly makes it easier to degrade. Another factor that could improve the biodegradation of the fresh algal biomass is that it was cultivated under alkaline conditions in contrast to the cultivation of *Spirulina* which is normally done at neutral pH. Growth of *Spirulina* at neutral pH and subsequent dehydration can cause *Spirulina* to aggregate due to ionic interaction which can make the digestion process more complex.

An interesting result was obtained when wheat straw was used as substrate. As can be seen in Figure 5.1a the alkaline conditions of the inoculum (pH~10 and around 100 g $CaCO_3$ L^{-1}) apparently acted as a pretreatment for the digestion of wheat straw and, its biodegradability reached 63.2 % of its theoretical methane potential (Table 5.2). The BMP and the percentage of conversion of untreated wheat straw digested at mesophilic pH conditions (pH~7.5) ranges from 100 mL CH_4 g VS^{-1} and 24% conversion to 242 mL CH_4 g VS^{-1} and 57% (Demirbas, 2006; Motte et al., 2014; Song et al., 2014). Therefore, the obtained production was higher or similar to that obtained at normal pH conditions.

It is worth noting that when the wheat straw was submitted to an external pretreatment (incubation with $Ca(OH)_2$) the biomethane potential and the biodegradability were extremely low when compared to the non-treated wheat straw (Figure 5.1a; Table 5.2). This result was unexpected as, under mesophilic conditions, the pretreatment with $Ca(OH)_2$ usually increases the BMP of wheat straw (Song et al., 2013; Song et al., 2014). One possible explanation for this reduction in biogas is that the bacterial population was inhibited by the presence of Ca^{2+}. It is known that in Soda lakes, in order to have such a high pH and alkalinity, Ca^{2+} and Mg^{2+} have to

absent from the water (Ulukanli and Rak, 2002). In this context, the soda lake microbial community might be sensitive to Ca^{2+} and thus its presence might have caused inhibition of the hydrolytic bacteria or the methanogenic community (Chen et al., 2008).

The digestion of all tested substrates, except pretreated wheat straw, seemed to take place in three distinguishable phases (Figure 5.1). In each case however, the three phases could be attributed to different causes:

- Wheat straw: In the first phase, from day 0 to day 10, 12% (35 mL) of the total biogas was produced (Figure 5.1a). In this phase the hydrolytic bacteria rapidly consumed all the easily available sugars. After day 10, an adaptation phase took place which lasted for 30 days in which only 12 mL of biogas were produced (Figure 5.1b). Wheat straw is mainly composed of cellulose, hemicellulose and lignin in low amounts (Zahoor and Tu, 2014) and, during this “lag” period, the alkaline conditions of the medium contributed to degradation by disrupting its crystalline structure and making cellulose and hemicellulose more accessible for enzyme attack (Kumar et al., 2009). After this “pretreatment” period, cellulose and more simple sugars were released and made accessible for hydrolytic bacteria which resulted in a second biogas production phase, from day 40 to day 200, where 75% of the total biogas was produced (Figure 5.1a). After this, a plateau was reached, from day 200 to 325 in which 22 mL of biogas were produced. This low biogas production indicated that the majority of the biodegradable biomass was consumed and only lignin and minor amounts of complex sugars remained undigested.

- Avicel: A similar trend was observed when microcrystalline cellulose (Avicel) was used as substrate and three distinct phases could be identified (Figure 5.1). In this case however, an initial lag, or adaptation phase took place during the first 16 days. In this period the biogas production was extremely low with a production of 0.2 mL of biogas per day (Figure 5.1b). The fact that during the first 16 days the biogas production was low corroborates the findings in the wheat straw tests where the initial biogas was originated from easily degradable sugars. After this adaptation period, and as in the case of the wheat straw, the alkaline medium contributed to the disruption of the cellulose molecules and the hydrolytic bacteria were able to start to degrade it resulting in a sharply increased of the biogas production from day 16 to 90. During this period, the daily production was 1.4 mL of biogas. The third phase

started at day 90 and ended at day 200 in which 60% of the total biogas was produced. After this phase a plateau of almost no biogas production was reached (Figure 5.1a).

Considering that with both cellulose rich substrates, wheat straw and Avicel, most of the biogas produced was obtained after a relatively long lag phase it is reasonable to state that the fermentation of cellulolytic material, including disruption of the crystalline structure by the alkaline medium and subsequent release of sugars and easily degradable molecules, is a relatively slow process.

- *Spirulina*: In this case, a rapid biogas production was observed during the first 16 days, after which a very long lag phase occurred, 154 days (Figure 5.1a). During this lag phase only 4.0 mL of biogas were produced. The long lag phase could be attributed to a period of adaptation in order to overcome a possible inhibition by ammonia accumulated during the first degradation phase, which temporarily reduced the activity of the methanogenic bacteria. After the lag phase, or adaptation phase, from day 170 onwards, biogas production was resumed, and 50 mL of biogas, (60% of total produced biogas) were obtained.

- Fresh algal biomass: In the case of fresh algal biomass the initial production phase lasted from day 0 to 16 where 23% of the total biogas was produced. This phase was again followed by a 74 days lag phase where almost no biogas was produced (Figure 5.1a). After this period, the biogas production was resumed until day 325. During this time, 153 mL of biogas were produced, 69% of the total produced gas. In this case, however, and in contrasts to what occurred in the *Spirulina* batch test, the 74 days lag phase could not be attributed to an adaptation to ammonia inhibition as the levels of ammonia at the end of the experiment were low (Table 5.2). A possible explanation for the observed lag phase could be the mixture of fresh algae used. We can only speculate, but it is possible that during the first 16 days only the easily accessible parts of both microalgae were rapidly digested, and at the same time, the alkaline medium acted relatively slowly on the more complex molecules, causing the lag phase. Once the breakdown of the organic matter by the alkaline conditions led to the release of compounds that could be further degraded by the microbial community, the biogas production was resumed (Figure 5.1). One observation that points to this possibility is that the lag phase was much shorter in the fresh algal biomass, 74 days, when compared to the lag phase in

the *Spirulina* tests, 154 days. This could indicate that, as speculated, the lag phase was due to a slow breakdown of the substrate rather than to an adaptation to inhibitory conditions.

The results obtained with the Alk-BT2 batch tests indicate firstly, that in order to obtain a high biogas production at alkaline conditions it is best to use fresh inoculum or, inoculum that has not suffered from harsh inhibitory conditions. It is also worth noting, that fresh algal biomass has a greater biogas potential than freeze dried *Spirulina*. These results suggest that a biogas system working at alkaline conditions and using fresh cyanobacterial biomass as substrate might be an interesting option for the production of biogas. Another interesting application of biogas at alkaline conditions might be the option of using wheat straw as a substrate. As was shown here, the alkaline medium acts as a pretreatment that breaks down the complex cellulolytic material which then can be converted into biogas. This approach opens the possibility to produce biogas from cellulose biomass in a single step process.

4. Conclusions

The results presented are the first attempt of biomethane potential (BMP) from *Spirulina*, fresh algal biomass, and wheat straw at alkaline conditions using the sludge originated form an alkaline anaerobic reactor. The results show that fresh algal biomass has a higher BMP potential when compared to freeze dried *Spirulina*. These results make fresh algal biomass a possible substrate for an alkaline anaerobic digester. BMP of un-pretreated wheat straw at alkaline conditions was high, achieving 63% of biodegradability. The presented results open the possibility of using un-pretreated wheat straw as substrate for biogas production at alkaline conditions in one single step.

[6] Metagenome analysis of the microbial population from an alkaline anaerobic digester fed with the microalga *Spirulina*

V Nolla-Ardèvol

1. Introduction

Metagenomics has become a commonly used tool to study taxonomy, gene composition and gene function in microbial communities (Simon and Daniel, 2011). So far, a number of metagenome studies from several biogas producing plants and lab scale anaerobic digesters have been performed (Jaenicke et al., 2011; Krause et al., 2008; Li et al., 2013; Schlüter et al., 2008; Wirth et al., 2012). In a previous work high throughput DNA sequencing was used to study the microbial community involved in the anaerobic digestion of the microalga *Spirulina* at mesophilic pH conditions [3].

Several studies have applied molecular approaches and sequencing technologies to study the microbial diversity of alkaline or hypersaline environments (Kanekar et al., 2008; Ochsenreiter et al., 2002; Rees et al., 2004). Detection of the methanogenic marker gene *mcrA* has also been used to study the methanogenic population of hypersaline and soda lakes (García-Maldonado et al., 2012; Nolla-Ardèvol et al., 2012). However, few metagenomic approaches have been performed to study the microbial community of alkaline lakes or hypersaline environments. López-López et al., 2013, studied the archaeal diversity in a hypersaline mat by sequencing selected fosmids and Lanzén et al., 2013 studied the prokaryotic diversity in a Kenyan Soda lake using the GS-FLX Titanium sequencer. The only known work in which a metagenomic approach was applied to study an anaerobic microbial community at high pH was recently performed by Wong et al., 2013 in which waste activated sludge was pretreated at pH 10 and subsequently used as inoculum for an anaerobic digester.

The anaerobic digestion of organic matter is a complex process that involves the participation of both bacteria and archaea (Schlüter et al., 2008; Wirth et al., 2012).

Table 6.1 DNA extraction protocols
Modifications made to the Zhou et al., 2006 DNA extraction protocol to increase DNA extraction yields.

Sample	A1	A2	A3	A4
Protocol Modifications	Zhou et al., 2006		Zhou et al., 2006	
Wash	-	NaCl[a]	NaCl	NaCl
$AlNH_4(SO_4)_2$ (mM)	-	-	100	200
Sequenced	No	No	No	Yes

a; 1.0 M solution

Under alkaline conditions this likely also applies but to date, the different functional groups have only been addressed individually (Antony et al., 2012; Kivistö and Karp, 2011; Sorokin and Kuenen, 2005b). In this work, the metagenomic analysis of the anaerobic community of an alkaline reactor digesting *Spirulina* is discussed with regards to taxonomy and function. Elucidating the microbial composition of the biogas alkaline digester and understanding the function and relationships of the different microorganisms could help to improve the biogas production at alkaline conditions.

2. Material and methods

DNA for metagenomic analysis was extracted from the Alk-Sed-2 alkaline reactor on day 111 of operation [4].

2.1. DNA extraction

Due to the harsh conditions of the sludge, high pH and high alkalinity and to possible presence of inhibitory substances such as humic acids and PCR inhibitors, different protocols for DNA extraction were compared. DNA was extracted from four samples (A1 – A4) following the Zhou et al., 1996 protocol with several modifications in order to optimize the DNA extraction (Table 6.1).Extraction of DNA from sample A1 was done following the Zhou et al., 1996 protocol without any extra modification.

The remaining samples, A2, A3 and A4 were first washed three times with a 1.0 M NaCl solution to reduce the alkalinity by centrifuging 10 minutes at 4,600 rpm. DNA from sample A2 was then extracted as in sample A1. For samples A3 and A4 an extra modification was made. During the Lysozyme incubation period, 200 µL of 100 and 200 mM $AlNH_4(SO_4)_2$ solution was added to samples A3 and A4 respectively to precipitate humic acids (Braid et al., 2003; Foti et al., 2008).

Extracted DNA from all four samples was purified with an ion exchange column (Macherey-Nagel, Germany) and re-suspended in TE buffer. Concentration of extracted and purified DNA was determined with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The absorption ratios at 260nm/230nm (DNA/humic acid) and 260nm/280nm (DNA/protein) were determined and used to assess the purity of the extracted DNA. A A260 to A280 ratio higher than 1.8, indicates the successful removal of proteins, while an A260 to A230 ratio greater than 2, indicates high purity of DNA regarding the absence of humic acids and other PCR inhibitory substances.

2.2. DNA sequencing

2.5 µg of purified DNA obtained from the A4 sample and were used to prepare a 400 bp insert size sequencing libraries for the Ion Torrent Personal Genome Machine (PGM) platform (Life Technologies, USA) and sequenced on a 318™ Chip as in [3]. Automated quality control of the sequence reads was performed with the Torrent Suite™ Software v3.2 using default settings. Additional quality filtering was done using the Trimmomatic tool v3 (Lohse et al., 2012) with settings for removal of trailing bases of a q-value lower than 20, and removal of reads shorter than 100 bp and longer than 450 bp.

2.3. Removal of reads belonging to *Spirulina*

Quality trimmed reads were uploaded to the MGX platform, a metagenomics platform currently being developed at the CeBiTec, for a rapid prescreening of the reads. A preliminary taxonomic analysis revealed over 30% of reads assigned to *Spirulina* which were removed as follows: First, 3,125 sequences, from all genomes and curated sequences (RefSeq) from the NCBI database (November 2013) classified as *Spirulina*/*Arthrospira,* were downloaded. Next, quality trimmed reads were blasted against the 3,125 sequences in order to identify reads that could potentially belong to *Spirulina*. Blast was performed with an e-value of 1e-10, a maximum of one target per sequence and with a minimum of 98% of identity. Any read that had a blast hit to any of the downloaded sequences was identified as belonging to *Spirulina* and therefore removed from the dataset. The remaining reads, quality trimmed with no *Spirulina* reads, were used for functional and taxonomical computational analysis.

2.4 Analysis of sequencing reads

2.4.1 Direct analysis of sequencing reads

Reads were uploaded to the MG-Rast metagenome analyzer (Meyer et al., 2008) with default settings and searched for functional genes with the Subsystem hierarchical classification option with an e-value of 1e-5, 50% minimum identity and 15 amino acid minimum length.

A second functional analysis was performed by identifying specific protein domains (Pfam) related to cellulose degradation, and protein and amino acid degradation. Pfam identification was done as in [3] with minor modifications. In this case, the Translatedna v 1.75 script (www.mbari.org/staff/haddock/scripts/), with the "1" option, print best ORF, was used to obtain the best possible ORF for each read. According to the scripts user manual, the criteria to select the best ORF are (i) length of longest ORF and (ii) the number of individual ORFs.

2.4.2 Analysis of assembled reads

In total, two assemblies (A and B) were performed with the Genome Sequencer De Novo Assembler Software v2.6 (Roche Applied Science, Germany); assembly A was done with default settings for genomic DNA, and assembly B was done with more stringent settings, according to Fan et al., 2012, for better assembly of 16S rDNA sequences. Metawatt v1.7, with settings as in [3], was used to perform the binning of contigs from assembly A for genome-centric analysis. Automatically generated bins with a consistent phylogenetic signature were manually selected and annotated (16S rDNA, tRNA and genome completeness) as in [3].

2.4.3. Phylogeny of assembled 16S rDNA sequences

To identify 16S rDNA sequences among the assembled contigs, all contigs from assemblies A and B were submitted to a blastn search against the RDP database (v11-2) (Cole et al., 2014). Sequence parts with a hit were extracted and aligned parts with a minimum length of 1000 bp for bacteria and archaea or 500 bp for Bacteroidetes were used to create phylogenetic trees as in [3] with minor modifications. Blastn searches were done against the current (May 2014) NCBI nucleotide collection (nr/nt), and reference RNA sequences (refseq_rna) with default settings.

3. Results and discussion

3.1. Optimization of a DNA extraction protocol

DNA for metagenomic analysis was extracted from alkaline sludge of the Alk-Sed-2 anaerobic reactor [4]. Due to the harsh conditions of the sludge, pH 10, high alkalinity (100 g $CaCO_3$ L^{-1}), high salt concentration, high presence of both degraded and undegraded organic matter, the extraction efficiency of DNA was low when using standard protocols. The main problem associated with the high amount of organic matter might have been the presence of humic acids and other PCR inhibitors which co-precipitate with the extracted DNA and impede further enzymatic processing for PCR or high throughput sequencing library preparation (Braid et al., 2003; Foti et al., 2008). In the first DNA extractions attempts (data not shown), the total DNA extracted was high but contaminated with high amounts of humic acids (A260/A230 ration ~ 1.0). In these trials, after DNA purification, only a small fraction of the original DNA remained, in most cases with concentrations which were too low for metagenomic DNA library preparation Therefore, it was decided to optimize the protocol from Zhou et al 1996 and adapt it to the harsh conditions of the sludge. The modifications consisted in adding an initial washing step with NaCl in order to lower the alkalinity and in the addition of $AlNH_4(SO_4)_2$ to increase the precipitation of humic acids and other PCR inhibitors (Braid et al., 2003; Foti et al., 2008).

