Université de Montréal

Anaerobic Digestibility of Microalgae

Fate and Limitations of Long Chain Fatty Acids in the Biodegradation of Lipids

par

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Anaerobic Digestibility of Microalgae

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Abstract

Anaerobic digestion is a biological process in which an anaerobic microbial consortium converts organic matter into biogas, primarily methane and carbon dioxide. Among the organic substrates, lipids are the most productive of methane in comparison to carbohydrates and proteins; but their degradation is very difficult, due to their hydrolysis which can be the limiting step. Algae can be an important source for methane production because of their potentially high content of lipids.

The objective of this study was, therefore, to evaluate the methane production of microalgae using the Biochemical methane Potential (BMP) technique and to identify the limit of biodegradation of lipids in the anaerobic digestion.

The experimentation plan was divided into the following stages: 1) Compare the energy potential in methane of macroalgae versus microalgae. 2) Screen different species of freshwater and marine microalgae to compare their methane potential. 3) Determine the impact of mild pretreatment of targeted microalgae on the methane production. 4) Identify the limits of biodegradation of algal lipids in the anaerobic digestion by studying kinetics limiting steps of lipids and individual LCFA (Long Chain Fatty Acids).

The results showed that microalgae produce more methane than macroalgae. The BMP of freshwater and marine microalgae showed no difference in terms of methane yield. The results of pretreatment showed that the thermal (microwave) pretreatment seemed to be more effective than the chemical (alkaline) pretreatment. A BMP control test done on palm oil, macadamia oil and fish oil showed that the hydrolysis of oils in glycerol and LCFA was not the limiting step in the production of methane. The addition of fat in the samples of defatted *Phaeodactylum* increased the methane yield and this augmentation was correlated to the quantity of fat added.

Keyswords: anaerobic digestion, microalgae, long chain fatty acids, inhibition

Résumé

La digestion anaérobie est un processus biologique dans lequel un consortium microbien complexe fonctionnant en absence d'oxygène transforme la matière organique en biogaz, principalement en méthane et en dioxyde de carbone. Parmi les substrats organiques, les lipides sont les plus productifs de méthane par rapport aux glucides et aux protéines; mais leur dégradation est très difficile, en raison de leur hydrolyse qui peut être l'étape limitante. Les algues peuvent être une source importante pour la production de méthane à cause de leur contenu en lipides potentiellement élevé.

L'objectif de cette étude était, par conséquent, d'évaluer la production en méthane des microalgues en utilisant la technique du BMP (Biochemical méthane Potential) et d'identifier les limites de biodégradion des lipides dans la digestion anaérobie.

Le plan expérimental a été divisé en plusieurs étapes: 1) Comparer le potentiel énergétique en méthane des macroalgues par rapport aux microalgues. 2) Faire le criblage de différentes espèces de microalgues d'eau douce et marines afin de comparer leur potentiel en méthane. 3) Déterminer l'impact des prétraitements sur la production de méthane de quelques microalgues ciblées. 4) Identifier les limites de biodégradation des lipides algaux dans la digestion anaérobie, en étudiant les étapes limitantes de la cinétique des lipides et de chacun des acides gras à longues chaines.

Les résultats ont montré que les microalgues produisent plus de méthane que les macroalgues. Les BMP des microalgues d'eau douce et marines n'ont montré aucune différence en termes de rendement en méthane. Les résultats des prétraitements ont montré que le prétraitement thermique (microonde) semblait être plus efficace que le prétraitement chimique (alcalin). Les tests de contrôle du BMP faits sur l'huile de palme, l'huile de macadamia et l'huile de poisson ont montré que l'hydrolyse des huiles en glycérol et en acides gras à longues chaines n'était pas l'étape limitante dans la production de méthane. L'ajout de gras dans les échantillons de *Phaeodactylum* dégraissée a augmenté le rendement de méthane et cette augmentation a été corrélée à la quantité de matières grasses ajoutées.

Mots-clés : digestion anaérobie, microalgues, acides gras à longues chaines, inhibition.

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Dedication

To my father, Abdou Hamani, and my mother, Haoua Keita: your advices, teachings and support, despite the distance that separates us, have been an inspiration to me. This work is entirely dedicated to you.

To my husband, Bachir Ganda: thank you for your love and support. You were always present to share my joys, take care of our children, comfort and help me regain confidence in difficult times. This work is also yours.

To my daughters, Hayrah Aminata and Naila Awa: thank you for your patience when I could not kiss you good night, find through this work all my affection and love.

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Abbreviations and notations

AD: Anaerobic digestion

BMP: Biochemical Potential Methane

sCOD: Soluble chemical oxygen demand

tCOD: Total chemical oxygen demand

EPA: Eicosapentaenoic acid

FID: flame ionization detector

GC: gas chromatography

HPLC: high performance liquid chromatography

HRT: Hydraulic retention time (day)

LCFA: Long chain fatty acid

OLR: Organic loading rate (gVS /*l*/day)

SS: Suspended solids (g/kg)

TCD: thermal conductivity detector

TS: Total solids (g/kg)

TVS: Total Volatile solids (g/kg)

UASB: Up flow anaerobic sludge blanket reactor

VFA: Volatile fatty acid (mg/*l*)

VSS: Volatile suspended solids (g/kg)

Chapter One - Introduction: Literature

I. Microalgae

Microalgae constitute a large and diverse group of prokaryotic and eukaryotic photosynthetic microorganisms. Unlike higher plants, these microscopic plants lack vascular systems for nutrient and water transport, but compensate that by their very large surface to volume ratio (Van Harmelen and Oonk, 2006). They are found in all ecosystems: aquatic, terrestrial and can be produced rapidly under difficult climatic conditions due to their unicellular or simple multicellular structure (Li et al., 2008). It is estimated that 50,000 species of microalgae have been discovered, but only 30,000 have been studied and analyzed (Richmond, 2004). Microalgae are capable of reproducing themselves by photosynthesis using solar energy, water and carbon dioxide (Chisti, 2008). It is estimated that the biomass productivity of microalgae could be 50 times more than that of switchgrass, which is the fastest growing terrestrial plant (Li et al., 2008).

II. Chemical Composition of Microalgae

The chemical composition of algae is not constant and the proportions of the different constituents depend on many environmental factors such as temperature, illumination, pH value of the medium, mineral nutriments, and CO_2 supply. A desired proportion of the constituents of algae can be obtained by varying the culture conditions, like the nitrogen or phosphorus depletion in the medium or by changing the physical factors, for instance, osmotic pressure, radiation intensity, population density, light or dark growth (Becker, 1994). In their study, Spoehr and Milner (1949) described the effects of environmental conditions and the effects of changing nitrogen supply on the lipid and chlorophyll content of chlorella and some diatoms. Some of the constituents found in algae are: proteins, amino acids, carbohydrates, vitamins, pigments (chlorophyll, carotenoids, and phycobiliproteins) and lipids.

II-1 Proteins and Amino Acids

The high protein content of many microalgal species was one of the most important reasons for considering them as an unconventional source of proteins (Cornet, 1998; Soletto et al., 2005). As it can be seen in Table I, they contain more proteins than the other vegetable sources such as rice, wheat, vegetables; but some algae have fewer proteins than animal sources of protein such

as milk, egg and meat (Mata et al., 2010). The nutritional quality of a protein depends on the content, proportion and availability of the amino acids (Becker, 1994). While plants can synthesize all amino acids, animals and humans are capable of synthesizing only the non-essential amino acids, and look for the essential ones in their food (Guil-Guerrero et al, 2004). The composition of amino acids in the different species of microalgae is then important because they are primarily considered as source of proteins.

II-2 Carbohydrates

In microalgae, carbohydrates are in form of starch, glucose, sugars and other polysaccharides. They have a high digestibility, thus the dried whole microalgae can be used in large amounts in food or feeds (Becker, 2004). Table I gives the chemical composition of carbohydrates in different algae.

Table I: ***Gross Chemical Composition of Human Food Sources and Different Algae (% of Dry Matter) (Becker, 1994)***

Commodity	**Protein**	**Carbo-hydrates**	**Commodity**	**Protein**	**Carbo-hydrates**
Baker's yeast	39	38	*Spirogyra sp.*	6-20	33-64
Rice	8	77	*Dunaniella bioculata*	49	4
Egg	47	4	*Dunaliella salina*	57	32
Milk	26	38	*Euglena gracilis*	39-61	14-18
Meat muscle	43	1	*Prymnesium parvum*	28-45	25-33
Soya	37	30	*Tetraselmis maculata*	52	15
Scenedesmus obliquus	50-56	10-17	*Porphyridium cruentum*	28-39	40-57
Scenedesmus quadricauda	47	-	*Spirulina platensis*	46-63	8-14
Scenedesmus dimorphus	8-18	21-52	*Spirulina maxima*	60-71	13-16
Chlamydomonas rheinhardii	48	17	*Synechococcus sp.*	63	15
Chlorella vulgaris	51-58	12-17	*Anabaena cylindrica*	43-56	25-30
Chlorella pyrenoidosa	57	26			