Table 6.2 shows the DNA concentration and the purity achieved with each of the different extraction methods. As can be seen, the original Zhou et al., 1996 method (sample A1) yielded the lowest DNA concentration and both ratios that determine the purity of the extracted DNA were far from their optimal values. A A/260/A280 ratio lower than 1.8 is indicative of protein contamination while a A260/A230 ratio below indicates the presence of humic acids and other contaminants (Siddhapura et al., 2010).

The use of the modified protocol had a positive effect both on the amount of extracted DNA and in the reduction of contaminants (Table 6.2). The ratios A/260/A280 and A260/A230 were still below their optimal values but were higher than the ones obtained with the original DNA extraction procedure. The results show that washing with NaCl plus the addition of $AlNH_4(SO_4)_2$ is a valid strategy to extract high

Table 6.2 DNA obtained with the different extraction protocols
Amount and quality of the total and purified DNA extracted from 15 mL of alkaline sludge originating from the Alk-Sed-2 reactor according to the different used protocols.

Sample	A1	A2	A3	A4
Protocol	Zhou et al., 2006	NaCl[a]	NaCl 100 mM[b]	NaCl 200 mM
Raw DNA				
µg total DNA	1.84	50.56	27.47	20.5
A260/A280**	1.65	1.59	1.75	1.74
A260/A230**	0.49	0.87	0.94	1.01
Purified DNA				
µg total DNA	N.P	4.17	4.59	4.84
A260/A280	N.P	1.95	1.84	2.06
A260/A230	N.P	2.22	3.44	4.78
Sequenced	No	No	No	Yes

a: Wash with NaCl 1.0 M; b: mM of $AlNH_4(SO_4)_2$; N.P: Not performed
**A260/A280 ratio <1.8 is indicative of protein contamination.
**A260/A230 ratio >2 is indicative of high purity of DNA.

molecular DNA from alkaline sludge and might be applicable to other types of extreme environmental samples.

3.2. Analysis of the sequenced metagenome

A DNA sample was extracted from the alkaline reactor Alk-Sed-2 on day 111 of operation where the biogas production was 70 mL biogas day^{-1} with 92% methane content [4]. The sequencing run produced 4,602,427 reads and after quality trimming and removal of reads that potentially belonged to *Spirulina,* 2,032,005 reads remained.

3.2.1. Taxonomic analysis of assembled reads

Contigs obtained from assembly A were processed by binning into provisional whole genome sequences of abundant community members (Strous et al., 2012) and annotation of these whole genome sequences. From the initial automatic generate bins nine were manually identified, based on consistent phylogenetic profiles, presence of a complete set of encoded tRNA molecules and presence of a near complete set of conserved single copy genes, to contain a provisional whole genome sequence belonging to an abundant population. Fragments of 16S rDNA sequences were assigned to each of the bins based on correlation of the phylogenetic profile of the bins with the phylogenetic affiliation of the identified 16S sequences. 60% of the sequenced reads were mapped to these data, indicating that together these

Table 6.3 Selected microbial bins
Characteristics and 16S rDNA taxonomical classification of the 9 selected bins obtained from the alkaline metagenome.

Bin characteristics									
Bin	**Contigs (#)**	**Size (Mb)**	**N50 contig length (kb)**	**GC (%)**	**Cov (X)**	**tRNA (#)**	**Conserved Genes* (#)**	**Abun (%)**	**16S rDNA taxonomic classification****
A	703	2.6	7.3	49.6	21.2	32	128/139	21.2	Bacteroidetes
B	334	1.9	23.1	42.4	9.0	11	103/139	11.1	Clostridiales
C	1,900	2.2	1.8	36.9	5.0	24	164/139	7.4	Halobacteroidaceae
D	2,851	1.6	0.6	35.2	2.2	4	38/139	2.3	Halanaerobiaceae
E	2,693	2.9	1.6	51.2	3.2	9	111/139	6.0	Bacteroidetes
F	2,217	2.4	1.9	51.0	2.8	30	97/139	4.5	Methanocalculus
G	6,796	3.7	0.6	38.6	2.1	43	232/139	5.1	Haloanaerobiales
H	1,750	1.2	0.7	46.5	2.9	7	20/139	2.2	Clostridiales
I	1,671	0.8	0.5	61.8	2.3	3	55/139	1.3	Rhodobacteraceae

Cov: Coverage
Abun: Abundance
*Number of Conserved Single Copy Genes detected (out of a set of 139). Numbers higher than 139 indicate the presence of DNA originating from more than a single population in the bin. Numbers lower than 139 indicate the provisional genome sequence associated with the bin may be incomplete.
**Taxonomical assignment based on the SILVA and RDP maximum coincidence level. See Table 6.4 for assignment details.

populations accounted for 60% of the microbial community present in the alkaline reactor at day 111 (Table 6.3).

The inoculum used for the anaerobic reactor consisted of sediments from a haloalkaline lake, which have not been studied extensively at molecular level and, therefore, it was not surprising that a high percentage of the assembled contigs (~40%) could not be assigned to any particular taxon by blastn. This difficulty in the assignment of taxa indicates that a great number of the bacteria and archaea present in the alkaline reactor were, as expected, relatively unrelated to reference organisms with sequenced genomes.

As in the case of the mesophilic digester microbial community [3], Bacteria clearly dominated with 8 of the 9 bins representing over 95% of the binned populations. Only a single of the binned populations belonged to Archaea and was related to *Methanocalculus* (4.5% abundance; (Table 6.3).

Interestingly, and in contrast to previous studies where Clostridia dominated the bacterial population of anaerobic digesters (Jaenicke et al., 2011; Kovács et al., 2013; Li et al., 2013; Schlüter et al., 2008; Wirth et al., 2012), here Bacteroidetes were predicted to be the dominant. Firmicutes accounted for 28.1% of the identified

microbial population while Bacteroidetes represented 27.2%. Proteobacteria were also present but in much lower abundance, 1.3% (Table 6.3). As expected, most of the identified 16S rDNA populations were assigned to halotolerant and alkaliphilic groups such as *Halanaerobium* and other members of the Halanaerobiaceae family (Baumgarte, 2003; Kapdan and Erten, 2007; Kivistö and Karp, 2011; López-López et al., 2013), *Anaerobranca* (Grant, 2006; Humayoun et al., 2003; Yang et al., 2013), *Orenia* (Kivistö and Karp, 2011; Rainey et al., 1995; Wuchter et al., 2013) and the methanogen *Methanocalculus* (Garcia et al., 2000; Lanzén et al., 2013; McGenity, 2010; Nolla-Ardèvol et al., 2012; Surakasi et al., 2007), which have previously been identified in alkaline lakes and saline habitats (Table 6.4).

3.2.1.1 Taxonomy of the fermentative bacteria

The first three steps of the anaerobic digestion process, hydrolysis, acidogenesis and acetogenesis are performed, at mesophilic pH conditions, mainly by bacteria of the phyla Firmicutes, Bacteroidetes and Thermotoga among others (Jaenicke et al., 2011; Pavlostathis, 2011; Schlüter et al., 2008; Al Seadi et al., 2008). In the case of the anaerobic digestion at alkaline conditions these groups of bacteria also appear to be responsible for these processes but in different proportions than previously found (Table 6.3).

(i) Cytophaga-Flavobacterium-Bacteroides group (Bacteroidetes)

The most abundant population, 21.2%, corresponding to bin A (Table 6.3), contained three contigs, contig08670, contig11253 and contig24233, with 16S rDNA sequences that were all assigned to “ML635J-40 aquatic group” by the SILVA classifier, and to Bacteroidetes Incertae Sedis, Flavobacteria and Bacteroidia by the RDP classifier, all members of the Cytophaga-Flavobacterium-Bacteroidetes group (CFB) (Table 6.4).

Contig08670, assigned by SILVA to the “ML635J-40 aquatic group” and by RDP to Bacteroidia was closely related (97% blastn identity) with “clone ML635J-56”, an uncultured Bacteroidetes identified in soda lake sediments (Humayoun et al., 2003) (Figure 6.1). 16S rDNA sequences assigned to “ML635J-40 aquatic group” and to “ML635J-56” have previously been identified in Mono lake (California, USA) (Humayoun et al., 2003) and Lonar crater lake sediments (Maharastra, India) (Wani et al., 2006), both saline soda lakes. This group of uncultured bacteria is currently classified as members of the Bacteroidetes phylum. The class Bacteroidia includes

only one family, Marinilabiaceae, which is known to include a halophilic anaerobic fermentative bacterial species, *Anaerophaga thermohalophila* (de la Haba et al., 2011). This same contig, contig08670, was assigned to *Anaerophaga* by the RDP classifier but only with 47% confidence at family level and 25% at genus level (data not shown). Contig08670 was also closely related to contig05582 from assembly B which could be classified by RDP as Bacteroidia with 100% identity (Table 6.4) and was also classified as *Anaerophaga* (25% identity; data not shown). Even though the RDP classification of both contigs suggested that they belonged to *Anaerophaga*, their phylogenetic classification places them closer the "ML635J-40 aquatic group" (Figure 6.1). Contig11253, also assigned to "ML635J-40 aquatic group" by SILVA, was classified as Flavobacteria by RDP (*Belliella* with 25% identity; data not shown).

Phylogenetically it was closely related to cloneCSS133, (99% blastn identity) another uncultured Bacteroidetes sequenced from coastal soil (Keshri et al., 2012; direct submission) and both were also placed into the "ML635J-40 aquatic group" clade (Figure 6.1). The third contig, contig24233, could only be assigned at class level as a Bacteroidia Incertae Sedis by RDP (*Marinifilum* 18% identity; data not shown) and again to "ML635J-40 aquatic group" by SILVA. These results indicate that bin A was composed of DNA sequences from an uncultured Bacteroidetes of the CFB group phylogenetically close to the "ML635J-40 aquatic group".

A similar result was obtained in bin E, 6% abundance (Table 6.3), where all four 16S rDNA sequences were also assigned to "ML635J-40 aquatic group" by SILVA and to Flavobacteria and Sphingobacteria, by RDP (Table 6.4). Contig05178, assigned to "ML635J-40 aquatic group" by SILVA, was assigned to *Alkaliflexus* by RDP (27% identity; data not shown), an alkaliphilic Bacteroidetes of the CBF group (Zhilina et al., 2004). Phylogenetically was closely related (99% blastn identity) to an uncultured Bacteroidetes clone WN-HWB-154 isolated from Wadi An Natrun alkaline lake in Egypt (Mesbah et al., 2007a) and to contig13353 from assembly B, which was assigned by RDP to *Paludibacter* (29% data not shown), another Bacteroidales (Figure 6.1; Table 6.4). The "ML635J-40 aquatic group" (Baumgarte, 2003), *Anaerophaga* (Denger et al., 2002), *Belliella* (Brettar et al., 2004), Marinifilum (Na et al., 2009) and *Alkaliflexus* (Zhilina et al., 2004) belong to what is known as the Cytophaga–Flavobacterium–Bacteroides group.

Table 6.4 Contigs containing 16S rDNA sequences of the 9 selected bins

16S rDNA taxonomical assignment of binned and unbinned contigs from assembly A and contigs from assembly B performed with the SILVA and RDP databases. Assignment was done with 80% minimum identity. Numbers in brackets indicate the highest percentage of identity. No number indicates 100% identity.

Ass*	Bin	Contig	DB	Phylum	Class	Order	Family	Genus
A	A	Contig08670	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes	Bacteroidia (61)			
		Contig11253	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes	Flavobacteria (63)			
		Contig24233	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes (99)	Incertae sedis (24)			
A	B	Contig02281	SILVA	Firmicutes	Clostridia	Clostridiales		
			RDP	Firmicutes	Clostridia	Clostridiales	Clostridiaceae (74)	
A	C	Contig01919	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halobacteroidaceae	
			RDP	Firmicutes	Clostridia	Halanaerobiales	Halobacteroidaceae	*Orenia (76)*
A	D	Contig03844	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
			RDP	Firmicutes	Clostridia	Halanaerobiales (98)	Halanaerobiaceae (91)	*Halocella (52)*
		Contig21218	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	
			RDP	Firmicutes	Clostridia	Halanaerobiales (99)	Halanaerobiaceae (98)	*Halothermothrix (64)*
		Contig25077	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
			RDP	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae (98)	*Halanaerobium (61)*
A	E	Contig05178	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes	Flavobacteria (55)			
		Contig25151	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes	Flavobacteria (38)			
		Contig26930	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes (98)	Sphingobacteria (31)			
		Contig29659	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes (91)	Flavobacteria (71)			
A	F	Contig01776	SILVA	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Incertae Sedis	*Methanocalculus*
			RDP	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Incertae Sedis	*Methanocalculus*

* Ass: Assembly used. See Material and Methods section for details

Table 6.4 Continuation

Ass*	Bin	Contig	DB	Phylum	Class	Order	Family	Genus
A	G	Contig11185	SILVA	Firmicutes	Bacilli	Bacillales	Bacillaceae	
			RDP	Firmicutes (97)	Clostridia (86)	Halanaerobiales (61)		
		Contig12844	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
			RDP	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
		Contig14076	SILVA	Firmicutes	Clostridia			
			RDP	Firmicutes (89)	Clostridia (73)			
		Contig18213	SILVA	Firmicutes				
			RDP	Firmicutes (68)				
		Contig18995	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
			RDP	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
		Contig25642	SILVA	Firmicutes	Clostridia	Halanaerobiales		
			RDP	Firmicutes (90)	Clostridia (81)	Halanaerobiales (73)		
		Contig25685	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
			RDP	Firmicutes (86)	Clostridia (73)			
		Contig26525	SILVA	Firmicutes				
			RDP	Firmicutes (91)	Clostridia (89)	Halanaerobiales (70)		
		Contig27756	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
			RDP	Firmicutes (84)	Clostridia (78)			
		Contig29794	SILVA	-				
			RDP	Firmicutes (76)				
A	H	Contig01782	SILVA	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
			RDP	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Tindallia*
		Contig02379	SILVA	Firmicutes	Clostridia	Clostridiales	Incertae Sedis XIV	*Anaerobranca*
			RDP	Firmicutes	Clostridia (99)	Clostridiales (98)	Incertae Sedis XIV (88)	*Anaerobranca (88)*
A	I	Contig04714	SILVA	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
			RDP	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Rhodobaca (52)*
		Contig08033	SILVA	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
			RDP	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae (99)	*Rhodobaca (45)*

* Ass: Assembly used. See Material and Methods section for details

Table 6.4 Continuation

Ass*	Bin	Contig	DB	Phylum	Class	Order	Family	Genus
A	Un**	Contig01627	SILVA	Firmicutes	Clostridia	Natranaerobiales	Natranaerobiaceae	
			RDP	Firmicutes	Clostridia	Natranaerobiales	Natranaerobiaceae	*Natranaerobius*
B	Un**	Contig02658	SILVA	-				
			RDP	Firmicutes	Clostridia	Natranaerobiales	Natranaerobiaceae	*Natronovirga*
		Contig03146	SILVA	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
			RDP	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	*Halanaerobium*
		Contig05582	SILVA	-				
			RDP	Bacteroidetes	Bacteroidia	Bacteroidales (43)		
		Contig05874	SILVA	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Salinarimonas*
			RDP	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	*Chelatococcus*
		Contig07456	SILVA	-				
			RDP	Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae (73)	
		Contig13353	SILVA	Bacteroidetes	Bacteroidia	Bacteroidales	ML635J-40 aquatic group	
			RDP	Bacteroidetes	Bacteroidia	Bacteroidales (65)		

* Ass: Assembly used. See Material and Methods section for details

** Un: Unbinned contigs

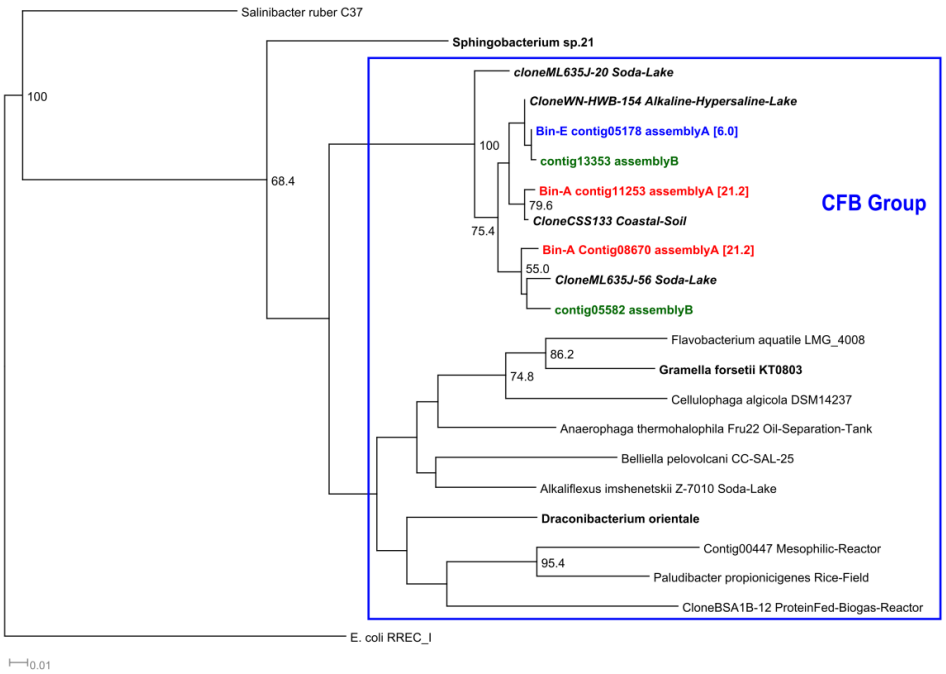

Figure 6.1 16S rDNA Cytophaga-Flavobacterium-Bacteroides phylogenetic tree
16S rDNA phylogenetic tree of the contigs assigned to members of the Cytophaga-Flavobacteria-Bacteroides group (CFB) by the RDP and SILVA classifiers. Although the binning was performed with contigs of assembly A, the tree also includes those contigs that were obtained from assembly B and were not assembled in assembly A. Minimum contig length of 500bp. Colored: sequences obtained from metagenomic reads. Assignment to Metawatt bins and percentage of bin abundance is indicated if applicable. Reference sequences in **bold:** top hits in blast search against NCBI reference RNA sequences database; ***bold+italics****:* top hits in blast search against NCBI non-redundant nucleotide collection. Additional reference sequences tree represent genera detected in other alkaline environments or anaerobic digesters. 16S rDNA sequence of *E. coli RREC_I* was chosen as outgroup. Bootstrap values at nodes are obtained from 500 replicates and are only shown for branches with at least 50% support (values > 49.9). The scale bar represents 0.01 nucleotide substitutions per site. Accession numbers of reference sequences are available in Suppl. Table 6.1

This is an important group of bacteria present in aquatic and haloalkaline environments (Humayoun et al., 2003; Rees et al., 2004) which participate in the degradation of organic matter and complex polysaccharides (Brettar et al., 2004) Some of their members are able to utilize proteins as substrate (Chen and Dong, 2005) and all the identified organism can grow in the presence of salt (Brettar et al., 2004; Denger et al., 2002; Na et al., 2009).

Members of the phylum Bacteroidetes have been previously identified in several biogas reactors but at lower abundance (Jaenicke et al., 2011; Rivière et al., 2009; Schlüter et al., 2008). The relatively high abundance of these populations in the data presented here, 21% bin A, and 6% bin E (Table 6.3), is in accordance with the findings of several studies from alkaline and saline environments where CFB bacteria

were found to dominate microbial communities. For example Mouné et al., 2003 found that CFB dominated in anoxic sediments below cyanobacterial mats from Mediterranean hypersaline ponds while Boutaiba et al., 2011 detected 40% abundance of Bacteroidetes in the sediments of Sidi Ameur hypersaline lake (Algeria). Bacteroidetes were also the dominant phyla in the waters of lake Chaka (China) an athalassohaline lake with a 32% salinity (Jiang et al., 2006). In addition, Bacteroidetes have also been found to be present in anaerobic reactors fed with protein rich substrates like *Spirulina* [3], casein and pig blood (Kovács et al., 2013). The ability to degrade proteins and the relatively high abundance of members of the CFB group observed in these habitats might be indicative that this particular group plays an important role in the degradation of protein rich substrates at alkaline conditions.

(ii) Clostridiales, Natranaerobiales and Halanaerobiales (Firmicutes)

Clostridiales (bin B and bin H) were the second most abundant identified populations, together accounting for 13% of the identified community (Table 6.3). Contig02281 from bin B was assigned, with 62% identity (data not shown) to the genus *Natronincola* by the RDP classifier while SILVA could only assign the contig to the order Clostridiales (Table 6.4). Contig02281 was phylogenetically related to an uncultured bacterium clone x216 identified in sediments from soda lake Xiarinur in Inner Mongolia (Cao et al., 2009; direct submission) and to *Clostridium difficile 630* (Figure 6.2). Members of the genus *Clostridium* are ubiquitous, and capable of growing both at neutral and alkaline pH (up to pH 11), and some require sodium. *Clostridium* can utilize yeast extract as carbon and energy source and they produce acetate as the main fermentative product (Yutin and Galperin, 2013).

The 16S rDNA sequences detected in Bin H could be assigned to *Anaerobranca* and *Tindallia*, both Clostridiales, which are alkaliphilic bacteria, that is, members of this group can grow optimally at pH above 9.0 (Yumoto, 2002) (Table 6.4). Contig02379 was assigned to *Anaerobranca* by both classifiers. Phylogenetically, the 16S rDNA sequence was closest to *Proteinovarax tanatarense* (96% blastn identity against NCBI NR database, assigned to genus *Anaerobranca* in the RDP database) isolated from a decaying algal bloom obtained from the Tanatar soda lake (Russia) (Kevbrin et al., 2013) (Figure 6.2). Contig01782 was assigned to *Tindallia* by RDP. As can be seen in Figure 6.2 its closest related 16S rDNA sequence originates from *Clostridium*

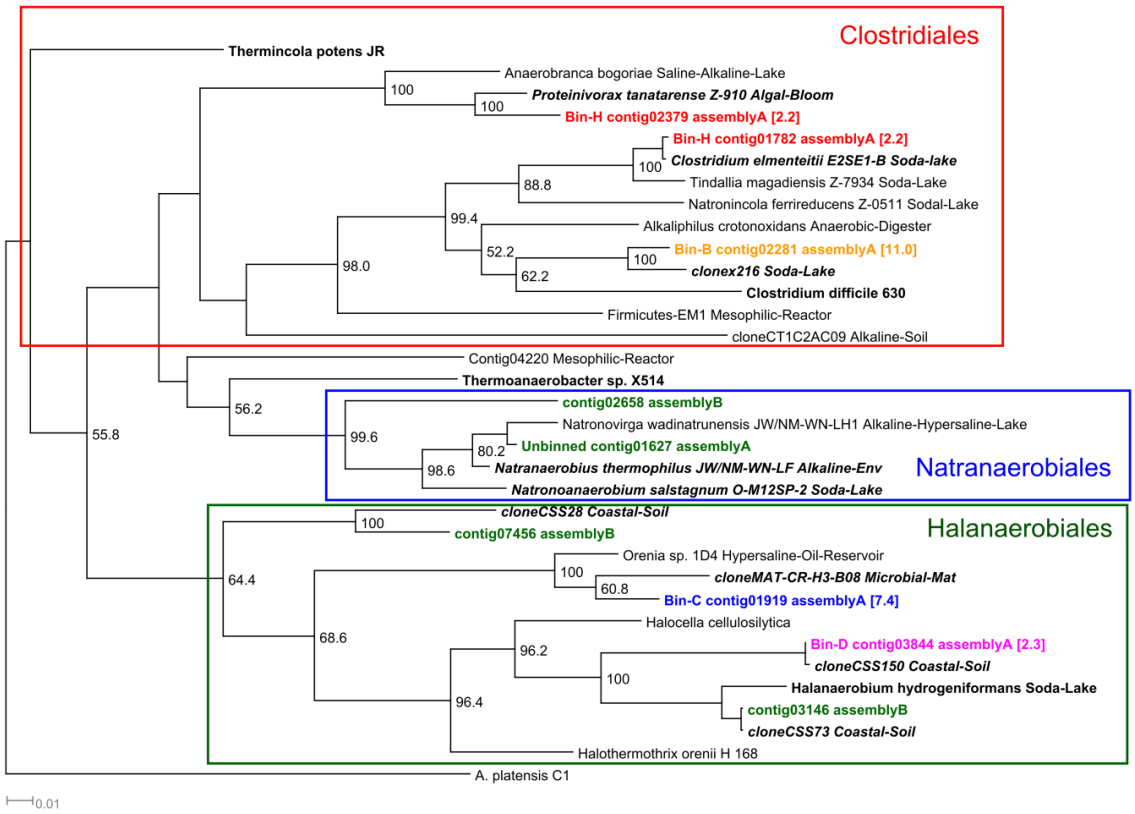

Figure 6.2 16S rDNA Firmicutes phylogenetic tree
16S rDNA phylogenetic tree of the contigs assigned to members of the Firmicutes phyla by the RDP and SILVA classifiers. Although the binning was performed with contigs of assembly A, the tree also includes those contigs that were obtained from assembly B and were not assembled in assembly A. Minimum contig length of 1000bp. Colored: sequences obtained from metagenomic reads. Assignment to metawatt bins and percentage of bin abundance is indicated if applicable. Reference sequences in **bold**: top hits in blast search against NCBI reference RNA sequences database; ***bold+italics:*** top hits in blast search against NCBI non-redundant nucleotide collection. Additional reference sequences tree represent genera detected in other alkaline environments or anaerobic digesters. 16S rDNA sequence of *Arthrospira platensis* was chosen as outgroup. Bootstrap values at nodes are obtained from 500 replicates and are only shown for branches with at least 50% support (values > 49.9). The scale bar represents 0.01 nucleotide substitutions per site. Accession numbers of reference sequences are available in Suppl. Table 6.1

elmenteitii (99% blastn identity against NCBI NR database, assigned to genus *Tindallia* in the RDP database) an organism identified in soda lakes (Jones et al., 1998).

16S rDNA clones related to *Anaerobranca* and to *Proteinovarax* have already been detected in several soda lakes (Humayoun et al., 2003; Kevbrin et al., 2013). *Anaerobranca* was also among the most abundant Clostridia in an anaerobic reactor used for the production of short chain fatty acids at alkaline conditions (Zhang et al., 2010). *Anaerobranca* requires Na^+ for growth and members of this genus are capable of degrading proteins and carbohydrates to acetate CO_2 and H_2 at pH values between 6.0 and 10.5 (Engle et al., 1995). *Proteinivorax* is also an anaerobic

alkaliphilic bacterium which can grow at pH up to 10.8 and can only use proteinaceous substrates such as albumin, peptone and yeast extract as carbon, nitrogen and energy sources (Kevbrin et al., 2013).

The first representative pf the genus *Tindallia*, was isolated from a Kenyan alkaline lake, Lake Magadi by Kevbrin et al., 1998 and more recently similar clones have been again identified in sediments from the same lake (Baumgarte, 2003). Members of the genus *Tindallia* belong to the group of organisms called acetogenic ammonifers, and are able to ferment amino acids such as arginine and ornithine to acetate, propionate, H_2 and ammonia. They are also unable to utilize carbohydrates or polymeric compounds (Kevbrin et al., 1998; Oren, 2005). *Tindallia* use arginine and ornithine as substrates, and because they have been isolated from soda lakes where significant blooms of cyanobacteria occur, it has been suggested that these organisms use cyanophycine, an amino acid polymer composed of aspartic acid and arginine which can be found in most Cyanobacteria, as the substrate (Kevbrin et al., 1998).

As can be seen in Figure 6.2, bin H appears to be composed of sequences originating from two different organisms, one encoding the 16S rDNA sequence of contig02379, most similar to *Anaerobranca*, and the other of contig 01782, most similar to *Tindallia* (contig01782) (Table 6.4). This could be explained by the fact that for both the contigs have similar sequence coverage (25.1X for contig02379 and 19.7X for contig01782), and they both have similar tetranucleotide patterns which causes them to cluster together in the same bin. As a result, it can be speculated that, bin H contains sequence information from two different populations of related Clostridia.

Multiple clostridial clones have been identified in several soda lakes (Baumgarte, 2003; Jones et al., 1998; Kivistö and Karp, 2011; Mesbah et al., 2007a), but the specific function is still unknown. In mesophilic pH anaerobic digesters members of the order Clostridiales are known to play a major role in the decomposition of organic matter and are among the most abundant orders (Jaenicke et al., 2011; Kovács et al., 2013; Li et al., 2013; Schlüter et al., 2008). With the scarce information regarding the physiology of this type of bacteria adapted to alkaline conditions, their function in the anaerobic digestion process at alkaline conditions can only be a speculation.

Bins, C, D and G, were assigned to members of the order Halanaerobiales, families Halobacteroidaceae and Halanaerobiaceae (Table 6.3 and 6.4). Bin C contained one contig encoding a 16S rDNA sequence, contig01919 (1495 bp; 13X coverage; data not shown), which was assigned to the halotolerant family, Halobacteroidaceae. The SILVA classifier could only classify the contig to family level, while the RDP classifier assigned the contig at genus level to *Orenia* (76% identity) (Table 6.4). Phylogenetically, the 16S sequence was most similar to that of an uncultured organism, clone MAT-CR-H3-B08 (assigned to genus *Orenia* in the RDP database), isolated from a microbial mat originating in a saltern pond (Puerto Rico) (Isenbarger et al., 2008), and also closely related to an *Orenia* 16S sequence obtained from a hypersaline oil reservoir (Joulian 2012; Direct submission) (Figure 6.2). Members of the genus *Orenia* were first isolated from the hypersaline lake Dead Sea and are known to be moderately halophilic, they can ferment glucose to formate, acetate, butyrate, CO_2 and H_2 and they have also been identified in sediments of lake Magadi (Kenya) (Baumgarte, 2003; Kivistö and Karp, 2011; de la Haba et al., 2011). Even though the function of *Orenia* in the anaerobic digestion process is not known, members of the family Halobacteroidaceae are also known to be able to ferment sugars and amino acids (Oren, 2008).