II-3 Lipids

The lipids are a large and diverse group of molecules with different structures, principally composed of hydrogen, carbon and oxygen. They are soluble in non-polar organic solvents (ether, chloroform, benzene acetone) and insoluble in water. Commonly known as fats or oils,

lipids are one of the major components of organic matter in waste or wastewaters (Li et al., 2002). These compounds are glycerol bounded to long chain fatty acids (LCFA), alcohols or other groups with an ester linkage (Cavaleiro et al., 2008). Fats contain saturated LCFA, and oils are normally composed of unsaturated fatty acids (Alves et al., 2009). The triacylglycerols also called neutral fats are the most abundant family of lipids (Cavaleiro et al., 2008). Algae have a high content of lipids, which varies between 1 and 70%. These rates can reach 90% of dry matter under the influence of certain factors (Mata et al., 2010). Some microalgae such as *Chlorella, Crypthecodinium, Cylindrotheca, Dunaliella, Isochrysis*, and *Nannochloris* have lipid levels between 20 and 50%. *Brotryococcus braunii* contains up to 75% of lipid and *Porphyridium cruentum* contains 60.7%. Table II shows the lipid content of different microalgae. An important factor is also the fatty acid composition of different species of microalgae. Algal lipids are composed of glycerol, sugars or bases esterified to fatty acids. These fatty acids may be saturated or unsaturated with 12-22 carbon atoms (Mata et al., 2010). Some cyanobacteria, especially the filamentous ones, have a larger amount of polyunsaturated fatty acids (25-60% of the total). Eukaryotic algae have more saturated and monosaturated fatty acids (Becker, 1994). The most common storage lipids are triacylglycerol which constitute up to 80% of the total lipids fraction (Sheehan et al., 1998). Many nutritional and environmental factors can affect the quantity of fatty acids. Piorreck et al. (1984) showed the effects of different nitrogen regimes on lipid content of different algae: two green algae *Chlorella vulgaris* and *Scenedesmus obliquus* and four blue-green algae *Anarystis nidulans, Microcystis aeruginosa, Oscillatoria rubescens* and *Spirulina platensis.* The study showed that low nitrogen levels enhanced the percentage of lipid (45% of the biomass) and 70% of these were neutral lipids such as triacylglycerols (containing mainly 16:0 and 18:1 fatty acids). However, at high nitrogen levels, the percentage of total lipids dropped to about 20% of the dry weight and the predominant lipids were polar lipids containing polyunsaturated C_{16} and C_{18} fatty acids. Besides nitrogen, silicon deficiency can increase the amount of lipids in diatoms. Light increases the formation of polyunsaturated C_{16} and C_{18} fatty acids in *Chlorella* and a low temperature favors the synthesis of polyunsaturated C_{18} fatty acids in some algae; it also changes the fatty acids composition of *Dunaliella* (Becker, 1994). Fatty acids can differ depending on the type of algae. Table III gives the fatty acid composition of lipids in five different algae. Linolenic acid (18:3) is common in *Chlorophycee* (*Scenedesmus obliquus, Dunaliella bardawil*), whereas, in *Bacillariophyceae,* palmitic acid ($C_{16:0}$), hexadecenoic acid ($C_{16:1}$), and polyenoic acid (C_{20}) are

the major fatty acids. In *Rodophyceae* such as *Porphyridium sp*., the most abundant fatty acids found are arachidonic acid ($C_{20:4}$), palmitic, oleic ($C_{18:1}$) and linoleic acids ($C_{18:2}$) (Becker, 1994).

Table II: *Lipid Content of Different Microalgae (Mata et al., 2010; Becker, 1994)*

Algae	Lipid Content (% of dry matter)	Algae	Lipid Content (% of dry matter)
Anabaena cylindrica	4-7	*Monallanthus salina*	20.0-22.0
Ankistrodesmus sp.	24-31	*Nannochloris sp.*	20.0-56.0
Botryococcus braunii	25-75	*Nannochloropsis oculata*	22.7-29.7
Chlamydomonas reinhardtii	21	*Nannochloropsis sp.*	12.0-53.0
Chlorella emersonii	25-63	*Neochloris oleoabundans*	29-65
Chlorella protothecoides	14.6-57.8	*Nitzschia sp.*	16.0-47.0
Chlorella sorokiniana	19-22	*Oocystis pusilla*	10.5
Chlorella vulgaris	5-58	*Pavlova salina*	30.9
Chlorella sp.	10-48	*Pavlova lutheri*	35.5
Chlorella pyrenoidosa	2	*Phaeodactylum tricornutum*	18.0-57.0
Chlorella	18-57	*Porphyridium cruentum*	9.0-18.8/60.7
Chlorococcum sp.	19.3	*Scenedesmus obliquus*	11-55
Dunaliella salina	6.0-25.0	*Scenedesmus quadricauda*	1.9-18.4
Dunaliella primolecta	23.1	*Scenedesmus sp.*	19.6-21.1
Dunaliella tertiolecta	16.7-71.0	*Skeletonema sp.*	13.3-31.8
Dunaliella sp.	17.5-67.0	*Skeletonema costatum*	13.5-51.3
Ellipsoidion sp.	27.4	*Spirogyra sp.*	11-27
Euglena gracilis	14-20	*Spirulina platensis*	4-16.6
Haematococcus pluvialis	25	*Spirulina maxima*	4-9
Isochrysis galbana	7.0-40.0	*Thalassiosira pseudonana*	20.6
Isochrysis sp.	7.1-33	*Tetraselmis suecica*	8.5-23.0
Monodus subterraneus	16	*Tetraselmis sp.*	12.6-14.7

Table III*: Analytical Data on Fatty Acid Composition of Lipids of Different Algae (Becker, 1994)*

Fatty Acid C:D	*Spirulina platensis*	*Spirulina maxima*	*Scenedesmus obliquus*	*Chlorella vulgaris*	*Dunaliella bardawil*
12:0	0.4	tr	0.3	-	-
14:0	0.7	0.3	0.6	0.9	-
14:1	0.2	0.1	0.1	2.0	-
15:0	tr	tr	-	1.6	-
16:0	45.5	45.1	16.0	20.4	41.7
16:1	9.6	6.8	8	5.8	7.3
16:2	1.2.	tr	1.0	1.7	-
16:4	-	-	26.0	-	3.7
17:0	0.3	0.2	-	2.5	-
18:0	1.3	1.4	0.3	15.3	2.9
18:1	3.8	1.9	8.0	6.6	8.8
18:2	14.5	14.6	6	1.5	15.1
18:3	0.3	0.3	28.0	-	20.5
18:3	21.1	20.3	-	-	-
20:2	-	-	-	1.5	-
20:3	0.4	0.8	-	20.8	-

Note: tr: traces C: number of atoms D: number of double bounds

II-4 Hydrocarbons

There are only a few species of microalgae that contain a large amount of hydrocarbons: *Dunaniella sp*. as source of carotenoids and *Botrycoccus braunii* as a source of a mixture of unique C_{17}-C_{34} hydrocarbons. *Botrycoccus braunii* has 20% of hydrocarbon during exponential growth (Becker, 1994).

II-5 Vitamins

Microalgae constitute an important source of all essentials vitamins (A, B_1, B_2, B_6, B_{12}, C, E, Nicotinate, Biotin, Folic acid). The quantity of vitamin in algae varies with environmental factors and growth conditions. The drying processes decrease the amount of vitamins B_1, B_2, C and nicotinic acid found in fresh material. The detection of vitamin B_{12} in microalgae is

surprising because it is usually not found in plants. *Spirulina* is considered as a microalga rich in vitamin B_{12} (Becker, 1994).

II-6 Pigments

II-6-1 Chlorophyll

One important feature of algae is their color. Algae contain one or more types of chlorophyll. Chlorophyll-a is the primary photosynthetic pigment in all algae and it is the only chlorophyll in the cyanobacteria (blue-green algae). Chlorophylls –b, -c, -d, -e can also be found in marine algae and fresh water diatoms. Chlorophylls amount are usually about 0.5 to 1.5% of dry weight (Becker, 1994).

II-6-2 Carotenoids

Carotenoids are the second important pigment found in algae. They are yellow, orange or red lipophilic pigments of aliphatic or alicyclic structure composed of eight, five-carbon (isoprenoid) units, which are linked so that the methyl groups nearest the center of the molecule are in the 1, 5-positions, whereas all other lateral methyl-groups are in the 1, 6-positions. Certain carotenoids are found in most algal classes, whereas others occur only in few classes of algae. The average concentration of carotenoids in algae is 0.1-2% of dry weight. However, when certain algae like *Dunaliella bardawil* are grown under favorable conditions (high light intensity), their amount of ß-carotene can vary from 2 to 14% (Becker, 1994).

II-6-3 Phycobiliproteins

Phycobiliproteins are deep colored water-soluble proteinaceous accessory pigments, which are components of a complex assemblage, the phycobilisomes.

III. Uses of Algae

The cultivation of algae and their use as a source of nutriments (such as lipids) started in large scale in Germany, during the World War II. The culture of the green alga *Chlorella* was initiated by a group of scientists at the Carnegie Institution of Washington. The production of microalgae and cyanobacteria is rapidly increasing throughout the world. Some country like Japan, USA, China, produce over 10,000 tons of microalgal biomass annually (Van Harmelen and Oonk, 2006). Over the past few years, many countries have successfully adapted the cultivation of algae and their various applications have received considerable attention:

1) in human nutrition: protein and dietary supplements. Four strains are the most commercially used: *Arthrospira (Spirulina*) *platensis* rich in proteins, with a high nutritive value (Soletto et al., 2005); *Chlorella* used as a food additive (Yamaguchi, 1996; Gouveia et al., 1996); *Dunaliella salina* used as an ingredient in dietary supplements;

2) in animal nutrition: microalgae can be used in the feed of animals. *Arthrospira* is used for farm animals and pets such as cats, dogs, birds (Spolaore et al., 2006). *Chlorella, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema and Thalassiosira* are the species most used in aquaculture (Apt and Behrens., 1999; Muller-Feuga, 2000; Borowitzka, 1997);

3) in therapeutics: β carotene is used in treatments for skin cancer, antibiotics for wound treatment, regulation of cholesterol synthesis, and as enzymatic hydrolyzate that promote skin metabolism;

4) as pigments: β carotene for food color and food supplement, phycobillins as food color, in diagnostics, cosmetics and analytical reagents;

5) as source of fine chemicals: glycerol is used in foods, beverages, cosmetics, and pharmaceuticals;

6) as hormones: auxins, gibberllins, cytokines (Becker, 1994).

IV. Algae as a Source of Energy

Microalgae are one of the promising sources of biomass in the energy field. Because their growth rate is high (10-30 g dry weight m^{-2} d^{-1}), they require an intensive culture, with high nutrients (Goldman, 1979). The use of microalgae as a source of energy increased with the oil crisis during the 1970s (Cornet, 1998). Several types of renewable biofuels can be obtained from microalgae: methane from anaerobic digestion of the algal biomass (Spolaore et al., 2006), biodiesel derived from microalgal oil (Gavrilescu and Chisti, 2005), ethanol and photobiologically produced biohydrogen (Ghirardi et al., 2000).

IV-1 Biodiesel and Bioethanol

Biodiesel and bioethanol are the most common biofuels; they can, respectively, replace diesel and gasoline, in today's cars with little or no modifications of vehicle engines (Mata et al., 2010). Bioethanol is an alcohol produced by fermentation of sugar from corn, wheat and sugar cane. Sugar cane is the most productive source of bioethanol (Chisti, 2008). Biodiesel is a mixture of fatty acid alkyl esters obtained by transesterification of vegetable oils or animal fats. These lipids are composed of triglycerides (90-98%), mono and diglycerides in small amounts, free

fatty acids (1-5%) and residual amounts of phospholipids, phosphatides, carotenes, tocopherols, sulfur compounds, and traces of water (Bozbas, 2008).

There are many advantages of using microalgae to produce biodiesel (Li et al, 2008): 1) the high growth rate of microalgae can satisfy the high demand of biofuels, while using limited land resources; 2) the cultivation of microalgae consumes less water than the cultivation of land crops; 3) when microalgae are used for biofuel production, there is a reduction in the emissions of nitrous oxide released; 4) microalgal farming could be potentially more cost effective than conventional farming. However, microalgae also present disadvantages for biofuel production, because of the low concentration of organic matter in their culture, induced by the limited access of light. This factor associated to the small size of cells makes the harvest of algae expensive. Microalgae have high levels of humidity, about 99 or 99.5 % (0.5- 1 g solid /*l*) (Minowa and Sanayama, 1999) compared to macroalgae: 87% (Chynoweth, 2002). Their high moisture content of harvested biomass would imply that the drying process would consume energy and would be more expensive (Li et al., 2008).

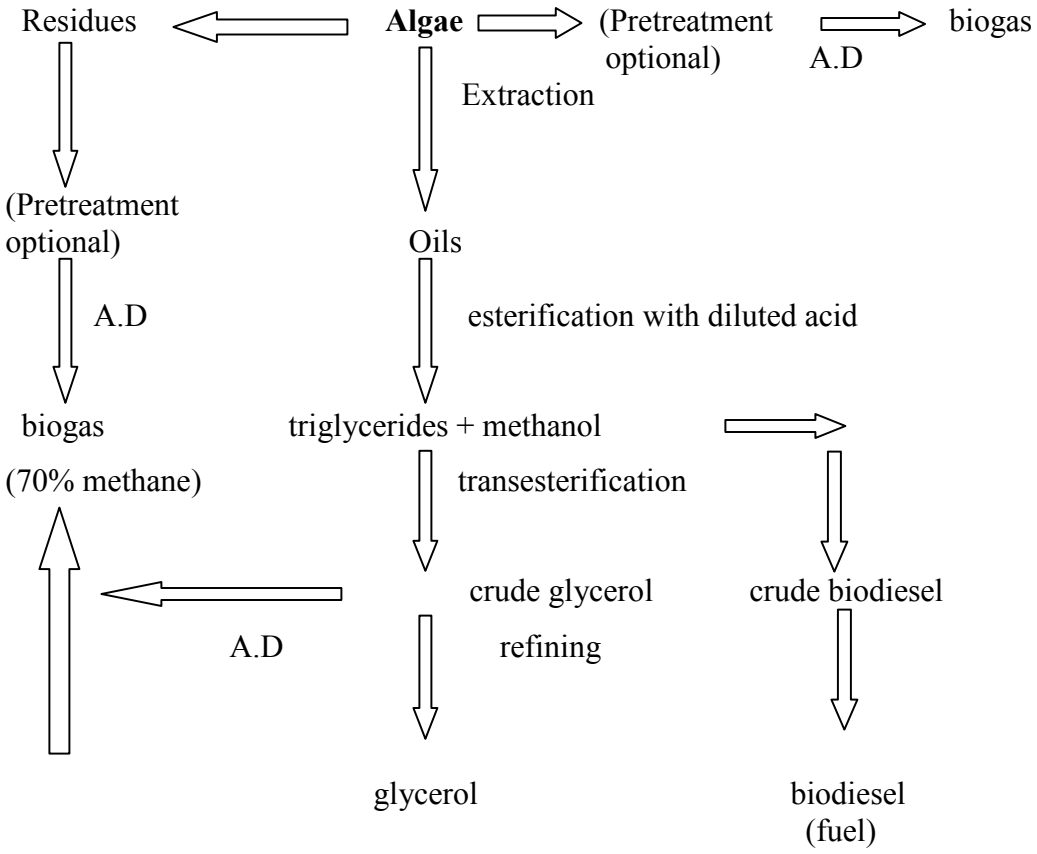
IV-2 Methane

Methane is the main constituent of biogas. It comes from the fermentation of organic matter from plant or animal origin in the absence of oxygen. Biogas is composed of 60-70% methane. Methane is a biofuel that can be substituted to natural gas (mainly composed of more than 95% methane). It is produced by methanogenic archaea that live in anaerobic environments. Methane is released naturally from low oxygen wetlands such as marshes. This gas can be used to replace gasoline in combustion engines. It can also be used in diesel engines (Kangmin and Ho, 2006).

IV-3 Techniques for Obtaining Biodiesel and Methane from Algae

One of the possibilities of obtaining algal fuel is by the direct extraction of lipids and their processing as a diesel-fuel substitute (biodiesel). Oils are obtained from algae by cold pressing, crushing or/and chemical treatment (e.g. solvent extraction) (Danielo, 2005). Biodiesel is produced by transesterification of oils with short-chain alcohols. The transesterification reaction consists of transforming triglycerides into fatty acid alkyl esters, in the presence of an alcohol, such as methanol or ethanol, and a catalyst, such as an alkali or acid, with glycerol as a byproduct (Mata et al., 2010).

Figure 1: ***Techniques for Obtaining Biodiesel and Methane from Algae (adapted from Danielo, 2005).***



A.D: anaerobic digestion

Most of the algal lipids require pretreatment and purification steps for a better performance. On the other side, methane can be obtained by anaerobic digestion of the glycerol and other residues or from the entire alga.

V. Anaerobic Digestion

Anaerobic digestion is a biological process of the conversion of organic matter into biogas consisting primarily of methane and carbon dioxide by an anaerobic microbial consortium (Moletta, 2008). It is a process widely used for wastewater biotreatment, for biosolids stabilization, and for conversion of organic waste and residues into energy. It is a natural transformation which occurs in all environments where there is organic matter in the absence of oxygen (marsh, lake bottoms, intestines of animals and engineered landfill). Several types of organic materials can be stabilized and simultaneously converted into methane, which is used as

a fuel. Because of its ability to produce methane, anaerobic digestion has become an effective process for the production of renewable energy.

V-1 Microbiology of the Anaerobic Digestion

During anaerobic digestion complex molecules are degraded into methane and CO_2 through enzymatic reactions This mineralization occurs in four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Moletta, 2008). The anaerobic microbial communities can be classified into two domains, *Bacteria* and *Archaea* (Demirel and Scherer, 2008).Three groups of micro-organisms are responsible for the methanisation: hydrolytic fermentative (acidogenic), acetogenic bacteria, and methanogenic archaea.

V-1-1. Hydrolysis and Acidogenesis

Hydrolysis is the first step of the anaerobic digestion. At this stage, complex molecules such as polysaccharides, lipids, proteins are degraded into monomers (monosaccharides, fatty acids, and amino acids).

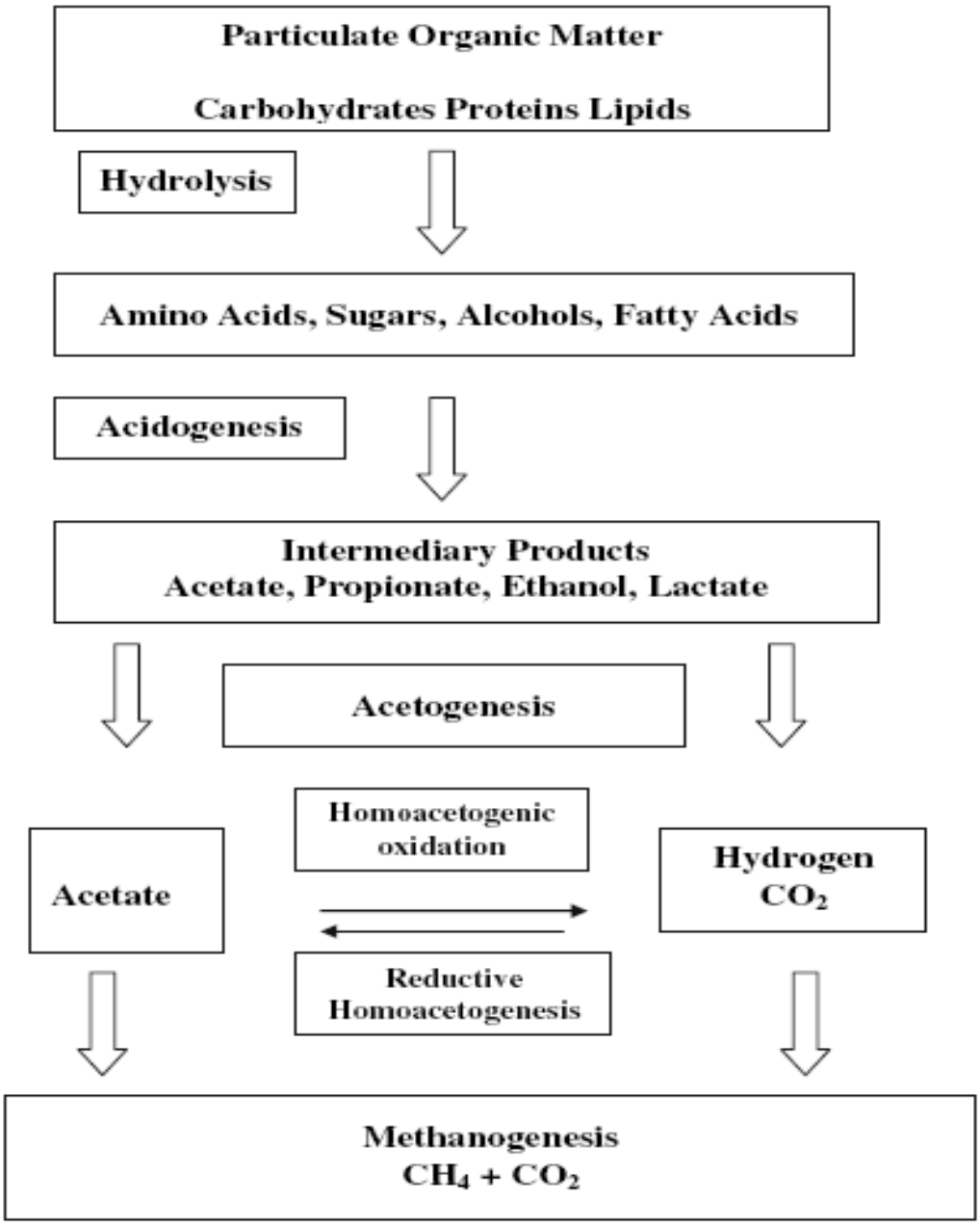
There are many species of bacteria responsible for this step and they can be strict or facultative anaerobic: *Clostridium, Bacillus, Anaerovibrio, Acetomicrobium*, and *Staphylococcus*. The microorganisms have rapid growth rates with doubling time of several hours; however, the stage of hydrolysis is the limiting step in the case of hardly hydrolysable compounds (Moletta, 2008).