Bin D contains three contigs with 16S rDNA sequences that were assigned to the same family, Halanaerobiaceae, but to different genera, *Halanaerobium*, *Halocella* and *Halothermothrix* (Table 6.4). Among the three contigs, only contig03844 contained a 16S rDNA sequence part longer than 1,000 bp and was included into the alignment for the phylogenetic tree. The 16S sequence part of contig03844 was almost identical (99% blastn identity) to clone CSS150, an uncultured Firmicutes detected in coastal soil of the Gulf of Khambhat (India) (Keshri et al., 2012; Direct submission) and fell into the same clade as *Halanaerobium* and *Halocella* (Figure 6.2). These results and considering that the 16S sequence of this contig was similar to the 16S sequence found on contig03146 from assembly B, which was also classified as *Halanaerobium* by SILVA and RDP (Table 6.4), suggest that bin D might represent a bacterium closely related to *Halanaerobium*.

The Halanaerobiaceae are moderate halophilic bacteria and the family consists of species of the genus *Halanaerobium, Halothermothrix* and *Halocella* (de la Haba et al., 2011). Members of this family are able to ferment glucose, fructose and pectin

among other substrates and they produce butyrate, acetate, propionate, H_2 and CO_2 (Kivistö and Karp, 2011). *Halanaerobium* 16S sequences and similar clones have been identified in alkaline lakes (Brown et al., 2011), in hypersaline ponds (Mouné et al., 2003) and in hypersaline sediments from Great Salt Lake (USA) (Ivanova et al., 2011) among other extreme environments. Interestingly, Kapdan and Erten, 2007 used a type of *Halanaerobium*, *H. lacusrosei*, to anaerobically treat saline wastewater using an up-flow anaerobic packed bed reactor which indicates that members of this group have biotechnological potential for the treatment of saline and alkaline wastewaters.

Bin G was difficult to assign to a particular organism by both 16S classifiers due to two main reasons: (i) bin G recruited a high number of short contigs (Table 6.3) and the identified 16S rDNA sequences were also short, less than 500 bp each (data not shown), and (ii) the number of tRNAs and conserved genes is the highest among all selected bins (Table 6.3) which indicates that the bin contains DNA originating from multiple organisms. Taking into account that most of the contigs that could be classified were assigned to *Halanaerobium* suggest that bin G belongs to the order Halanaerobiales which is in accordance to the type of inoculum utilized.

Two other contigs, an unbinned contig from assembly A, contig01627, and contig02658 from assembly B were assigned to members of the Natranaerobiales order (Table 6.4). Contig01627, assigned to *Natranaerobius* by RDP and to the family Natranaerobiaceae by SILVA was phylogenetically related to *Natronovirga wadinatrunensis* and to *Natranaerobius thermpohilus* both halophilic bacteria isolate from soda lakes (Mesbah and Wiegel, 2009; Zhao et al., 2011) (Figure 6.2). *N. thermophilus* is a polyextremophilic bacteria first isolated from Fazda soda lake (Wadi An Natrun, Egypt) by Mesbah et al., 2007b which is capable of growing under high salt concentrations (3.1 - 4.9 M Na^+), high temperature (35 - 56°C) and high pH (8.5 - 10.6) and can use peptone as carbon and energy source. *N. wadinatrunensis* also isolated from a Wadi An Natrun soda lake, requires the presence of yeast extract and tryptone to utilize glucose, fructose and other sugars as carbon and energy sources and it is unable to degrade cellobiose and other cellulose derived substrates (Mesbah and Wiegel, 2009). These nutritional characteristics plus the fact that members of the order Natranaerobiales have also been detected in a lab-scale

anaerobic reactor fed with casein (Kovács et al., 2013) might indicate that these bacteria play a role in the digestion of protein rich substrates.

(iii) Rhodobacterales and Rhizobiales (Alphaproteobacteria)

Alphaproteobacteria were also detected in the alkaline anaerobic reactor but their abundance was low, 1.3% (Table 6.3). Bin I was assigned by both classifiers to the purple non-sulfur bacteria *Rhodobaca* of the family Rhodobacteraceae (Table 6.4). Of the known 16S sequences, the one most similar to that of contig04714 was from an uncultured bacterium, clone TX4CB_152, isolated from sediments of former alkaline lake Texcoco (Mexico) (Valenzuela-Encinas et al., 2009). Other Rhodobacterales isolated from alkaline environments also had 16S sequences similar to that of contig04714 (Figure 6.3). The closest cultured organism was *Rhodobacter sp.* isolated from alkaline olive oil waste (Ntougias et al., 2006). *Rhodobacter* related bacteria are widespread in aquatic environments with abundant organic matter. They grow photoautotrophically under anaerobic conditions in the presence of sulfide or chemoorganoheterotrophically in aerobic conditions (Venkata Ramana et al., 2008; Wani et al., 2006). Members of the family Rhodobacteraceae have been identified and isolated from several soda lakes (Kompantseva et al., 2007; Kompantseva et al., 2010; Lanzén et al., 2013), from mesophilic pH biogas reactors (Krause et al., 2008; Nelson, 2011) and also from an anaerobic reactor treating dye wastewaters (Zhang et al., 2012b).

In assembly B, a contig containing a 16S sequence related to an alphaproteobacterium of the order Rhizobiales was also identified (Table 6.4). Contig05874 was classified as a *Salinarimonas* by SILVA and as a *Chelatococcus* by RDP. This 16S sequence showed 99% blastn similarity to cloneSA_118, identified in Lonar soda lake (India) (Figure 6.3). Rhizobiales and related members are known polyextremophilic and have been identified in other alkaline environments (Baumgarte, 2003; Valenzuela-Encinas et al., 2009) and in salt mines (Liu et al., 2010), as well as in mesophilic pH anaerobic reactors (Héry et al., 2010; Krause et al., 2008; Zhang et al., 2012b). *Chelatococcus* and *Salinarimonas*, the closest known organisms, are both halotolerant and can grow at high pH and they both require Tryptone Soya medium for growth which indicates that they need a source of amino acids to grow (Liu et al., 2010; Yoon et al., 2008).

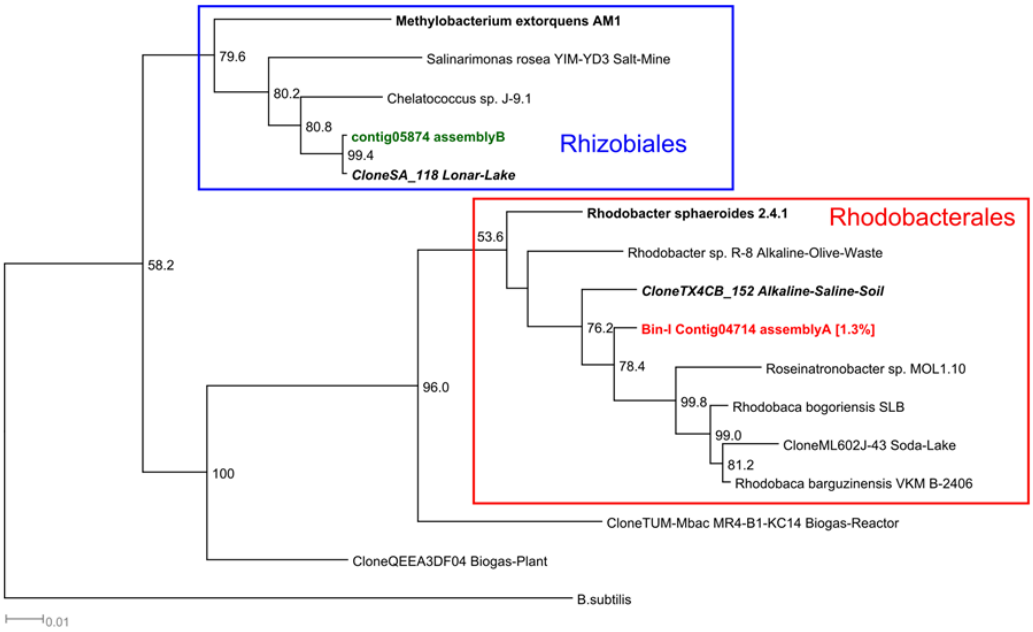

Figure 6.3 16S rDNA Alphaproteobacteria phylogenetic tree
16S rDNA phylogenetic tree of the contigs assigned to members of the Alphaproteobacteria class by the RDP and SILVA classifiers. Although the binning was performed with contigs of assembly A, the tree also includes those contigs that were obtained from assembly B and were not assembled in assembly A. Minimum contig length of 1000bp. Colored: sequences obtained from metagenomic reads. Assignment to Metawatt bins and percentage of bin abundance is indicated if applicable. Reference sequences in **bold**: top hits in blast search against NCBI reference RNA sequences database; ***bold+italics****:* top hits in blast search against NCBI non-redundant nucleotide collection. Additional reference sequences tree represent genera detected in other alkaline environments or anaerobic digesters. 16S rDNA sequence of *B. subtilis* was chosen as outgroup. Bootstrap values at nodes are obtained from 500 replicates and are only shown for branches with at least 50% support (values > 49.9). The scale bar represents 0.01 nucleotide substitutions per site. Accession numbers of reference sequences are available in Suppl. Table 6.1

Both, Rhizobiales and Rhodobacterales have been detected in anaerobic reactors and in alkaline environments but their ecological role has not yet been fully understood. As both, *Salinarimonas* and *Rhodobacter* can grow at aerobic and anaerobic conditions, it is possible that their relatively high presence is not only related to their capacity to grow in haloalkaline environments but to possible presence of oxygen in the anaerobic reactor due to the relatively low biogas production rate.

3.2.1.2 Taxonomy of the methanogenic archaea

The final step in the anaerobic digestion process is the production of methane and is carried out by methanogenic archaea. Methanogenesis in alkaline environments has been widely studied for the last decades (Grant, 2006; McGenity, 2010; Oremland et

al., 1982; Sorokin and Kuenen, 2005a; Zhilina and Zavarzin, 1994) but so far only one study by van Leerdam et al., 2008 described the methanogenic population in lab-scale anaerobic alkaline reactors. In that study, *Methanolobus oregonensis*, a methylotrophic member of the Methanosarcinales (Liu et al., 1999), was the dominant methanogen. In a previous work, several (halo)alkaliphilic methanogenic Archaea related to Methanobacteriales and Methanomicrobiales were identified from soda lake sediments based on the *mcrA* marker genes (Nolla-Ardèvol et al., 2012).

Apparently in the alkaline anaerobic reactor, a single population of methanogens, *Methanocalculus*, a Methanomicrobiales, dominated among the archaeal community (Table 6.3). Bin F contained one 16S rDNA sequence, contig01776, which was classified by both classifiers as *Methanocalculus* (Table 6.4). Phylogenetically, this sequence was closely related to *Methanocalculus sp. AMF-Bu2,* identified in sediments from soda lakes of the Kulunda Steppe (Altai, Russia), the same lake system from which the inoculum for the alkaline reactor was obtained (Sorokin 2012; direct submission), and to *Methanocalculus natronophilus*, isolated from sediments of soda lakes of the Tanatar II system, also in the Kulunda region (Zhilina et al., 2013) (Figure 6.4). As expected, the identified methanogen fell in the same clade as other halotolerant Methanomicrobiales and it is clearly distant from Methanomicrobiales isolated and identified in mesophilic pH anaerobic reactors (Figure 6.4). Members of the genus *Methanocalculus* have been identified in other alkaline environments such as Lonar crater lake in India (Antony et al., 2012; Surakasi et al., 2007; Wani et al., 2006), Ethiopian soda lakes (Lanzén et al., 2013), and an hypersaline oil reservoir (Ollivier et al., 1997) among others. Members of the genus *Methanocalculus* are hydrogenotrophic, and one of its members, *M. halotolerans* is the most halotolerant hydrogenotrophic methanogen identified so far. It can grow in the presence of up to 12.5% NaCl (Ollivier et al., 1997). All known members of this genus, including *M. halotolerans* have restricted pH growth ranges from 6.7 to 8.3 (Lai et al., 2002; Ollivier et al., 1997), with one exception, *Methanocalculus natronophilus. M. natronophilus* is so far the only known member of this genus to be strictly alkaliphilic, it can only grow at pH between 8.0 to 10.2, with an optimum between pH 9.0 and 9.5, it also requires between 0.5 and 1.6 M of carbonates and from 0.9 to 3.3 M of Na^+. *M. natronophilus* is an hydrogenotrophic methanogen and cannot use acetate as substrate for methanogenesis albeit it requires it for growth as carbon source (Zhilina et al., 2013).

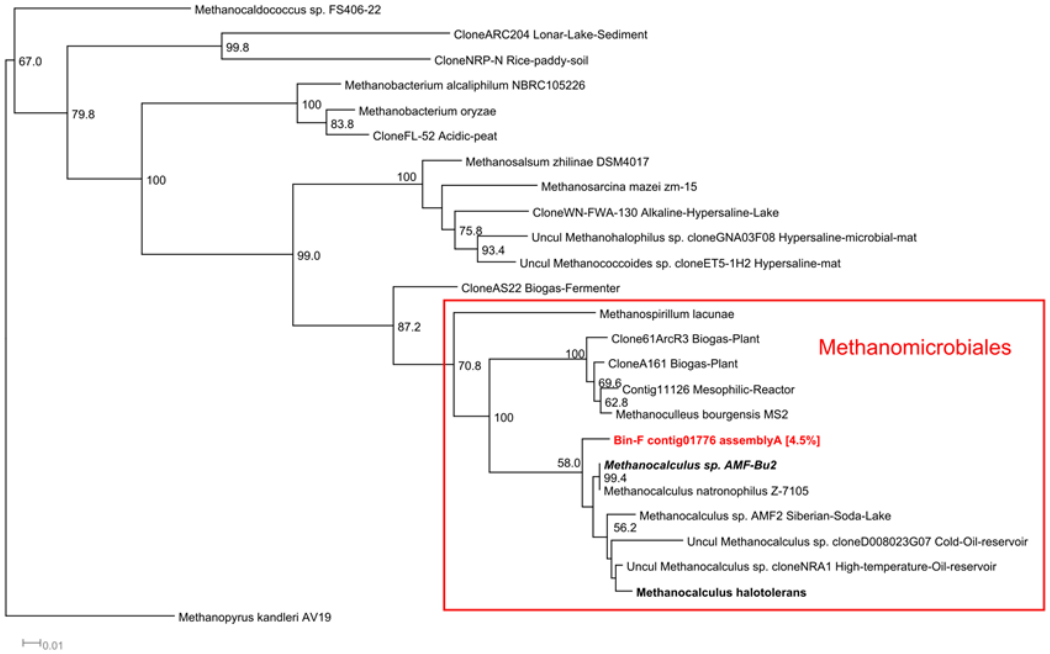

Figure 6.4 16S rDNA Methanogens phylogenetic tree
16S rDNA phylogenetic tree of the contigs assigned to methanogenic archaea by the RDP and SILVA classifiers. Minimum contig length of 1000bp. Colored: sequences obtained from metagenomic reads. Assignment to Metawatt bins and percentage of bin abundance is indicated if applicable. Reference sequences in **bold**: top hits in blast search against NCBI reference RNA sequences database; ***bold+italics:*** top hits in blast search against NCBI non-redundant nucleotide collection. Additional reference sequences tree represent genera detected in other alkaline environments or anaerobic digesters. 16S rDNA sequence of *M. kandleri* was chosen as outgroup. Bootstrap values at nodes are obtained from 500 replicates and are only shown for branches with at least 50% support (values > 49.9). The scale bar represents 0.01 nucleotide substitutions per site. Accession numbers of reference sequences are available in Suppl. Table 6.1.

At mesophilic pH conditions the methanogenesis step is generally performed mainly by Methanomicrobiales such as *Methanoculleus* (Ács et al., 2013; Schlüter et al., 2008; Sundberg et al., 2013; Wirth et al., 2012), and members of the order Methanosarcinales such as *Methanosarcina* (Li et al., 2013) and *Methanosaeta* (Rivière et al., 2009; Sundberg et al., 2013).

However, in the alkaline system, a *Methanocalculus* related methanogen appeared to be the dominant archaeal organism (Table 6.3). This could be explained by (i) the type of inoculum used, which was obtained from soda lakes where *Methanocalculus* related methanogens were present (Nolla-Ardèvol et al., 2012) and (ii) by the operational conditions from the reactor, pH~10 and 2.0 M Na^+, which fit the growth requirements of *M. natronophilus*, and thus likely also contributed to the prevalence

of the *M. natronophilus* related methanogen over other alkaliphilic and halotolerant methanogens

3.2.1.3 Microbial structure of the anaerobic digestion process at alkaline conditions

To date, anaerobic digestion has been performed generally at mesophilic pH conditions and the structure and composition of the microbial community has been relatively constant among the different reactors and substrates studied. Here, the breakdown of organic matter from complex substrates to acetate, volatile fatty acids, CO_2 and H_2 has been carried out mainly by bacteria of the order Clostridiales (Firmicutes), with abundances from 30 to 80% of the total bacterial population. Second in abundance have generally been Bacteroidetes and Bacilli (abundances around 10 - 15%) followed by multiple other classes such as alpha and gamma Proteobacteria, Actinobacteria, and Sphingobacteriia, Thermotogae among many others (Li et al., 2013; Rivière et al., 2009; Schlüter et al., 2008; Sundberg et al., 2013; Wirth et al., 2012; Ziganshin et al., 2013). At alkaline conditions, however, significant differences were observed, not only with regard to the type of bacteria present, mostly halotolerant, but also to their relative abundance (Table 6.3 and 6.4). The most remarkable difference was the high abundance of Bacteroidetes. At the alkaline conditions used in this study, the main breakdown of the organic matter seems to be performed not by members of the order Clostridiales, but by Bacteroidales related bacteria which dominate over the other orders with 27% of the total abundance (bins A and E) (Table 6.3 and 6.4). Clostridiales were still present but here only account for around 13% of the abundance (bins B and H) and were replaced by other Firmicutes such as members of the order Halanaerobiales ~15% (bins C, D and G). Alphaproteobacteria, mainly represented by Rhodobacterales, might have played a role in the degradation of organic matter at alkaline conditions (1.3% abundance) in contrast to mesophilic conditions where they are scarcely present. However, it has to be taken into account that the identified Alphaproteobacteria are both anaerobic facultative therefore, their presence could be related to possible oxygen leaks into the anaerobic reactor. With respect to the last step, the formation of methane, clear differences were observed regarding the involved taxa when compared to mesophilic pH reactors. At alkaline conditions *Methanocalculus*, a genus of methanogens which includes already isolated

alkaliphilic members, clearly dominates over the rest of methanogenic archaea (Table 6.3).

The results presented here suggest that at these alkaline conditions, pH 10 and 2.0 M Na^+, the microbial community responsible for the anaerobic digestion of organic matter differs, not only regarding the fact that the identified bacteria and archaea are mostly halotolerant and alkaliphilic but, also with regard to the relative abundance of the major players, Bacteroidetes, Clostridiales and Methanomicrobiales, when compared to mesophilic pH anaerobic microbial communities.

3.2.2. Direct analysis of sequencing reads

Two different approaches were used to study the function of the alkaline microbial community. In the first method, in order to get an insight into the different biological processes that took place in the alkaline anaerobic reactor, quality trimmed reads, not including *Spirulina* reads, were uploaded to the MG-Rast metagenome analyzer and functional proteins where annotated with the Subsystem hierarchical classifier. This is a categorization system which organizes functional categories of genes into a hierarchy with five levels of resolution. Each subsystem is comprised of a set of genes with functional roles that together implement a specific biological process or structural complex (Overbeek et al., 2005). In the second approach, all reads were translated into amino acids and blasted against the Pfam-A database to identify specific protein domains.

Of the 1,194,051 reads that were annotated to a protein by MG-Rast, 980,677 reads (82.1% of the annotated reads), were assigned to a Subsystem category (Figure 6.5). The subsystem that recruited the highest number of reads was “Clustering-based subsystems” with 151,597 hits (15% of the assigned reads). “Carbohydrates”, “Protein Metabolism” both with 9.9% and “Amino acid and derivatives” with 9.2% were the following most abundant subsystems (Figure 6.5). The “Clustering-based subsystems” includes genes related to bacterial cell division, DNA replication, RNA metabolism, carbohydrate related genes, and stress related proteins among many others. The “Carbohydrates” subsystem includes groups of proteins related to monosaccharide and polysaccharide metabolism, fermentation, C1 compounds metabolism and to the general central carbon metabolism, while the “Amino acid and derivatives” subsystem includes mainly proteins involved in amino acid metabolism.

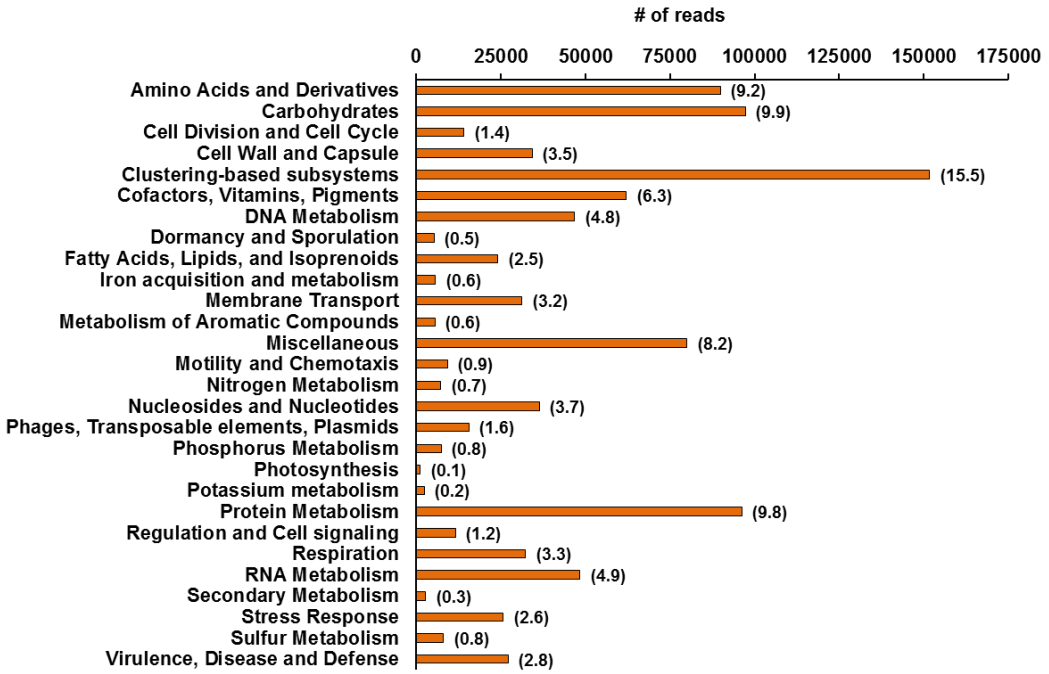

Figure 6.5 Subsystem hierarchical classification of functional reads
Number of reads assigned to each individual Subsystem category by the MG-Rast metagenome analyzer. Value in brakes corresponds to the percentage of total assigned reads to the Subsystems categories. Assignment was done with an e-value of $10e^{-5}$, 50% minimum identity and 15 amino acid minimum length.

Categories related to the general function of an active community also recruited a high number of reads. For example, subsystems “DNA metabolism” and “RNA metabolism” both recruited over 45,000 hits (Figure 6.5). Within the first one, genes related to DNA replication, recombination and repair were detected while in the latter, proteins related to RNA processing, modification and transcription could be identified. Other general metabolic functions were also detected in the annotated reads. Subsystem “Respiration”, which includes genes encoding basic functions such as ATP synthases, hydrogenases, carbon monoxide dehydrogenases and enzymes related to anaerobic respiration such as anaerobic dehydrogenases recruited over 30,000 hits. As expected, most of the identified functions were related to the basic metabolism of organic macromolecules such as synthesis of proteins and amino acids as well as general housekeeping mechanisms all of which are required for an appropriate performance of an active microbial community.

So far, most of the metagenomic functional analyses of biogas reactors focus their search on enzymes related to the degradation of complex sugars derived from the degradation of cellulosic material (Li et al., 2013; Schlüter et al., 2008; Wong et al.,

2013). In the present work, however, as the substrate used for the alkaline reactor is a protein rich substrate which does not contain cellulose related compounds, enzymes related to the degradation of proteins and peptides were analyzed. Subsystems “Amino acids and derivatives” and “Protein metabolism” contain enzymes which participate in the metabolism of peptides, while the “Carbohydrate” subsystem contains the cellulose degradation enzymes such as xylanases and cellulosome related proteins. In the alkaline metagenome 74,000 reads of the subsystems “Amino acid and derivatives” and “Protein metabolism” were assigned to enzymes that could be related to protein and amino acid degradation while only 18,000 reads of subsystem “Carbohydrate” were assigned to enzymes directly related to the degradation of cellulose (Table 6.5). A similar result was obtained when the number of detected protein specific domains (Pfams) related to the degradation of proteins, 29,000, was compared to the number of detected Pfams related to the degradation of cellulose, 5,000 (Table 6.6).

As proteases, peptidases and other enzymes involved in the degradation of protein and amino acids are ubiquitous and present in all bacteria it is difficult to directly couple the higher abundance of these enzymes to the type of substrate used. Nevertheless and as already seen in [3], the higher relative abundance of these enzymes, combined with the low abundance of cellulolytic enzymes might indicate that the microbial community does adapt to the type of substrate used by increasing the abundance of enzymes able to degrade the supplied substrate. The results obtained here are the outcome of the first observations of the functional potential of this alkaline community and more work is necessary to determine if the microbial community really adapts to the type of substrate used.

Multiple enzymes involved in the adaptation strategies of the halotolerant and haloalkaline bacteria present at these extreme conditions could also be identified among several subsystems. The general strategy of halotolerant and haloalkaline microorganisms to adapt to such a high salt concentration is to maintain their intracellular pH and salt concentration at “mesophilic” levels (Ventosa, 2006). In this sense, most alkaliphilic and halophilic bacteria require Na^+, K^+, Cl^- and other ions for growth and to compensate the intracellular osmolarity (Kivistö & Karp, 2011; Mesbah, Hedrick, Peacock, Rohde, & Wiegel, 2007; Ulukanli & Rak, 2002). Several genes encoding proteins related to Na^+ and K^+ transporters and related proteins could also

Table 6.5 Specific Subsystem features

Specific Subsystem features from categories “Amino acid and Derivatives”, “Carbohydrates” and “Protein Metabolism” related to amino acid, protein and carbohydrate degradation obtained with the MG-Rast Subsystem hierarchical classification. Assignment was done with an e-value of $10e^{-5}$, 50% minimum identity and 15 amino acid minimum length.

Amino acid	Hits
Anaerobic oxidative degradation of L-Ornithine	2160
Arginine and Ornithine degradation	5961
Aromatic amino acid degradation	1275
Branched chain amino acid degradation	4574
Creatine and Creatinine degradation	454
Glutamate and Aspartate uptake	379
Glycine and Serine utilization	8164
Glycine cleavage system	3460
Histidine degradation	3222
Isoleucine degradation	5401
Leucine degradation	4582
Lysine degradation	2202
Methionine degradation	6664
Proline, 4-hydroxyproline uptake	1372
Threonine degradation	2183
Valine degradation	4869
Total	**56922**

Carbohydrate	Hits
Beta-Glucoside Metabolism	594
Cellulosome	1967
D-allose utilization	8
D-galactarate, D-glucarate catabolism	226
D-galactonate catabolism	24
L-Arabinose utilization	339
L-fucose utilization	260
L-rhamnose utilization	575
Maltose and Maltodextrin utilization	7899
Mannose metabolism	1704
Predicted carbohydrate hydrolases	538
Sucrose utilization	102
Trehalose uptake and utilization	1217
Unknown carbohydrate utilization	872
Xyloglucan utilization	51
Xylose utilization	811
Fructooligosaccharides(FOS) utilization	919
Melibiose utilization	272
Total	**18378**

Proteins	
Aminopeptidases (EC 3.4.11.-)	1049
Dipeptidases (EC 3.4.13.-)	647
Metallocarboxypeptidases (EC 3.4.17.-)	815
Metalloendopeptidases (EC 3.4.24.-)	39
Proteasome bacterial	3914
Protein degradation	2474
Proteolysis in bacteria, ATP-dependent	7860
Serine endopeptidase (EC 3.4.21.-)	206
Total	**17004**

be detected. For example a “Na^+/H^+ antiporter” gene of the “Miscellaneous” subsystem recruited 1,400 reads while an osmosensitve K^+ channel gene was detected among the “Potassium metabolism” subsystem sequences. Genes encoding several sodium symporters such as sodium/glycine symporter, proline/sodium symporter and sodium/choline symporter, which participate in the re-entry of Na^+ into the cell’s cytoplasm, were also identified. NAD-dependent isocitrate dehydrogenase is an halophilic enzyme, that requires salt for its correct activity and stability, which is present in several extremely halophilic bacteria such as *Salinibacter* (Ventosa, 2006). Several NAD-Isocitrate dehydrogenase genes were also detected among the functionally annotated reads of the “Carbohydrate” subsystem. The

Table 6.6 Identified Protein Domains (Pfams)
Pfams related to amino acid, protein and cellulose degradation identified in the alkaline dataset. Pfams were identified with the hmmscan tool with a 1.0 E-value cutoff.

Initial # of reads	2,032,005	
Initial # of ORFs	3,622,083	
Total identified Pfams	2,503,581	
	# Pfams	**% identified Pfams**
Amino acid degradation	8,537	0.34
Proteases	20,922	0.84
Cellulase	5,118	0.20
Methanogenesis	2,246	0.09

accumulation of organic compounds and compatible solutes which function as osmoprotectants is another strategy applied by halotolerant bacteria to cope with high salt concentrations (Ma, Galinski, Grant, Oren, & Ventosa, 2010; Mesbah & Wiegel, 2012; Ventosa, 2006). Accordingly, genes encoding several enzymes related to the use of ectoins, betaines and choline were detected which recruited over 3,800 reads. In the “Osmotic stress” cluster within the “Stress response” subsystem an L-ectoin synthase and an ectoin hydrolase gene were detected. Further detected genes encoded enzymes related to betaine and choline uptake and biosynthesis such as multiple glycine betaine ABC transportes, several osmotically activated L-carnitine/choline ABC transporters, an ATP-binding protein, a choline ABC transport system and one high-affinity choline uptake protein BetT.