At the stage of acidogenesis, the molecules from the previous step are degraded into volatile fatty acids (VFA) (acetic, propionic, butyric acids), alcohol (ethanol), organic acids (lactic acid) hydrogen and carbon dioxide. This step is faster than the other steps and 30-40 times faster than hydrolysis. The bacteria involved in this step may be strict or facultative anaerobes with short doubling times. The most encountered ones are strictly anaerobic bacteria of the genus *Clostridium*. There are also the genus *Bacteroides, Bacillus, Pelobacter*, *Acetobacterium* and *Ulyobacter* and the family of *Enterobacteriaceae* (Moletta, 2008).

V-1-2. Acetogenesis

During the acetogenesis, the different compounds (acids, alcohols) obtained during the hydrolysis and acidogenesis are converted to acetate, hydrogen and carbon dioxide which are direct precursors of methane (Moletta, 2008). Two main pathways may be used: the heterofermentative pathway that produces carbon dioxide and hydrogen from VFA such as butyrate and propionate, and the homoacetogenic pathway which only produces acetate from organic molecules (Tholen and Brune, 1999).

Figure 2: ***Anaerobic Conversion of Biomass to Methane (extracted from Demirel and Scherer, 2008)***



Three groups of bacteria are responsible for the transformation of organic molecules to acetate, hydrogen and carbon dioxide: the obligate hydrogen producing acetogens (which are syntrophic bacteria), the homo-acetogenic bacteria and the sulfate-reducing bacteria that may have one or both functions. The syntrophic acetogenic bacteria are of the genus *Syntrophobacter, Syntrophomonas, Syntrophus* (Moletta, 2008).

The accumulation of hydrogen indicates a dysfunction and leads inevitably to the inhibition of acetogenesis. This implies the need of a constant elimination of the hydrogen produced.

This elimination can be achieved through the hydrogenetrophic archea, which will consume the hydrogen produced to reduce the carbon dioxide into methane, and the sulfate-reducing bacteria which will consume the hydrogen to reduce the sulfates into sulfides (Hanaki, 1981).

V-1-3. Methanogenesis

Methanogenesis is the transformation of acetate, hydrogen and carbon dioxide in methane. It is also possible to have traces of hydrogen and nitrogen as a result of methanogenesis. The methane can be obtained by two pathways: from carbon dioxide and hydrogen and from acetate and hydrogen (Moletta, 2008). The methanogens are strict anaerobic archaea and are represented by two categories: 1) Hydrogenophilic methanogens: they produce methane from carbon dioxide and hydrogen. They are represented by the genres *Methanobacterium, Methanobrevibacter, Methanospirillum, Methanocorspusculum* (Moletta, 2008; Demirel et al., 2008); 2) Acetoclastic (or acetotrophic) methanogens produce methane from acetate. The most encountered genus are *Methanosarcina* and *Methanosaeta* (or *Methanothrix*) (Morgan et al. 1991; Moletta, 2008). The theory gives a value of production of methane of up to 350 *l* per kilogram of chemical oxygen demand (COD) eliminated (under normal temperature and pressure).

V-1-4. Other Reactions

The sulfate-reduction is the transformation of volatile fatty acids and ethanol using sulfate as an energy source (Moletta, 2008). The reactions associated with nitrogen are the denitrification and the anaerobic ammonium oxidation (Moletta, 2008).

V-2. Physical and Chemical Parameters Influencing the Anaerobic Digestion

V-2-1. Temperature

One important factor in the anaerobic digestion is the temperature. Compared with thermophilic temperature (50-70°C), mesophilic temperature (25-45°C) appeared to be an optimal condition for a maximal methane yield (Cecchi et al., 1996).

V-2-2. pH

pH is the most important factor impacting methane proportion in biogas. The CO_2 dissolved in water (CO_2 [*l*]) is in equilibrium with the CO_2 content in the gas phase (headspace of the reactor or the test bottle) (CO_2 [*g*]) according to the Henry law.

On the other side, the dissolved CO_2 is in chemical equilibrium with its carbonated forms: CO_2 [*l*] + H_2O ^{TM} H_2CO_3 ^{TM} HCO_3^- +H^+ ^{TM} $CO_3^=$ +$2H^+$. Therefore the concentration of CO_2 [*l*] depends on the pH. In alkaline water, the reaction is displaced to the right which *in fine* increases the fraction of CO_2 which dissolves in water, resulting in a lower content of CO_2 [*g*] and a higher percentage of methane.

The overloading of the digester results in the accumulation of acids including acetic acid and a diminution of the pH. The decrease of the pH causes the dissociation of acetic acid – acetate to acetic acid, characterized by the following reaction: CH_3-COOH or HAc ↔ CH3-COO^- or Ac^-. It is the undissociated form of acetic acid HAc that inhibits methanogenesis. It is also under this form that the substrate migrates within the cell by diffusion through the cell membrane. The increase in the HAc extra cellular concentration from the accumulation of acids and the decrease of the pH increase their transmembrane diffusion rate and their intracellular concentration. To maintain the neutral pH of the medium, the HAc is then dissociated in acetate and protons (HAc→ Ac^- + H^+). The cell has to continually evacuate its additional protons in order to maintain its intracellular pH. This results in a significant and continuous expenditure of energy at the expense of the cell that is no longer viable. A similar process occurs in the inhibition of methanogenesis by ammoniac, NH_3. At alkaline pH, the dissociation NH_4^+ ↔ NH_3^+ + H^+ is shifted towards NH_3 with an increase of its intracellular concentration. To maintain the neutral pH of the intracellular medium, the NH_3 will react with the intracellular protons to form NH_4^+. The cell will have to take the H^+ from the extracellular medium to restore its internal pH and reject cations as potassium K^+ to compensate for the increased intracellular proton. This leads to an intracellular K^+ deficiency that is fatal to the cell.

A pH between 6.6 and 7.5 is optimum for an increased methane production (Hu and Yu, 2006; Hu et al., 2006; Zheng-Bo et al., 2007; Ihrig et al., 2008; Vergara-Fernandez et al., 2008). On the other hand, at a pH inferior at 6, methane production is negatively affected (Vergara-Fernandez et al., 2008; Hu et al., 2006; Hu and Yu, 2006). The pH is essentially related to the presence of volatile fatty acids.

V-2-3. The Hydraulic Retention Time (HRT)

The HRT is a key parameter in anaerobic process. In a completely stirred tank reactor (CSTR), the HRT must be greater than the generation time of microorganisms, to avoid the microorganisms to be washed out of the reactor. This is particularly critical for the retention of methanogens, which are the slowest growing microorganisms in the anaerobic consortium.

When the process is operated at a HRT of up to 33 days, the methane is constant and maximal (Cecchi 1996; Minowa and Sanayama, 1999; Sialve et al., 2009).

V-2-4. Nutrients

In addition to the organic matter which serves as a source of carbon and energy to micro-organisms, there are nutritional requirements for macronutrients (nitrogen and phosphorus mainly) (Moletta, 2008). Iron, nickel, magnesium, calcium, sodium, cobalt are essential oligo elements that are also necessary for good enzymatic activity. Their deficiency may affect a proper functioning of the trophic chain (Rajeshwari et al., 2000).

V-2-5. Agitation

The stirring system should be efficient enough to maintain a uniform temperature and to liberate the biogas formed. An efficient stirring of the substrates will also result in a better contact between the microorganisms and the substrates.

V-2-6. The Presence of Toxic Compounds and Inhibitors

The presence of ammonia, volatile fatty acids, long chain fatty acids, and hydrogen can inhibit the anaerobic digestion, particularly the methanogenesis (Moletta, 2008). In this section, only the inhibition due to volatile fatty acids and long chain fatty acids will be discussed.