The methanogenesis related gens, which recruited over 4,000 reads, are clustered in the “One carbon metabolism” (“Carbohydrates” subsystem). Among these, the gene with the highest number of hits, 570, was the CoB-CoM heterodisulfide reductase gene, encoding an enzyme that regenerates co-enzyme M and co-enzyme B, the first a product of methyl-coenzyme M reduction (Hedderich, Hamann, & Bennati, 2005). Methyl-coenzyme M reductase, the enzyme that catalyzes the final step of methanogenesis (Ermler, 1997) was assigned to almost 200 reads. Methanogens can produce methane via two main routes, the hydrogenotrophic pathway by reducing CO_2 with H_2 to form CH_4, or they can split acetate into CH_4 and CO_2, using the acetotrophic pathway (Madigan, Martinko, Bender, Buckley, & Stahl, 2012). Of the two pathways, 896 reads were assigned to the specific enzymes of the hydrogenotrophic pathway, while only 86 reads were assigned to specific enzymes of the acetotrophic pathway. Formylmethanofuran dehydrogenase recruited 433 of the hydrogenotrophic pathway reads. This enzyme catalyzes the first step in the

reduction of CO_2 to CH_4, the reduction of CO_2 to formyl-methanofuran (Madigan et al., 2012). The search of Pfams which group enzymes from the methanogenesis pathways showed similar results and Pfams from enzymes which participate in the hydrogenotrophic pathway were more abundant than Pfams from enzymes of the acetoclastic pathway. The most abundant Pfam identified among the methanogenesis related Pfams was tetrahydromethanopterin S-methyltransferase Subunit-G" (MtrG) (PF04210), an enzyme involved in the production of methane from CO_2 (Sauer, 1986). Moreover, five other subunits from the same enzyme, MtrA, MtrB, MtrC, MtrF and MtrH were also identified. In addition, other enzymes from the methanogenesis pathways were also detected. For example, three of the detected Pfams, PF02240, PF02241 and PF02249 represent the three subunits, alpha, beta and gamma, of the Methyl coenzyme M reductase, while PF04029 represents a 2-phosphosulpholactate phosphatase which participates in the biosynthesis of Coenzyme M (CoM), an essential cofactor for the production of methane (Graham, Graupner, Xu, & White, 2001)**.**

These results suggest that the hydrogenotrophic pathway might be the pathway mainly used by the methanogens present in the reactor, which is in accordance with the fact that *Methanocalculus*, a hydrogenotrophic methanogen, was the most abundant methanogen detected in the alkaline metagenome (Table 6.3). They also indicate that, as already reported in other studies (Nolla-Ardèvol, Strous, Sorokin, Merkel, & Tegetmeyer, 2012; Sorokin et al., 2014), in alkaline environments the hydrogenotrophic methanogenesis is dominant over the acetotrophic methanogenesis which is practically absent.

5.4. Conclusions

The metagenome analysis of the haloalkaline anaerobic microbial community responsible for the anaerobic digestion of biomass at alkaline conditions reveals differences when compared to the microbial community present in the mesophilic pH anaerobic reactor. Two main differences were observed, the type of bacteria which, as expected, were mostly halotolerant and alkaliphilic and the relative abundance of each microbial group. Among the bacteria, in contrast to traditional anaerobic digesters where Clostridia class clearly dominate, at alkaline conditions a co-dominancy between Clostridia and Cytophaga-Flavobacteria-Bacteroides (CFB) was

observed. At lower taxonomical levels, this difference was more acute and CFB bacteria such as the uncultured “ML635J-40 aquatic group” clearly dominated over Clostridiales, Halanaerobiales and Rhodobacterales. Differences between the alkaline and the mesophilic archaeal community could also be seen. At alkaline conditions, the alkaliphilic methanogen *Methanocalculus* was the clear dominant. This is a hydrogenotrophic methanogen which might indicate that at these extreme conditions methane is mainly produced from CO_2 and H_2.

Functional analysis of the sequenced reads allowed the identification of several gene clusters related to the strategies utilized by halotolerant bacteria to cope with the extreme environmental conditions. Genes and specific protein domains involved in the degradation of proteinaceous compounds were identified in higher number compared to genes and proteins related to the degradation of cellulosic material which might indicate that the microbial community adapts to the type of substrate present in the anaerobic reactor.

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9. Appendix: Supplementary figures and tables

Publication [3]: Metagenome analysis and biogas production from the anaerobic digestion of the protein rich microalga *Spirulina*

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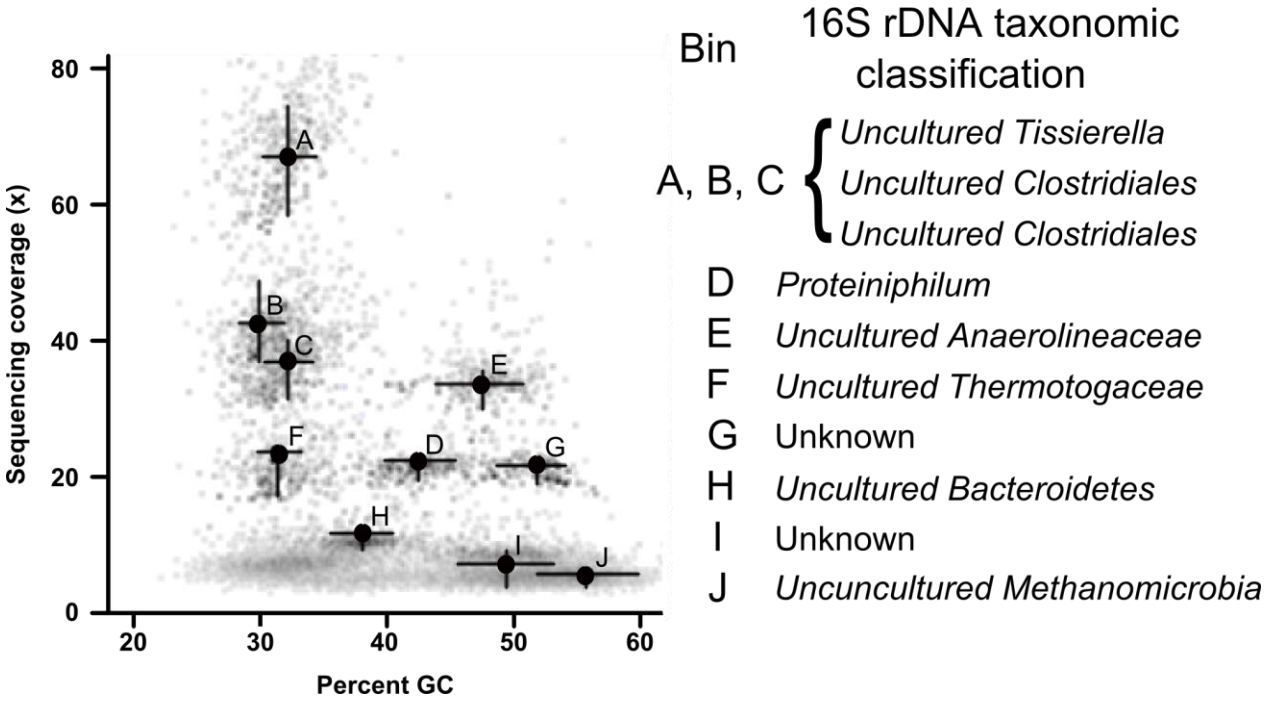
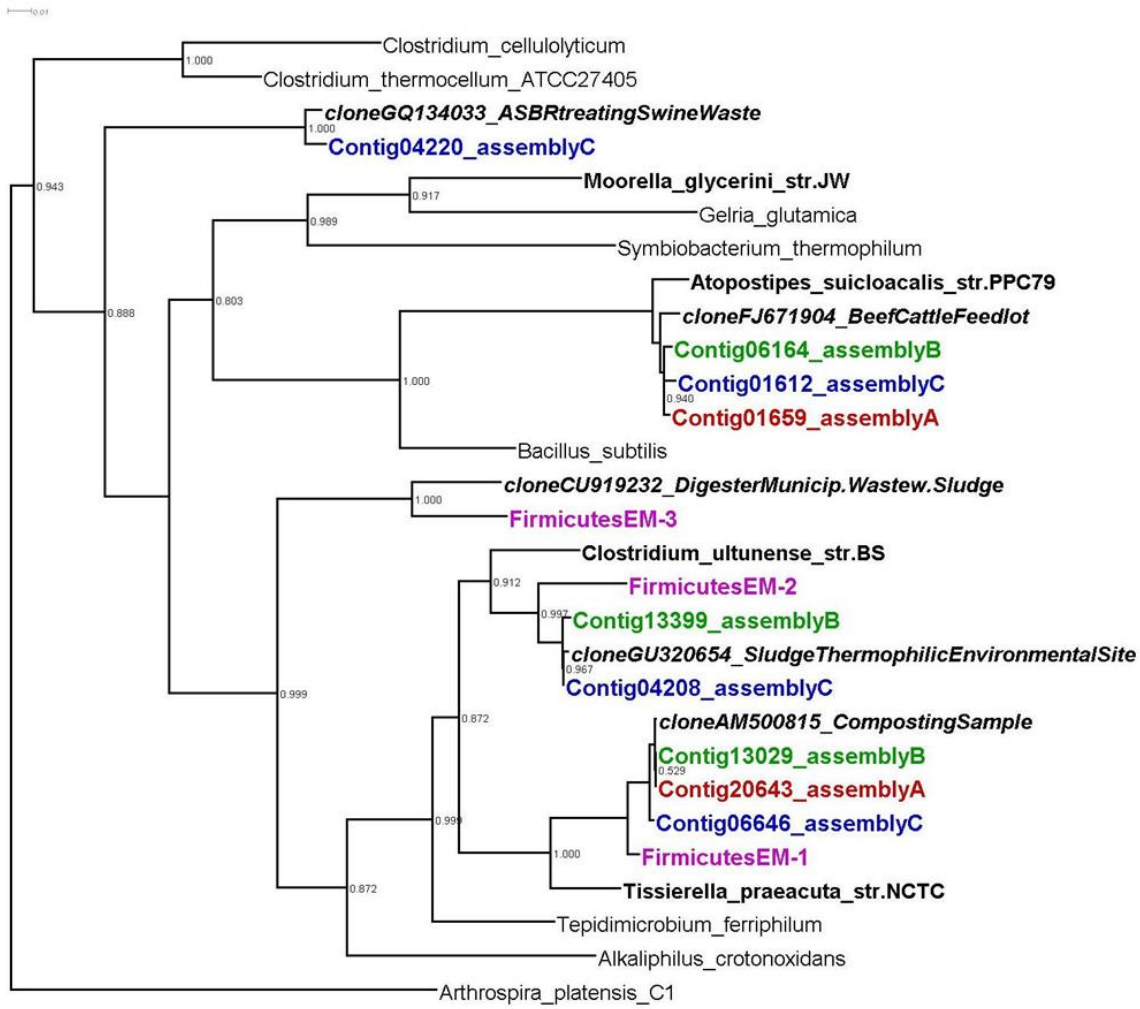
Additional File-1 Supplementary Figures



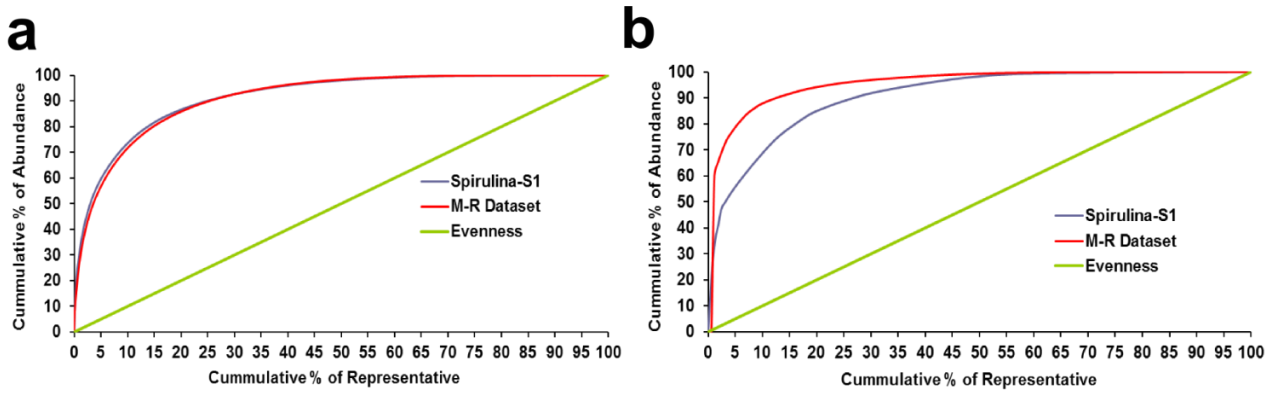
Suppl. Figure 1 Contig coverage, GC content and taxonomic classification of selected bins

Dot plot graph generated with the Metawatt v1.7 pipeline representing all the assembled contigs (grey) and the 10 selected bins (represented by black dots in the center of the bins, including bars showing contig distribution in each bin.)



Suppl. Figure 2 16S rDNA Firmicutes phylogenetic tree

Includes the sequences of the Firmicutes branch in Figure 2a and additionally the unbinned contigs listed in Suppl. Table 2 that are not included in Figure 2a. Values at nodes are from the FastTree default Shimodaira-Hasegawa test.



Suppl. Figure 3 Microbial population evenness

Lorenz curves for the estimation of population evenness at species level from the *Spirulina*-S1 and the Maize-Rye datasets. **a)** Bacterial evenness curves. **b)** Archaeal evenness curves.

Additional File-2 Supplementary tables

Suppl. Table 1 RDP and SINA classification results for assembled and EMIRGE detected 16S rDNA sequences

Classification of 16S rDNA sequences with RDP and SINA sequence classifiers. Sequences are assigned with a minimum of 80% confidence for the RDP, for SINA with a minimum of 80% identity to reference sequences and 10 LCA neighbors.

Sequence name	16S rDNA fragment length [bp]	Detected by/in	RDP classification (lowest classified rank)	SINA classification (lowest classified rank)
FirmicutesEM-1	1425	EMIRGE	Clostridiales: 1 / Clostridiales Incertae Sedis XI: 0.99 / *Tissierella* (genus): 0.99	Clostridiales; Family XI Incertae Sedis (family)
FirmicutesEM-2	1450	EMIRGE	Clostridiales: 1 / Incertae Sedis XI: 0.87 / Clostridium XII (genus): 0.87	Clostridiales; Family XI Incertae Sedis; *Tepidimicrobium* (genus)
FirmicutesEM-3	1357	EMIRGE	Clostridia: 1 / Clostridiales: 0.98 /Clostridiales_Incertae Sedis XI: 0.95 / *Tepidimicrobium* (genus): 0.85	Clostridia (class)
Contig01659	1559	Assembly A	*Atopostipes* (genus): 1	*Atopostipes* (genus)
Contig04220	1061	Assembly C	Bacteria (Domain): 1	Firmicutes; OPB54 (class)
Contig03630	1528	Assembly A	Thermotogaceae: 1 / *Petrotoga* (Genus): 1	Thermotogaceae (family)
Contig05284	1442	Assembly B	Anaerolineaceae (family): 1	Anaerolineaceae (family); uncultured
Contig00447	1512	Assembly C	Porphyromonadaceae: 1 / *Proteiniphilum* (genus): 0.99	Porphyromonadaceae (family)
Contig03141	1272	Assembly A	Bacteroidetes: 1 / Flavobacteria: 0.82 / Flavobacteriales: 0.82 / Cryomorphaceae (family): 0,81	Bacteroidetes; Sphingobacteriia; *Sphingobacteriales* (order)
Contig06470	925	Assembly A	Methanomicrobia: 1/ Methanosarcinales: 1 / *Methanosarcina* (genus): 1	Methanomicrobia (class)
Contig11126	551	Assembly B	*Methanoculleus* (genus): 1	*Methanoculleus* (genus)

Suppl. Table 2 Assembly depth values and classification results of selected contigs encoding 16S rDNA sequences

Contig classification according to RDP and SINA classifiers. Similarity to EMIRGE detected 16S rDNA sequences is based on alignment (see phylogenetic tree topology in Additional File-1 Suppl. Figure 2).