V-3. Biomethanisation of Lipids

Wastes or wastewaters with a high fat content are an attractive source for the production of methane, although lipids are a group of organic compounds with a difficult degradation in biogas. Among the organic substrate, lipids are the most productive of methane (0.99 L_{STP} CH_4/g substrate) when compared to other compounds: carbohydrates (0.42 L_{STP} CH_4/g substrate) and protein (0.63 L_{STP} CH_4/g substrate) (Alves et al., 2009). Theoretically, under normal pressure and temperature, 1 g of oleate (unsaturated long chain fatty acid) gives 1.01 *l* of methane while 1 g of glucose produces 0.37 *l* of methane (Kim et al., 2004). Because of their high content of lipids, algae are an important source for the production of methane. During the anaerobic digestion, lipids are rapidly hydrolyzed to glycerol and long chain fatty acids by extracellular lipases. The glycerol is degraded by acidogenesis, while long chain fatty acids are degraded to acetate, hydrogen and carbon dioxide through β oxidation process. Acetate and hydrogen are finally converted into biogas (Angelidaki and Ahring, 1992, Palatsi et al., 2009).

V-4. Role of Volatile Fatty Acids (VFA) in the Anaerobic Digestion of Lipids

The VFA (pyruvate, butyrate, and propionate) are the most important intermediate of the anaerobic digestion. During acetogenesis, these products are converted to acetate and hydrogen, which subsequently give methane. Their accumulation in the digester leads to an increase of hydrogen and a decrease in pH. These metabolites can inhibit methanogenic Archea and acetogenic bacteria and halt the degradation process (Moletta, 2008). The presence of species such as methanogenic Archea and sulfate reducing bacteria which keeps the hydrogen partial pressure at a low level is then necessary for a good anaerobic digestion (Hanaki, 1981).

V-5. Role and Inhibition potential of Long Chain Fatty Acids (LCFA) in the Anaerobic Digestion of Lipids

Usually, the anaerobic digestion of fats and oils to glycerol and LCFA proceeds rapidly, resulting in the accumulation of LCFA in the wastewater (Angelidaki and Ahring, 1992). At neutral pH, LCFA are ionized and it is appropriate to refer to them according to their carboxilate form; example, oleate and palmitate instead of oleic and palmitic acids (Alves et al., 2009). Studies have shown that LCFA have an inhibitory or limited effect on anaerobic digestion (Neves et al., 2009; Angelidaki and Ahring, 1992; Palatsi et al., 2009; Hwu et al., 1998). Several factors are the cause of this limitation: 1) Lipids are complex molecules and their hydrolysis is the limiting step in the anaerobic digestion; 2) absorption of thin lipid layers around biomass particles causes biomass flotation and washout: when the reactors are overloaded, a severe washout caused by flotation occurs; 3) lipid containing wastes often have low content nutrients and low alkalinity; 4) the carbon chain length and saturation. The anaerobic digestion of the LCFA is accomplished by syntrophic communities of anaerobic bacteria and methanogenic archaea (Alves et al., 2009). This degradation can be restrained because the LCFA have inhibitory effects on many bacteria involved in this process (Koster and Cramer, 1987). Both acetoclastic and methanogenic microorganisms are affected by LCFA; however methanogens are more affected by these compounds (Alves et al., 2001b). The inhibition of methanogenic archaea will result in an accumulation of organic acids which are intermediary metabolites, leading to a decrease of pH and so-called “sour digester” in which the methanogens cannot survive. At low concentrations, LCFA have been reported to be inhibitory for gram-positive microorganisms. Since methanogens have a cell wall similar to that of gram-positive microorganisms, they can be expected to be susceptible to inhibition by LCFA as well (Koster and Cramer, 1987). The effect

of neutral lipids on the anaerobic digestion depend on the degree of biomass adaptation, whereas the addition of free LCFA above a certain concentration may directly lead to a process failure because of the permanent toxic effect of these LCFA towards acetogenic bacteria and methanogenic archaea (Angelidaki and Ahring, 1992). Lalman and Bagley (2002) reported that unsaturated LCFA are more inhibitory than saturated LCFA. Hanaki (1981) showed that the toxicity of a mixture of LCFA is greater than the toxicity of an individual LCFA. Koster and Cramer (1987) confirm this in their study, where a concentration of lauric acid below its minimum inhibitory concentration (MIC) or toxicity threshold level (concentration level below which the maximum specific acetoclastic methanogenic activity was not affected by the presence of the LCFA) strongly increased the toxicity of capric acid and myristic acid. Many studies done on the toxic and inhibitory effect of LCFA showed that they inhibit anaerobic microorganisms at very low concentrations, at mesophilic temperatures (Palatsi et al., 2009). The anaerobic degradability and inhibitory effect of oleic acid ($C_{18:1}$) was studied by Angelidaki and Ahring (1992) and Salvador et al. (2007). They found that this compound had an initial inhibitory concentration of 0.1-0.2 g/*l* and 0.5 g/*l*, respectively, at 37°C. At lower temperature (30°C) Koster and Cramer (1987), as well as Salvador et al. (2007), found an initial inhibitory concentration of 2.4 mM (0.68 g/*l*). At 25°C, Galbraith et al., (1971) found that this concentration was 0.05 mM (0.014 g/*l*) and that oleate was the most inhibitory of ten fatty acids tested in pure culture. Linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids seem to be more toxic with initial inhibitory concentration of 0.02 mM (0.0056 g/*l*) at 25°C (Galbraith et al. 1971). In their study, Lalman and Bagley (2000) showed that linoleic acid at 21°C and a concentration of 30 (0.03 g/*l*) inhibits acetoclastic methanogenesis and this inhibition is enhanced by the cosolvent diethyl ether, but hydrogenotrophic methanogenesis is only slightly inhibited by 30 (0.03 g/*l*) at 21°C.

As mentioned, saturated LCFA appeared to be less inhibitory than unsaturated LCFA. Stearic acid ($C_{18:0}$) has been found to be less toxic with an initial inhibitory concentration of 0.5g/*l* compared to oleic acid ($C_{18:1}$) at 1-0.2 g/*l* (Angelidaki and Ahring, 1992). Galbraith et al. (1971) showed a concentration of 0.4 mM (0.11 g/*l*) for inhibition by stearic acid ($C_{18:0}$) compared to a concentration of 0.05 mM (0.01 g/*l*) for oleic acid ($C_{18:1}$). Koster and Cramer (1987) studied the effects of four saturated long-chain fatty acids: caprylic ($C_{8:0}$) capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$) at 30°C. They found that the MIC was 6.75 mM (0.97 g/*l*), 2.6 mM (0.45 g/*l*), 1.6 mM (0.32 g/*l*), 2.6 mM (0.59 g/*l*), for the four acids respectively. At 25°C Galbraith et al.

(1971) found inferior values with 2 mM (0.29 g/*l*) for caprylic acid ($C_{8:0}$), 1 mM (0.17g/*l*) for capric ($C_{10:0}$), 0.15 mM (0.03 g/*l*) for lauric ($C_{12:0}$), 0.15 mM (0.03 g/*l*) for myristic ($C_{14:0}$), 0.3 mM (0.08 g/*l*) for palmitic acid ($C_{16:0}$). From these experiments, it can be concluded that the susceptibility of the acetoclastic methanogens varies with the type of microorganisms, that lauric acid is the stronger inhibitor of saturated fatty acids and that the inhibition is more correlated to the concentration, although adhesion of LCFA around the bacterial cell wall has been suggested as the mechanism of inhibition, preventing the passage of nutrients through the membrane (Alves et al., 2001 a, b; Hwu et al., 1998). Previous work suggested that LCFA exerts a permanent toxic effect on anaerobic digestion (Angelidaki and Ahring, 1992). However, inhibition caused by LCFA is a reversible process (Palatsi et al., 2009; Alves et al., 2009; Galbraith et al., 1971; Salvador et al., 2007). The acetogenesis and methanogenesis have not been irreversibly damaged since the rate of methane production increased rapidly soon after the LCFA-biomass associated degradation had recommenced (Pereira et al., 2005; Pereira et al., 2003). Several methods can be used to overcome the inhibition of the LCFA. Continuous or pulse exposure in the reactors results in a faster recovery system, improvement of the methane yield and consumption rates of acetate, suggesting an increase tolerance of LCFA (Alves et al., 2001a; Cavaleiro et al., 2008; Hwu et al.,1997). Discontinuous feeding of the system can also promote the development of the anaerobic bacteria communities, able to efficiently convert lipid-rich effluents (Cavaleiro et al., 2008). Other methods such as co-digestion (Fernandez et al., 2005), the addition of absorbents (Angelidaki et al., 1990) or the addition of easily-degradable co-substrates such as glucose and cysteine (Kuang et al., 2006) have been used to overcome LCFA inhibition. Compounds like calcium ions precipitate the LCFA and reduced their inhibitory effect. Cholesterol and ergocalciferol reversed the inhibition of lauric and linoleic acid, but magnesium reversed the inhibition of lauric acid only (Galbraith et al., 1971). The inhibition of LCFA is reversible between 1000 and 5000 mg LCFA/g VSS (Alves et al., 2009).