Bin	16S contig	Assembly	Cov*	RDP	SINA	EMIRGE	Bin Cov (X)
Unbinned	01659	A	11.4	*Atopostipes*	*Atopostipes*	EM-1	n.a
	06164	B	8.4	*Atopostipes*	*Atopostipes*		
	01612	C	7.1	*Atopostipes*	*Atopostipes*		
	20643	A	158.9	Clostridiales, Inc. Sed. XI	Clostridiales, Inc. Sed. XI		
	13029	B	76.5	*Tissierella*	Clostridiales, Inc. Sed. XI		
	06646	C	102.3	*Tissierella*	Clostridiales, Inc. Sed. XI		
	13399	B	141.4	Clostridiales, Inc. Sed. XI	*Tepidimicrobium*	EM-2	
	04208	C	164.2	Clostridiales, Inc. Sed. XI	*Tepidimicrobium*		
	04220	C	33.4	Clostridia	Firmicutes; OPB54		
D	03740	A	38.2	*Proteiniphilum*	*Proteiniphilum*		22.3
	03861	B	25.8	*Proteiniphilum*	*Proteiniphilum*		
	00447	C	18.5	*Proteiniphilum*	Porphyromonadaceae		
E	05284	B	24.6	Anaerolineaceae	Unc. Anaerolineaceae		33.3
	02468	B	25	Anaerolineaceae	Unc. Anaerolineaceae		
F	03630	A	50.3	*Petrotoga*	Thermotogaceae		23.4
	03105	B	34.4	*Petrotoga*	Thermotogaceae		
	03129	C	26.1	*Petrotoga*	Thermotogaceae		
H	03141	A	8.9	Cryomorphaceae	Sphingobacteriales		11.5
	10506	B	6.9	Flavobacteria	Bacteroidetes		
	02933	C	5.5	Cryomorphaceae	Sphingobacteriales		

* Assembly depth value from Newbler output
Unc: Uncultured

Suppl. Table 3 Taxonomic classification of metagenomic reads

Classification of reads from datasets "*Spirulina*-S1" and "MR" with the MG-Rast metagenome analyzer. Listed are the 10 most abundant bacteria and 3 most abundant archaea of both datasets based on the M5NR database with a $1e^{-5}$ E-value cutoff, a 60% minimum identity and 15 bp minimum length reads.

***Spirulina*-S1 dataset**				
Class	**Order**	**Family**	**Genus**	**Hits**
Bacteria				
Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	89870
Clostridia	Clostridiales	Clostridiaceae	*Alkaliphilus*	39255
Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	25932
Clostridia	Clostridiales	Peptostreptococcaceae	*Peptostreptococcaceae*	21781
Bacilli	Bacillales	Bacillaceae	*Bacillus*	20918
Thermotogae	Thermotogales	Thermotogaceae	*Petrotoga*	20262
Clostridia	Clostridiales	Fam XI Insertae Sedis	*Peptoniphilus*	12495
Clostridia	Thermoanaerobacterales	Thermoanaerobacteraceae	*Caldanaerobacter*	11096
Anaerolineae	Anaerolineales	Anaerolineaceae	*Anaerolinea*	8033
Clostridia	Clostridiales	Peptococcaceae	*Desulfitobacterium*	6833
Archaea				
Methanomicrobia	Methanosarcinales	Methanosarcinaceae	*Methanosarcina*	5122
Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	*Methanoculleus*	3997
Methanococci	Methanococcales	Methanococcaceae	*Methanococcus*	431
Maize-Rye dataset				
Bacteria				
Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	114140
Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	47051
Unclassified	Unclassified	Unclassified	*Candidatus Cloacamonas*	36032
Bacilli	Bacillales	Bacillaceae	*Bacillus*	32446
Clostridia	Clostridiales	Clostridiaceae	*Alkaliphilus*	18065
Bacteroidia	Bacteroidales	Porphyromonadaceae	*Parabacteroides*	16365
Clostridia	Clostridiales	Peptococcaceae	*Desulfotomaculum*	16053
Clostridia	Clostridiales	Peptococcaceae	*Desulfitobacterium*	13461
Clostridia	Clostridiales	Peptococcaceae	*Pelotomaculum*	12639
Bacilli	Bacillales	Bacillaceae	*Geobacillus*	11970
Archaea				
Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	*Methanoculleus*	54153
Methanomicrobia	Methanosarcinales	Methanosarcinaceae	*Methanosarcia*	4768
Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	*Methanoplanus*	4062

Suppl. Table 4 Identified Pfams related to cellulose degradation

Number of cellulose degradation Pfams identified with the hmmscan tool with a 1.0 E-value cutoff in each dataset.

Pfam	ID	Function	*Spirulina*-S1 dataset	M-R dataset*
PF00150	Cellulase	Hydrolyses glycosidic bond	61	304
PF00331	Glyco_hydro_10	Hydrolyses glycosidic bond	34	429
PF00404	Dockerin_1	Cellulosome structure	188	316
PF00457	Glyco_hydro_11	Hydrolyses glycosidic bond	2	36
PF00553	CBM_2	Carbohydrate binding module	15	80
PF00703	Glyco_hydro_2	Carbohydrate degradation	56	254
PF00704	Glyco_hydro_18	Hydrolyses glycosidic bond	150	309
PF00722	Glyco_hydro_16	Carbohydrate degradation	41	100
PF00734	CBM_1	Carbohydrate binding domain	78	139
PF00759	Glyco_hydro_9	Hydrolyses glycosidic bond	3	81
PF00840	Glyco_hydro_7	Hydrolyses glycosidic bond	3	11
PF00933	Glyco_hydro_3	Carbohydrate degradation	358	908
PF00942	CBM_3	Carbohydrate binding module	17	27
PF01183	Glyco_hydro_25	Carbohydrate degradation	31	37
PF01270	Glyco_hydro_8	Hydrolyses glycosidic bond	11	56
PF01341	Glyco_hydro_6	Hydrolyses glycosidic bond	1	16
PF01464	SLT	Carbohydrate degradation	169	320
PF02011	Glyco_hydro_48	Hydrolyses glycosidic bond	4	18
PF02013	CBM_10	Carbohydrate binding domain	24	34
PF02015	Glyco_hydro_45	Hydrolyses glycosidic bond	3	2
PF02018	CBM_4_9	Carbohydrate binding module	31	446
PF02055	Glyco_hydro_30	Hydrolyses glycosidic bond	14	67
PF02156	Glyco_hydro_26	Hydrolyses glycosidic bond	7	38
PF02836	Glyco_hydro_2_C	Hydrolyses glycosidic bond	202	483
PF02837	Glyco_hydro_2_N	Hydrolyses glycosidic bond	166	529
PF02839	CBM_5_12	Carbohydrate binding module	74	104
PF03422	CBM_6	Carbohydrate binding module	29	249
PF03442	CBM_X2	Carbohydrate binding module	5	79
PF03443	Glyco_hydro_61	Hydrolyses glycosidic bond	2	1
PF04616	Glyco_hydro_43	Hydrolyses glycosidic bond	72	1008
PF12891	Glyco_hydro_44	Hydrolyses glycosidic bond	4	21
PF14587	Glyco_hydr_30_2	Hydrolyses glycosidic bond	10	20
PF14600	CBM_5_12_2	Carbohydrate binding module	16	32

* M-R: Maize-Rye dataset

Suppl. Table 5 Identified Pfams related to protein degradation

Number of protein degradation Pfams identified with the hmmscan tool with a 1.0 E-value cutoff in each dataset.

Pfam	ID	Function	*Spirulina*-S1 dataset	M-R dataset*
PF00082	Peptidase_S8	Peptide degradation	770	900
PF00089	Trypsin	Protein degradation	198	425
PF00246	Peptidase_M14	Peptide degradation	117	207
PF00326	Peptidase_S9	Peptide degradation	496	1019
PF00413	Peptidase_M10	Peptide degradation	36	97
PF00450	Peptidase_S10	Peptide degradation	10	31
PF00557	Peptidase_M24	Peptide degradation	586	1054
PF01400	Astacin	Degradation of polypeptides	9	12
PF01431	Peptidase_M13	Peptide degradation	47	164
PF01434	Peptidase_M41	Peptide degradation	672	847
PF01447	Peptidase_M4	Peptide degradation	3	18
PF01546	Peptidase_M20	Peptide degradation	878	1276
PF01551	Peptidase_M23	Peptide degradation	801	1273
PF02031	Peptidase_M7	Peptide degradation	2	47
PF02868	Peptidase_M4_C	Peptide degradation	10	9
PF03070	TENA_THI-4	Secretion extracellular	10	31
PF03575	Peptidase_S51	Peptide degradation	37	78
PF04389	Peptidase_M28	Peptide degradation	219	531
PF05134	T2SL	Secretion of proteases	20	14
PF05257	CHAP	Peptidoglycan hydrolysis	31	60
PF05342	Peptidase_M26_N	Peptide degradation	12	32
PF05362	Lon_C	Peptide degradation	363	852
PF05543	Peptidase_C47	Peptide degradation	6	3
PF05548	Peptidase_M11	Peptide degradation	4	7
PF05569	Peptidase_M56	Peptide degradation	268	230
PF05576	Peptidase_S37	Peptide degradation	2	13
PF05577	Peptidase_S28	Peptide degradation	33	34
PF05649	Peptidase_M13_N	Peptide degradation	31	156
PF06480	FtsH_ext	Membrane bound protease	88	223
PF07502	MANEC	Protease activator and	11	15
PF07580	Peptidase_M26_C	Peptide degradation	2	4
PF07687	M20_dimer	Peptide degradation	610	584
PF08548	Peptidase_M10_C	Peptide degradation	6	7
PF12388	Peptidase_M57	Peptide degradation	8	28
PF13365	Trypsin_2	Peptide degradation	288	539
PF13485	Peptidase_MA_2	Peptide degradation	245	370
PF13529	Peptidase_C39_2	Peptide degradation	109	129
PF13574	Reprolysin_2	Peptide degradation	40	43
PF13582	Reprolysin_3	Peptide degradation	51	93
PF13583	Reprolysin_4	Peptide degradation	19	43

* M-R: Maize-Rye dataset

Suppl. Table 6 Identified Pfams related to amino acid degradation

Number of amino acid degradation Pfams identified with the hmmscan tool with a 1.0 E-value cutoff in each dataset.

Pfam	ID	Function	*Spirulina*-S1 dataset	M-R dataset*
PF00056	Ldh_1_N	Lactate/malate dehydrogenase	146	390
PF00070	Pyr_redox	Oxidoreductases	1254	2574
PF00155	Aminotran_1_2	Aminotransferase	1372	2613
PF00185	OTCace	Transferring one-carbon groups	282	482
PF00205	TPP_enzyme_M	Thiamine binding	134	460
PF00208	ELFV_dehydrog	Glutamate catabolism	590	882
PF00291	PALP	Amino acid metabolism	934	1024
PF01053	Cys_Met_Meta_PP	Amino acid metabolism	1383	1166
PF01212	Beta_elim_lyase	Degradation of amino acids	767	1391
PF01571	GCV_T	Degradation of amino acids	334	375
PF02254	TrkA_N	Transporter	671	1608
PF02347	GDC-P	Degradation of amino acids	598	713
PF02729	OTCace_N	Transferring one-carbon groups	302	362
PF02812	ELFV_dehydrog_N	Glutamate catabolism	304	289
PF02852	Pyr_redox_dim	Oxidoreductases	377	367
PF02866	Ldh_1_C	Lactate/malate dehydrogenase	37	111
PF03313	SDH_alpha	Degradation of amino acids	462	337
PF03315	SDH_beta	Degradation of amino acids	126	135
PF04898	Glu_syn_central	Amino acid metabolism	9	54
PF04960	Glutaminase	Amino acid metabolism	188	156
PF05995	CDO_I	Amino acid metabolism	16	22
PF07085	DRTGG	Amino acid metabolism	111	196
PF08669	GCV_T_C	Degradation of amino acids	90	183
PF12544	LAM_C	Amino acid metabolism	141	162

* M-R: Maize-Rye dataset

Suppl. Table 7 Accession numbers of reference 16S rDNA sequences in phylogenetic trees

Accession numbers of reference 16S rDNA sequences for which in the phylogenetic trees (Fig 2) only organism names are shown.

Organism name	Accession
Arthrospira platensis C1	gi|423061881:1247729-1249205
Tepidimicrobium ferriphilum strain SB91	gi|343198615|ref|NR_043077.1|
Atopostipes suicloacalis strain PPC79	gi|265678531|ref|NR_028835.1|
Clostridium ultunense strain BS	gi|219846939|ref|NR_026531.1|
Tissierella praeacuta strain NCTC 11158	gi|343206268|ref|NR_044860.1|
Gelria glutamica strain TGO	gi|343201113|ref|NR_041819.1|
Alkaliphilus crotonoxidans	gi|19072573|gb|AF467248.1|
Bacillus subtilis subsp. subtilis str. BSP1	gb|CP003695.1|:compl(3947493-3945959)
Symbiobacterium thermophilum IAM 14863	gi|444439729|ref|NR_075044.1|
Clostridium_cellulolyticum H10 strain H10	gi|507147961|ref|NR_102768.1|
Clostridium thermocellum ATCC 27405	gi|444304204|ref|NR_074629.1|
Moorella glycerini strain JW/AS-Y6	gi|265678890|ref|NR_029198.1|
Petrotoga mobilis SJ95 strain SJ95	gi|444303977|ref|NR_074401.1|
Levilinea saccharolytica strain KIBI-1	gi|343200285|ref|NR_040972.1|
Proteiniphilum acetatigenes strain TB107	gi|343202727|ref|NR_043154.1|
Owenweeksia hongkongensis DSM 17368	gi|470466026|ref|NR_074100.1|
Anaerolinea thermophila UNI-1 strain UNI-1	gi|444303960|ref|NR_074383.1|
Thermotoga_lettingae TMO strain TMO	gi|444439636|ref|NR_074951.1|
Bacteroides paurosaccharolyticus JCM 15092	gi|166063925|dbj|AB298727.2|
Alistipes finegoldii DSM 17242	gi|507148137|ref|NR_102944.1|
Fluviicola taffensis DSM 16823	gi|444304123|ref|NR_074547.1|
Methanosarcina siciliae strain T4/M	gi|559795168|ref|NR_104757.1|
Methanoculleus marisnigri JR1 strain JR1	gi|470467424|ref|NR_074174.1|
Methanospirillum hungatei JF-1 strain JF-1	gi|470467480|ref|NR_074177.1|
Methanobacterium flexile strain GH	gi|304336835|gb|EU333914.2|
Methanosaeta concilii strain Opfikon	gi|254971324|ref|NR_028242.1|
Methanocorpusculum parvum strain DSM 3823	gi|343206139|ref|NR_044728.1|
Methanothermobacter marburgensis strain Marburg	gi|254971323|ref|NR_028241.1|
Methanolobus oregonensis strain WAL1	gi|254971319|ref|NR_028237.1|
Methanococcus maripaludis	gi|1145365|gb|U38484.1|
Methanopyrus kandleri AV19 strain AV19	gi|444304115|ref|NR_074539.1|

Additional File-3 Supplementary Material and Methods

1. Metagenome normalization and comparison

In order to compare our metagenomic data with a publicly available metagenome, (SRR034130.1) [1], normalization had to be applied to our data. Normalization of Dataset-1 was performed as in Jaenicke *et al.,* [1]. During the normalization procedure, reads shorter than 100 bp and longer than 309 bp were removed. From the initial 5,240,830 reads, after normalization, 2,486,976 reads remained. Subsequently from these 2.4 million reads, the same amount of reads as in the Jaenicke *et al.,* dataset, 1,019,333, were randomly selected in triplicates, *Spirulina*-S1, S2 and S3. To make sure that the three randomly generated datasets were not biased, they were imported and analyzed using MGX platform, a metagenomics platform currently being developed at CeBiTec, Bielefeld University. The MGX platform employs the Conveyor workflow engine [2] for executing all analysis tasks.

As can be seen in Figure 1a,b,c and d, no differences between the three randomly generated datasets were detected for any of the analyzed parameters. *Spirulina*-S1 dataset was chosen for the comparison with the public available metagenome. The two compared datasets, *Spirulina*-S1 and M-R were also analyzed with the MGX platform in terms of read length and GC content (Fig. 1e,f).

2. Generation of ORF and identification of specific protein domain (Pfam)

Specific protein domains (Pfams) were search in the randomly generated dataset *Spirulina*-S1 and the biogas plant dataset, Maize-Rye dataset (M-R). To do so, the following procedure was applied. First, all reads were translated to amino acids and searched for ORFs with the "Translate DNA" script (Translatedna v 1.75 www.mbari.org/staff/haddock/scripts/) with the "0" option, "print all possible ORF for each read". Second, to identify Pfams among the reads, the resulting ORFs were blasted against the Pfam-A database [3] with the hmmscan-v3 tool (http://hmmer.org/) with the –E and - -domE values set to 1.0. Third, Pfams identifiers of particular interest were obtained from two sources; (i) directly from the Pfam database (http://pfam.sanger.ac.uk/) with the search terms "extra cellular proteases", "cellulases" and "cellulosome" and (ii) Pfams from genes involved in the Protein, Amino acid and Cellulose degradation pathways from the MetaCyc Database of

metabolic pathways [4]. Subsequently the list of desired Pfams was searched amongst the Pfam domains identified in the hmmscan search.

References

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2. Linke B, Giegerich R, Goesmann A: **Conveyor: a workflow engine for bioinformatic analyses.** *Bioinformatics* 2011, **27**:903–11.

3. Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer ELL, Eddy SR, Bateman A, Finn RD: **The Pfam protein families database.** *Nucleic Acids Res* 2012, **40**(Database issue):D290–301.

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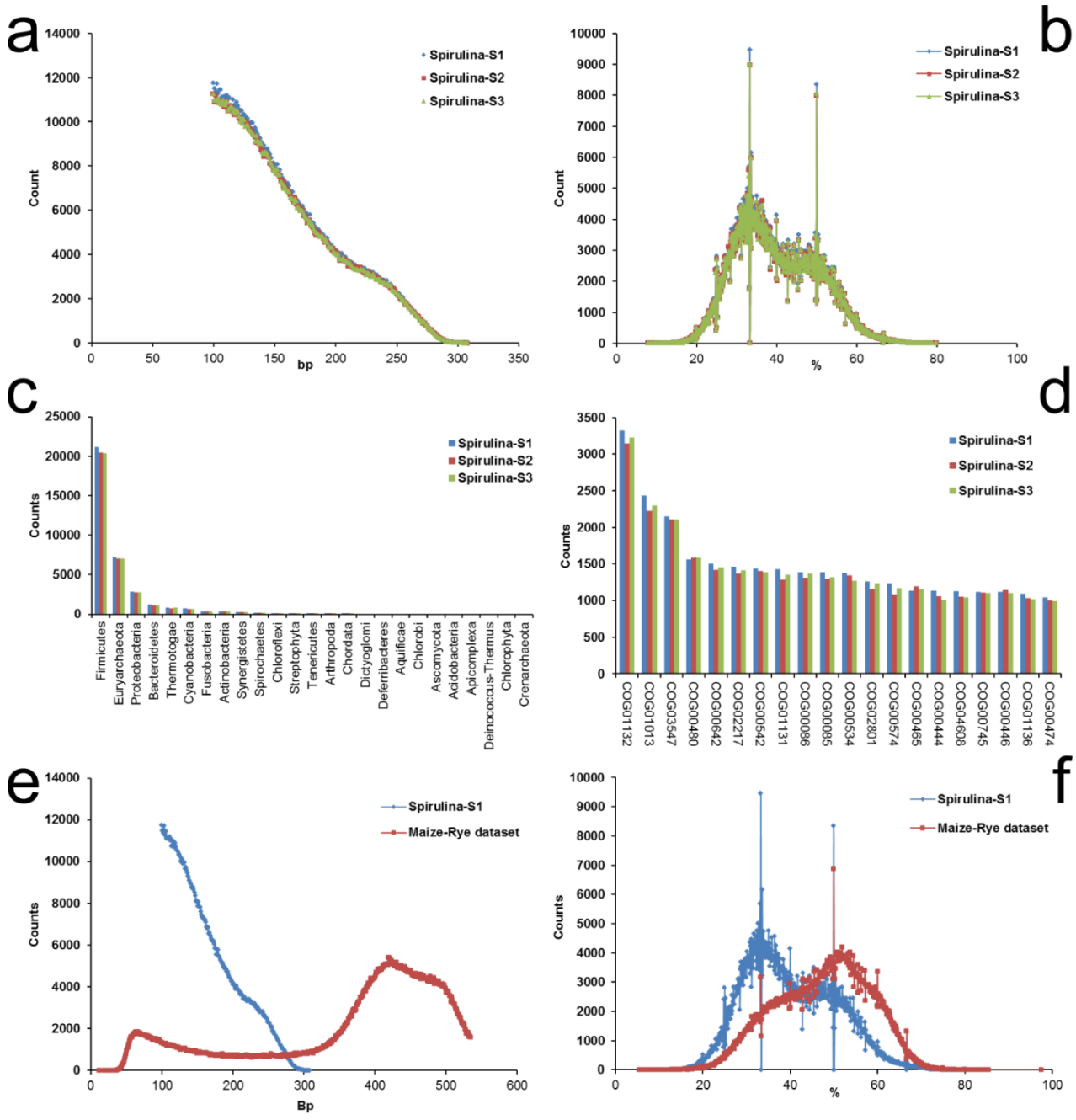
Supplementary Material and Methods Figure 1 Datasets comparison with the MGX platform

Comparison of the three randomly generated *Spirulina* datasets in terms of **(a)** Read length; **(b)** GC %; **(c)** 25 most abundant Phyla; **(d)** 20 most abundant COGs; and comparison of the *Spirulina*-S1 and the Maize-Rye dataset in **(e)** terms of read length and **(f)** GC %.



Unpublished result [6]: Metagenome analysis of the microbial population from an alkaline anaerobic digester fed with the microalga *Spirulina*

V. Nolla-Ardèvol

Suppl. Table 6.16S rDNA accession numbers

Accession numbers of reference 16S rDNA sequences for which in the phylogenetic trees (Figures 6.1; 6.2; 6.3 and 6.4) only clones and organism names are shown.

Organism name / Clone	Accession
Cytophaga-Flavobacteria-Bacteroides	
Salinibacter ruber C37	gb|KF668249.1|
Flavobacterium aquatile LMG_4008	ref|NR_118482.1|
Sphingobacterium sp.21	ref|NR_074508.1|
Gramella forsetii KT0803	ref|NR_074707.1|
Anaerophaga thermohalophila Fru22	emb|AJ418048.1|
Belliella pelovolcani CC-SAL-25	gb|EU685336.1|
Alkaliflexus imshenetskii Z-7010	emb|AJ784993.1|
Paludibacter propionicigenes	dbj|AB078842.2|
Cellulophaga algicola DSM14237	ref|NR_074452.1|
Draconibacterium orientale	ref|NR_121783.1|
Escherichia coli RREC_I	gb|AF527827.1|
CloneML635J-20	gb|AF507861.1|
CloneBSA1B-12	dbj|AB175366.1|
CloneML635J-56	gb|AF507862.1|
CloneWN-HWB-154	gb|DQ432348.1|
CloneCSS133	gb|JX240684.1|
Contig00447 Mesophilic-Reactor	JMBV00000000
Clostridiales	
Orenia sp. 1D4	gb|JQ690693.1|
Proteinivorax tanatarense Z-910	gb|JQ904541.1|
Tindallia magadiensis Z-7934	ref|NR_026446.1|
Halanaerobium hydrogeniformans	ref|NR_074850.1|
Clostridium elmenteitii E2SE1	emb|AJ271453.1|
Natranaerobius thermophilus JW/NM-WN-LF	ref|NR_074181.1|
Alkaliphilus crotonoxidans	gb|AF467248.1|
Clostridium difficile 630	ref|NR_074454.1|
Thermincola potens JR	ref|NR_074717.1|
Thermoanaerobacter sp. X514	ref|NR_074779.1|
Natronoanaerobium salstagnum O-M12SP-2	emb|AJ271450.1|
Anaerobranca bogoriae	gb|AF203703.1|
Natronincola ferrireducens Z-0511	gb|EU878275.1|
Halocella cellulosilytica DSM 7362T	emb|X89072.1|
Halothermothrix orenii H 168	ref|NR_074915.1|
Natronovirga wadinatrunensis JW/NM-WN-LH1	gb|EU338489.2|
Arthrospira platensis C1	gi|423061881|
CloneCSS28	gb|JX240605.1|
Clonex216	gb|GU083685.1|
CloneCT1C2AC09	gb|JQ427824.1|
CloneCSS150	gb|JX240699.1|
CloneCSS73	gb|JX240655.1|
CloneMAT-CR-H3-B08	gb|EU245154.1|
Contig04220 Mesophilic-Reactor	JMBV00000000
Firmicutes-EM1 Mesophilic-Reactor	No Accession #

Suppl. Table 6.1 Continuation

Organism name / Clone	Accession
Alphaproteobacteria	
Rhodobaca bogoriensis SLB	gb|EU908048.1|
Chelatococcus sp. J-9.1	emb|FR774565.1|
Rhodobacter sp. R-8	gb|AY914074.1|
Roseinatronobacter sp. MOL1.10	gb|KJ486297.1|
Rhodobaca barguzinensis VKM_B-2406	ref|NR_044285.1|
Rhodobacter sphaeroides 2.4.1	ref|NR_074171.1|
Methylobacterium extorquens AM1	ref|NR_074138.1|
Salinarimonas rosea YIM-YD3	ref|NR_116487.1|
Bacillus subtilis	emb|X60646.1|
CloneSA_118_	gb|JQ739039.1|
CloneTUM-Mbac-MR4-B1-KC14	gb|EU812964.1|
CloneQEEA3DF04	emb|CU918797.1|
CloneML602J-43_	gb|AF507829.1|
CloneTX4CB_152	gb|FJ153021.1|
Methanogens	
Methanoculleus bourgensis MS2	ref|NR_042786.1|
Methanosarcina mazei zm-15	gb|KF360023.1|
Methanocalculus sp. AMF2	gb|HM053969.1|
Methanocalculus sp. AMF-Bu2	gb|JQ724113.1|
Methanocalculus halotolerans	ref|NR_041843.1|
Methanocalculus natronophilus strain Z-7105	ref|NR_118529.1|
Methanobacterium oryzae	gb|AF028690.2|
Methanospirillum lacunae	dbj|AB517986.1|
Methanosalsum zhilinae DSM4017	ref|NR_102894.1|
Methanobacterium alcaliphilum NBRC105226	ref|NR_112910.1|
Methanocaldococcus sp. FS406-22	ref|NR_074228.1|
Uncultured *Methanohalophilus sp*. cloneGNA03F08	gb|EU731585.1|
Uncultured *Methanococcoides sp*. cloneET5-1H2	gb|EU585970.1|
Uncultured *Methanocalculus sp*. cloneNRA1	gb|HM041902.1
Uncultured *Methanocalculus sp*. cloneD008023G07	gb|GU179434.1|
Methanopyrus kandleri AV19	ref|NR_074539.1|
CloneARC204	gb|JN185089.1|
CloneFL-52	gb|DQ089009.1|
CloneWN-FWA-130	gb|DQ432522.1|
CloneNRP-N	dbj|AB243805.1|
Clone61ArcR3	gb|JF421670.1|
CloneA161	gb|FJ205789.1|
CloneAS22	gb|EU358672.1|
Contig11126 Mesophilic-Reactor	JMBV00000000

10. Curriculum Vitae

Vímac Nolla Ardèvol
Date of birth: 01-07-1979
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Education

April 2011 – present **PhD Candidate.** Cluster of Industrial Biotechnology, Universität Bielefeld; Bielefeld, Germany.
PhD Thesis: "Methane production from algae at high pH (~10)"
Supervisors: Prof. Dr. Marc Strous; Prof. Dr. Olaf Kruse

2009 – 2010 **MSc in Advanced Microbiology.** Universitat de Barcelona; Barcelona, Spain.
Master thesis: "Anaerobic co-digestion of pig manure and glycerin"
Supervisor: Dr. Joan Dosta; MSc. Sergi Astals Garcia

2004 – 2009 **Degree in Biotechnology**. Universitat de Vic; Vic, Spain.

2000 – 2002 **Laboratory Technician Professional formation** CE. Dolmen; L'Hospitalet de Llobregat, Spain.

Publications

Nolla-Ardèvol, V., Strous, M., Tegetmeyer, H.E. Metagenome analysis of the anaerobic microbial community from an alkaline anaerobic digester fed with the microalga *Spirulina*. (In preparation)

Nolla-Ardèvol, V., Peces, M., Strous, M., Tegetmeyer, H.E. Metagenome analysis and biogas production from the anaerobic digestion of the protein rich microalga *Spirulina* (Submitted)

Nolla-Ardèvol, V., Strous, M., Tegetmeyer, H.E. Anaerobic digestion of the microalga *Spirulina* at alkaline conditions (pH~10; 2.0 M Na^+) (Submitted)

Astals, S., Nolla-Ardevol, V., Mata-Alvarez, J., 2013. Thermophilic co-digestion of pig manure and crude glycerol: Process performance and digestate stability. J. Biotechnol. 166, 97–104.

Nolla-Ardèvol, V., Strous, M., Sorokin, D.Y., Merkel, A.Y., Tegetmeyer, H.E., 2012. Activity and diversity of haloalkaliphilic methanogens in Central Asian soda lakes. J. Biotechnol. 161, 167–173.

Astals, S., Nolla-Ardèvol, V., Alvarez-Mata; Joan, 2012. Anaerobic co-digestion of pig manure and crude glycerol at mesophilic conditions: Biogas and digestate. Bioresour. Technol 110, 63-70.

Research Experience

April 2011 – present **PhD thesis** "Methane production from algae at high pH (~10)"

This work focuses on the possibility of producing biomethane at alkaline conditions (pH ~10; 2.0 M Na^+) from the anaerobic digestion of the microalga *Spirulina* using alkaline extremophiles obtained from Soda lake sediments.

Feb 2010 - Sep 2010 **Master thesis "**Anaerobic co-digestion of manure and glycerin"

This work was part of the project "Probiogas: Development of sustainable production and use of agroindustrial biogas in Spain" (PS-120000-2007-6) and consisted in studying the co-digestion of pig manure with glycerin.

Oct 2005 - Oct 2008 **Research assistant**

Design of a synthetic culture medium for the growth of hydrogenotrophic methanogenic bacteria under the frame of the project CENIT SOST-CO2 "New sustainable industrial uses of CO2" (CEN-2008-1027).

Relevant Presentations and Posters at conferences

- **13th World Congress on Anaerobic Digestion. Recovering (bio) Resources for the world.** Santiago de Compostela, Spain, 25 – 28 June 2013.

Poster: "Anaerobic digestion of the microalga *Spirulina* and other substrates at high pH ~10 and high salt concentration."

- **17th International Pushchino School Conference of Young Scientist. "Biology. The science of the XXI century".** Pushchino, Russia, 21 – 26 April 2013.

Presentation: "Anaerobic digestion of *Spirulina* at alkaline conditions".

- **1st CLIB-GC Symposium "Patents in Biotechnology, Perspectives from Society and Industry".** Bielefeld, Germany, 11th March 2013.

Member of the organizing committee.

- **Biometa 2010.** Barcelona 19-20 April, 2010.

Organization assistance.

Declaration

I hereby declare that I have conducted the work presented here myself and have used only the specified sources and resources. All literature and supplemental sources were cited accordingly. All work from other authors, including images and figures, has been appropriately referenced, or reproduced with permission.

I declare that this dissertation has not been submitted in whole or in part, to another faculty with the aim to acquire an academic degree. I hereby apply, for the first time, for the degree of Doctor of Natural Sciences at the University of Bielefeld.

Vímac Nolla Ardèvol

Bielefeld, October 2014