VI. Biomethanization of Algae and Aquatic Plants

The co-digestion of two or more algae or aquatic plants in anaerobic digestion has been shown to give higher results in term of methane production (0.09-0.30 *l*/g VS) than the digestion of one single species (0.07-0.22 *l*/gVS) (Cecchi et al., 1996; Rigoni-Stern et al., 1990; Alvarez et al., 2008; Kerner and Hanssen,1991;Yang et al., 2009). The action of drying the biomass after

harvest tend to decrease (by 16% or more) the methane yield: 0.31 *l*/g VS for fresh plants vs 0.11-0.26 *l*/g VS for the dry biomass (Asinari Di San Marzano, 1982; Briand and Morand, 1997). Simple mechanical pretreatment also influence the yield of the methane. Briand and Morand (1997) showed that washing the plant decreases the potential of methane from 0.110 to 0.094 *l*/g VS, whereas grinding increases (by over 20%) the values from 0.145 to 0.177 *l*/g VS. One important factor in anaerobic digestion is the temperature. Thermophilic temperature can enhance the methane production rate (Sialve et al., 2009); while mesophilic temperature appear to be optimal conditions for maximal methane productivity (Cecchi et al., 1996; Chen, 1987). This is supported by our study literature where the mesophilic temperature is more widely used. The hydraulic retention time (HRT) and the organic loading rate (OLR) are key parameters in the anaerobic process. When the process is operated at high HRT (up to 33 days) and/or low OLR (0.91-4.1 gVS /*l*/d), the methane is constant and maximal between 0.22-0.32 /g/VS added (Cecchi, 1996; Hu et al., 2006; Siave et al., 2009), but Briand and Morand (1997) and Zheng-Bo et al. (2007) showed that a higher OLR concentration (5.3-7.5 gVS/*l*/d) allowed the microorganisms to grow rapidly and produced a higher efficiency than a lower OLR. However, the pH is the most important factor impacting CH_4 proportion in the biogas. If the pH is high, due to high alkalinity from NH_3 release, then the gas content will shift more to CH_4. A pH superior to 6 (6.6-7.5) is optimum for increased methane production: 0.11-0.35 *l*/gVS (Hu and Yu, 2006; Hu et al., 2006; Zheng-Bo et al., 2007; Ihrig et al., 2008; Vergara-Fernandez et al., 2008; Nagamani and Ramasamy, 1999), on the contrary, at a pH inferior to 6, methane production is negatively affected (Vergara-Fernandez et al., 2008, Hu and Yu, 2006, Hu et al., 2006). In the literature consulted, the reactor does not seem to influence the methane production, but we noted that the highest values of methane: (0.42-0.45 *l*/gVS added) were obtained with a batch system at 35°C, with an HRT of 28 days (Chen, 1987; Sialve et al., 2009). In our review, we identified one marine algae, *Dunaliella,* as having the potential for outstanding methane productivity up to 450 L_{CH4}/kg solid added (0.45 Lg^{-1}VS added) (Sialve et al., 2009).

Table IV: ***Methane Yield of Different Algae and Aquatic Plants***

Substrate	**Fermenter (*l*:liter)**	**T(°C) and pH**	**HRT (days)**	**OLR ($gV l^{-1}d^{-1}$)**	**Specific CH_4 production (liter $CH_4/g^{-1}VS$ added)**	**VS reduction (%)**	**References**
Spirulina maxima (Freshwater microalgae)	Semi-Continuous: 10 *l*	35	33	0.97	0.26	65.8	Samson and Leduy, 1982
Spirulina maxima	Batch 2 *l*	15-52	5-40	20-100	0.25-034	n.a	Samson and Leduy, 1986
Spirulina	Batch 11 l	35	28	0.91	0.32-0.31	n.a	Chen, 1987
Chlorella vulgaris (freshwater microalgae)	Batch 5 *l*	28-31	64	-	0.31-0.35	n.a	Sanchez and Travieso, 1993
Chlorella-Scenedesmus	Batch 11 *l*	35-50	3-30	1.44-2.8	0.17-0.32	n.a	Golueke et al., 1957
Chorella-Scenedesmus Co digestion *Chlorella-Scenedesmus* (50%) and waste paper (50%)	CSTR 4 *l* CSTR 4 *l*	35 35	10 10	2- 4 and 6 4	0.18- 0.58 and 0.82 1.17	n.a	Yen and Brune, 2007
Co-digestion of Sewage sludge (SS) and Macroalgae A (marine algae*:Ulva rigida* and *Gacilaria confervoides*)	Pilot plant $1m^3$ discontinuously fed twice a day	Period 1: 37.1 Period 2: 37.1 Period 3: 37.1 Period 4: 37.1 Period 5: 55.2 Period 6: 55	Period 1: 14.5 Period 2 :14.7 Period 3 :11.2 Period 4 :11.7 Period 5: 11.2 Period 6 :12.3	Period 1 (SS): 1.7 Period 2 (SS+A) :2.6 Period 3 (+SS) :4.4 Period 4 (+A) :4.2 Period 5: 5.3 Period 6 (fed without algae):5.5	Period 1: 0.14 Period 2: 0.22 Period 3: 0.17 Period 4: 0.16 Period 5: 0.01 Period 6: 0.12	A: 32 SS:49	Cecchi et al., 1996

Table IV: ***Methane Yield of Different Algae and Aquatic Plants (Continued)***

Substrate	**Fermenter**	**T°C and pH**	**HRT (days)**	**OLR ($gVS l^{-1}d^{-1}$)**	**Specific CH_4 production (litre $CH_4/g^{-1}VS$ added)**	**VS%**	**References**
Tetraselmis fresh (marine microalgae) *Tretraselmis* (dry)	CSTR 2-5 *l* CSTR 2-5 *l*	35 35	14 14	2 2	0.31 0.26	n.a	Asinari Di San Marzano et al., 1982
Waste Sludge from *Laminaria hyperborea , Foslie* and *Ascophylium nodosum* (marine algae)	Semi-continuous 8l Batch 8 *l*	35 and 7 35 and 7	23 and 16 30	6.5 *l* 6.5 *l*	0.07-0.28 0.10-0.15	40-50 20-40	Kerner and Hanssen., 1991
Ulva thalli (marine algae)	Stirred digester 30 *l*	35 and 7.3-7.5	algae : 15-20 Algae+manure: 15 Manure:15	algae: 1.7-1.8 Algae+manure: 5.3 Manure: 3.5	algae: 0.31-0.37 Algae+ manure: 0.93 Manure: 0.63	Ground: 50-58.6 Algae+manure: 38.8 Manure: 33.7	Briand and Morand, 1997
Ulva thalli	Batch 30 *l*	35	Non washed: 23 Washed: 44 Non-ground: 42 Ground: 4	Non washed: 0.95 Washed: 0.66 Non-ground: 1.97 Ground: 2.36	Non Washed: 0.110 Washed: 0.094 Non ground: 0.145 Ground: 0.177	50	Briand and Morand, 1997
Dunaliella (marine microalgae)	Batch 11 *l*	35	28	0.91	0.44-0.45	n.a	Siave et al., 2009
Algal biomass	Batch 11 *l*	35	28	1	0.42	n.a	Chen, 1987

Table IV: *Methane Yield of Different Algae and Aquatic Plants (Continued)*

Mixture *of Ulva rigida* (80-90%) and *Gracilaria confervoides* (20-10%) (marine algae)	Pilot Plant of 180	35±1	20	1	0.21	54-60	Rigoni-Stern et al., 1990
Mixture *of Quinoa stalk* (*Chenopodium quinoa* Wild), *totora (Schoenoplectus t0tora*) and 0-macrophytes (aquatic flora): (freshwater algae)	Semi continuous 10 *l*	25	30	1.8	-Unmixed feedstock (llama, cow, sheep manures, quinoa, omacrophytes):0.07-0.14 -co-digestion (mixtures of two cosubstrates): 0.09-0.2	14-43	Alvarez and Liden, 2008
Dried *Spartina alterniflora* (SA) and fresh potato (P)	Batch 2.5 *l*	35±1		1.5	- mono digestion (SA): 0.21 -Co digestion: 0.24 and 0.3 at SA:P of 4:1 and 6:1 respectively	6	Yang et al.,2009
Macrocystis pyrifera (dried and crushed): A *Durvillea Antarctica* (Dried crushed): B *Macrocystis* + *Durvillea* A+B (marine algae)	ASBR 2.5 *l* and UAF 4 *l* (two-phase anaerobic phase digestion system)	T^0 37 pH in ASBR: 5.5-5.7 pH in AUF: 6.8-7.2	31	3	A: 0.11 (±52.3) B: 0.11 (±80.2) A+B: 0.10 (±54.9)	n.a	Vergara-Fernández et al., 2008

HRT=hydraulic retention time, OLR=organic loading rate, VS=volatile solid, ASBR: anaerobic sequencing batch reactor, UAF: upflow anaerobic filter, n.a = not available

VII. Study Objectives

VII-1. Principal Objective

The objective of this study is to evaluate the Biochemical methane Potential (BMP) of microalgae and identify the limit of the biodegradation of lipids in the anaerobic digestion.

VII-2. Specific Objectives

As specific objectives, we will:

1) Compare macroalgae to microalgae in term of their energy potential in methane;

2) Screen different species of freshwater and marine microalgae to compare their methane potential;

3) Determine the impact that a mild pretreatment of identified microalgae may have on their methane production;

4) Identify the limits of biodegradation of algal lipids in the anaerobic digestion by studying limiting kinetics steps of lipids and individual LCFA;

   4.1) Hydrolysis test on oils: The purpose of this test is to verify that the oils are able to be hydrolyzed to fatty acids and thus confirm that hydrolysis is not the limiting step of the anaerobic digestion, in the setting of a BMP test performed on algae that contain those LCFA ;

   4.2) Activity test on LCFA: This test is to verify the activity or performance of our biomass (inoculum) on our substrate;

   4.3) BMP of microalgae at different concentrations of LCFA.

Chapter Two: Methodology

I. Physico-Chemical Analysis

These methods include the measurement of the pH, chemical oxygen demand, solids, volatile fatty acids, anions, cations. Analysis were performed on the substrate before the beginning of the experiment for the characterization of the samples (initial values), the day of the experiment ($t = 0$ or t_o analysis) and at the end of the experiment (final analysis). The t_o analysis gave the values of each component in the bottles at the beginning of the experiment.

I-1 pH

The pH is an important parameter used in water chemistry. In the anaerobic digestion, it needs to be evaluated because the microbial consortium requires a pH stable between 7 and 8. In the BMP bottles, this was done by the buffer solution. The measurement of the pH was done manually, using an Accumet AP61 portable pH meter equipped with a micro probe (Fisher, Fairlawn, USA)

I-2 Chemical Oxygen Demand (COD)

The chemical oxygen demand is the measure of the amount of oxygen required to oxidize the organic matter contained in a sample. It measures the reducing power of the substrate. Organic material contained in a sample is oxidized in a closed test tube by heating in a strongly acidic medium (H_2SO_4) with a known amount of potassium dichromate ($K_2Cr_2O_7$). There are two types of COD: soluble chemical oxygen demand (sCOD) and total chemical oxygen demand (tCOD). The sCOD was performed on the centrifuged sample. The sample was centrifuged at 10 000 RPM in the JA-20 rotor (Thermo Fisher Scientific, Asheville, NC, USA) for 10 minutes at 4°C. A known amount of sample was put in a Hach tube and distilled water was added to reach a total volume of 2 ml. Then 0.5 ml of digestion solution and 2.5 ml of acid solution were added with the automatic distributor. The tubes were then heated for 2 hours, at 150°C, in a Hach reactor. The absorbances of the tubes were read at 620 nm, using a Hach DRB 200 spectrophotometer (Hach Company, Loveland, USA). The concentrations were then calculated using a COD standard curve. Unlike the sCOD, tCOD was performed on the whole sample whether previously diluted or not. The same protocol was applied to the tCOD, except that the tubes

were heated for 4 hours, at 150°C, in a Hach reactor and the absorbances of the tubes were read at 620 nM, using a Hach DRB 200 spectrophotometer (Hach Company, Loveland, USA).

Table V: *COD Reagents*

Standard solution	637 mg potassium hydrogen phtalate. Add distilled water up to 500 ml
Digestion solution	8.5 g $HgSO_4$,24.5 g $K_2Cr_2O_7$, 250 ml distilled water, 85 ml H_2SO_4 complete to 500ml with distilled water
Acid solution	26.52 g Ag_2SO_4, 8.6 g $HgSO_4$ add H_2SO_4 to 1000ml

I-3. Solids

Solids refer to matter suspended or dissolved in water or wastewater. In a sample, based on particle size and characterization, solids were categorized into the following groups: Total Suspended Solids (TSS or SS), Total Dissolved Solids (TDS), Fixed Solids, and Total Solids (TS). Total solids were the total of all solids in a water sample. They included the total suspended solids and total dissolved solids. The total suspended solids were the amount of total solids retained by a filter and then dried at 105°C and the total dissolved solids were the portion that passes through the filter. The fixed solids were the residue of total, suspended or dissolved solids after ignition, and the solids lost on ignition (heating to 600°C) were the volatile solids. Volatile suspended solids (VSS) were the solids obtained by incineration of the dried total suspended solids (SS) at 600°C. The value of the VSS corresponded to the amount of organic matter in the sample. Total volatile solids (TVS) were the solids obtained by incineration of the TS at 600°C after drying it at 105°C (Fisher Scientific, Isotemp muffle Furnace 550 series, Pittsburgh, PA).

$$\text{TS or SS (g/kg)} = \frac{\text{dry weight (crucible+sample)} - \text{crucible weight}}{\text{Sample wet weight}} \times 1000$$

$$\text{TVS or VSS (g/kg)} = \frac{\text{dry weight} - \text{ash weight}}{\text{Sample wet weight}}$$

1-4. Volatile Fatty Acids (VFA).

The analysis of VFA included the measurement of acetic, propionic and butyric acids. The sample was centrifuged and a dilution was made, when needed, in order to have a final maximum concentration of 1000 mg/*l* of each volatile fatty acid. A specific amount of internal standard was added. The quantity of VFA was measured by gas chromatograph (Agilent 6890, Wilmington, DE) equipped with a FID. 0.2 ml of sample diluted 1:1 (V/V) with internal standard in 6% formic acid was directly injected on a glass column of 1m x 2mm Carbopack C (60-80 mesh) coated with 0.3% Carbowax 20 M and 0.1% H_3PO_4. The column was held at 120°C for 4 minutes. Helium at 20 ml/min was used as the carrier gas. The injector and the detector were both maintained at 200°C. Quantification was made, with iso-butyric acid as an internal standard.

Table VI: ***Fatty Acid Gas Chromatography Vial Preparation***

Dilution	Internal Standard (µl)	H_20 (µl)	Sample (µl)
none	350	-	350
1/2	350	175	175
1/5	350	280	70
1/10	350	315	35

I-5. Anions and Cations

The principal compounds studied are NH_4, K, Na, CL, NO_2, NO_3, PO_4 and SO_4. The samples were first centrifuged and the analyses were done on the supernatant. The total volume in the vial had to be 1 ml. For the determination of the anions (Cl^-, $NO2^-$, $NO3^-$, $HPO4^{-2}$, $SO4^{-2}$), the samples were injected on a Hamilton PRP-X200 column of 250 mm x 41mm while for the determination of the cations (Na^+, $NH4^+$, K^+) the samples were injected on a Hamilton PRP-X200 cation resin-based chromatography column (250 x 41mm O.D.). All the ions were measured on a High Performance Liquid Chromatograph (HPLC) from Thermo Separation Product AS3000- P4000 (Sunnyvale, CA, USA). Conductivity data were obtained by using a Waters Millipore detector model 432 (Milford, MA, USA).

Parameters of the gas chromatography:

Anions: mobile phase: 4.0 mM p-Hydroxybenzoic acid (pH 8.5 with 2.5 % methanol), injection: 100 µl, flow rate: 1.8 ml/min, temperature: 40 ^{o}C.

Cations: mobile phase: 4 mM nitric acid with 30% methanol, injection: 20 µl, flow rate: 1.8 ml/min, temperature: 40 ^{o}C. (Environmental Analytical Chemistry lab-BRI, Montreal, Quebec).

II. Biochemical Methane Potential (BMP)

The BMP test is a measure of substrate biodegradability determined by volumetrically monitoring biogas production and accumulative methane production from anaerobically incubated samples (Cornacchio et al., 1986; Owen et al., 1979 and Shelton and Tiedje, 1984). It gives important information about the potential of a given biomass to degrade certain substrates to methane.

II-1. Inoculum

The tests were conducted using a microbial inoculum in the form of wet granules (Figure 3), from an upflow anaerobic sludge blanket **(**UASB**)** reactor, provided by a food industry (A. Lassonde Inc, Rougemont, QC, Canada). The inoculum was pre-incubated in a solution of phosphate buffer for 2 days at 35°C. This step was necessary in order to eliminate the residual biodegradable organic material present in it.

Figure 3: *Inoculum in the Form of Granules of Bacteria*

II-2. Substrates

The macroalgae used in this study: *Ascophyllum nodosum* and *Fucus edentatus* were provided by Pro- Algue Marine inc. of St-Simon-de-Rimouski, Quebec, Canada.

We also received different strains of microalgae from the National Research Council Institute for Marine Biosciences (NRC-IMB) in Halifax, Nova Scotia, Canada. The samples were classified in two categories: freshwater microalgae (*Scenedesmus dimorphus, Scenedesmus sp. AMDD, Scenedesmus sp. PN2, Neochloris oleoabundans, Chlorella vulgaris, Chlorella sorokiniana, Chlorella sp. Island R., Chlamydomonas debaryana ambï, Chlamydomonas sp. AMLS1B, Microactinium sp. Rb1b*) and marine microalgae (*Phorphyridium aeruginosa, Phaeodactylum tricornutum, Thalassiosira weissflogii, Nannochloropsis gaditana*).

II-3. Reagents

II-3-1. Medium 5X

A defined medium (nutrients, micronutrients and vitamins) was added for nutrient supplementation and for optimal function of anaerobic microorganisms. The medium was prepared from the stock solutions in Table VII. The following stock solutions were mixed together: distilled water 900 ml, mineral I 10 ml, mineral II 1 ml, vitamins B 1 ml, phosphates 10 ml, resazurin 15 ml, 2-methyl-n-butyric acid 1 ml. The mixture was gased with N_2/CO_2 so as to maintain a neutral pH, boiled for 5 minutes and cooled at 35°C; then 3.4 g of $NaHCO_3$ were added. The vial was filled with distilled water to compensate for evaporation.

Table VII: ***Anaerobic Medium***

Solutions	**Component (concentration g/*l*)**
Mineral I	NaCl (50), $CaCl_2.2H_2O$ (10), NH_4Cl (189.4), $MgCl_2.6H_2O$ (10)
Mineral II	$(NH_4)_6Mo_7O_{24}.4H_2O$ (10), $ZnSO_4.7H_2O$ (0.1), H_3BO_3 (0.3), $FeCl_2.4H_2O$ (1.5), $CoCl_2.6H_2O$ (10), $MnCl_2.4H_2O$ (0.03), $NiCl_2.6H_2O$ (0.03), $AlK(SO_4)_2.12H_2O$ (0.1)
Vitamins B	nicotinic acid (0.1), cyanocobalamin (0.1), thiamin (0.05), p-aminobenzoic acid (0.05), pyridoxin (0.25), pantothenic acid (0.025)
Phosphates	KH_2PO_4 (50)
Resazurin	(0.1)
2-methyl-n-butyric acid	(102)

II-3-2. Sulfide Solution

The sulfide solution was used as a media reducing agent. 25 g $Na_2S.9H_2O$/*l* distilled water was prepared in small quantities with freshly boiled distilled water.

II-3-3. Dilution Water

Distilled water was deoxygenated by boiling and flushing under N_2/CO_2 during 20 minutes.

II-3-4. Bicarbonate Solution

The bicarbonate solution acted as a buffering solution. 42g of $NaHCO_3$ and 100g of $KHCO_3$ were dissolved in 1 liter of distilled water and flushed with N_2/CO_2.

II-3-5. Controls

The control tests gave an idea of the inoculum response toward the substrate and were done in triplicate for statistical significance. The methane production from the inoculum determined in the control assays was subtracted from the methane production obtained in the substrate assays. The composition of the controls was similar to the BMPs with the exception that the substrate was replaced by an equivalent volume of deoxygenated water.

II-3-6. Bottle Preparation

The tests were performed in triplicate for statistical analysis and to guarantee the reproducibility of the assays. The experiments were performed in serum bottles of 500, 160, 120 or 60 ml capacity, depending on the quantity of substrate available. N_2/CO_2 (80/20% as volume) was flushed continuously into the headspace of the bottles, before and during the transfer of the substrate and the inoculum, in order to maintain an anaerobic environment. The transfer of the inoculum was done by first draining the liquid from the granules. The basic media were described in Table VII and the gas mixture kept the pH at neutrality at the beginning of the assay. Finally, dilution water was added to bring the final volume in the bottles to 100 ml. In our experiment, we used an inoculum to substrate ratio (ISR) of 2:1. The calculation of the amount of substrate used in the BMP took into account the fact that there were 100g of TVS of inoculum in 1 kg of fresh (wet) inoculum. Had we used 20 g of inoculum for 500 ml bottle (the quantity of inoculum can change depending on the experiment), we would then have 2 g of TVS of inoculum. For an ISR of 2: 1, we needed to have 1 g of TVS of substrate. By using the same ratio X g of TVS of substrate in 1 kg of substrate (X g of TVS is the initial TVS value of the substrate), the quantity of substrate needed will then be 1000/ Xg of TVS.

1- g TVS of inoculum = g of inoculum x100/ 1000
2- g of substrate for 2:1 ISR = TVS of substrate x1000/TVS (g/kg) of substrate
3- g TVS of substrate = TVS (g/kg) of substrate x substrate (g)/1000

Table VIII shows an example of a preparation table for the macroalgae. After the transfer of the inoculum, defined media, sulfide solution and bicarbonate solution, the bottles were closed with

a butyl rubber stopper and an aluminum cap (Figure 4) and then weighted. The test bottles were incubated in a rotary shaker (New Brunswick Scientific Co., Edison, NJ, USA) at 35^0C at 100 rpm (Figure 5).

The weight of the bottle and cap was necessary for the calculation of the headspace (Table IX) which is the gas space above the sample, in the vial. The headspace enabled us to know the quantity of methane produced and the limit amount of gas that the bottle may contain, thus allowing us to predict the sampling dates.

Table VIII: ***Example of BMP Preparation: Macroalgae Ascophyllum nodosum and Fucus edentatus***

ID	#	Substrate	Inoculum	Medium 5X	Buffer	Na_2S solution	Dilution water	Total
BMP test		g	g	ml	ml	ml	ml	
Control	1	0	20	3	4.0	0.5	73	100
	2	0	20	3	4.0	0.5	73	100
	3	0	20	3	4.0	0.5	73	100
Ascophyllum nodosum	4	7	20	3	4.0	0.5	66	100
	5	7	20	3	4.0	0.5	66	100
	6	7	20	3	4.0	0.5	66	100
Fucus edentatus	7	11	20	3	4.0	0.5	62	100
	8	11	20	3	4.0	0.5	62	100
	9	11	20	3	4.0	0.5	62	100

Table IX: ***Calculation of the Headspace (Example)***

ID	#	Bottle (g)	Bottle + water (g)	Bottle + sample + cap (g)	Bottle + sample (g)	Headspace (g)
control	1	101.51	261.44	203.75	201.27	60.17
	2	100.70	262.46	203.40	200.92	61.54
	3	99.47	260.49	201.95	199.47	61.02

Bottle + sample = (bottle + sample + cap) – (cap average)
Headspace = (bottle + water) - (bottle + sample)

Figure 4: ***Vessel for Anaerobic Digestion Test (extracted from Angelidaki et al., 2009)***

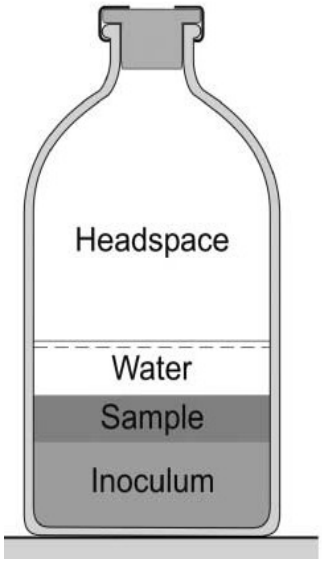


Figure 5: ***Rotary Shaker***

Figure 6: ***Burette for Biogas Measure***

Figure 7: ***Gas Chromatograph***

II-3-7. Biogas Monitoring

The production of biogas (hydrogen, nitrogen, oxygen, methane and carbon dioxide) was estimated by connecting a burette filled with water to a test bottle by using a syringe (Figure 6). The biogas produced passed from the test bottle to the burette and caused a drop in the water level. The volume of biogas produced corresponded to the volume of water displaced. A 300 µl gas sample was taken from the headspace of the test bottle using a micro-syringe (Hamilton Gastight no. 1750, Hamilton, NE, USA) at specific time intervals and injected in properly calibrated Gas Chromatograph (HP 6890 series, Hewlett Packard, Wilmington, DE) (Figure 7). The Gas Chromatograph (GC) was coupled with a thermal conductivity detector **(**TCD**)** and with a 3.5 m x 2 mm I.D mesh Chromosorb 102 column (Supelco, Bellefonte, Pennsylvania, USA). The column temperature was held at 50°C for 4 minutes. Argon was used as a carrier gas. The injector and detector were maintained at 125^{O}C and 150^{O}C respectively. Initial gas volume measurements were made after 24 hours (day 1). Subsequent measurements were made on day 2 and 3 and then approximately once a week, if needed, until the end of experiments, when methane production ceased, which lasted between 5 and 7 weeks depending on the microbial activity. Measurements of gas were made by transferring the area of each gas from the GC in the method 8 (Table X) which allowed a separation of H_2, N_2+O_2, CH_4 and CO_2. The volume of methane produced was obtained by first multiplying the headspace volume by the percentage of CH_4 in the headspace from the current sample compared with the previous value and the volume of gas measured in the burette multiplied by the current methane percentage. The values were calculated in standard temperature and pressure (STP) conditions: 0° C and 1 atm. All data were statistically analyzed using Excel.

Table X: ***In-House Template for Gas Calculation by Method 8***

METHOD 8		Gas Temperature (°C) :		35
		Sampling date		
		Sampling name		
Gas	Retention Time (min)	Area	Volume (uL)	Gas Fraction (%)
H2	1.358-1.476	**0**	0	**0.0**
N2	1.510-1.546	**4756**	121	**69.4**
CH4	1.905-2.091	**1283**	90	**3.5**
CO2	3.144-3.431	**2385**	75	**21.5**
H2O			17	**5.6**
		total volume :	**303**	**100**

The percentage of methane was transferred in an Excel table (Table XI) so as to calculate the volume of methane produced by the bottle.

Table XI: ***Calculation of the Methane Volume (Example)***

	Date Time	Cumulative (cumul)days	Headspace (ml)	Vol. biogas (ml)	% CH_4	CH_4 vol. (ml)	CH_4 cumul vol (ml)	CH_4 cumul-ctrl (ml)	CH_4 cumul -ctrl (ml STP/ g TVS in)	CH_4 cumul -ctrl (ml STP/ g substrate in)
t=0	7/4/2011 11:20	0.0	60.17	0	0.0	0.0	0.0	0.0	0.0	0.0
1st sampling	7/5/2011 8:43	0.9	60.17	43.8	17.4	18.1	18.1	15.4	27.3	21.3
2nd sampling	7/6/2011 12:52	2.1	60.17	36.8	33.5	22.0	40.1	34.9	61.9	48.4

III. Pretreatment

Pretreatments are methods implemented to improve the anaerobic digestion. The objectives were 1) to hydrolyse more organic matter and increase the production of biogas, 2) to increase the kinetic reaction and reduce the volumes of the reactors. In our study, we used two types of pretreatments: thermal and chemical.

III-1. Thermal Pretreatment

The purpose of the thermal pretreatment was to break the organic matter, thus allowing a better thermal hydrolysis and solubilization. Samples, consisting of 4.6 g of *Scenedesmus dimorphus* and 6.1 g of *Neochloris oleoabundans*, were vortexed separately in vials with 40 ml of water. The mixture was then transferred to a Teflon tube and irradiated in a microwave. The microwave used was a closed-vessel accelerated reaction system (MARS-5, CEM Corporation, Mattews, NC, USA) which run at 2450MHz with a power range between 400 and 1600 w, equipped with a turning carousel; holding a maximum of 12 vessels (XP-1500) of 100 ml each, with pressure and temperature probes. With *Neochloris oleoabundans*, the experiment started with an initial temperature of 23°C at T= 0 and reached the target temperature of 150 °C at 11.5 min, with an increase of the pressure at the 4.5^{th} min, reaching 53 PSI at the 11.5 min. For *Scenedesmus dimorphus*, the initial temperature was 35 °C and at 10.5 min, we reached the target temperature of 149°C. The pressure started to rise at 3.5 min and reached 55 PSI at 10.5 min.

III-2. Chemical Pretreatment

Chemical pretreatment is also a means of improving hydrolysis. In our case, we used sodium hydroxide as an alkaline reagent. 4.6g of *Scenedesmus dimorphus* and 6.1 g of *Neochloris oleoabundans* were separately put in two bottles. In each bottle, 40 ml of water and 0.1 g of sodium hydroxide were added and kept in a fume hood for 3 hours. The pH was adjusted to 7 by adding approximately 1 ml of phosphoric acid (H_3P0_4) prior to methane potential evaluation.

IV. Lipid Profile Activities

The study of lipid profile allows us to identify the limits of biodegradation of algal lipids in the anaerobic digestion by: 1) determining the anaerobic digestibility of microalgae rich in lipid and the degradation rates of various long chain fatty acids (LCFA) contained in the selected algae and 2) comparing with the kinetic study of individual pure LCFA found in the algae to see if the synergy effect plays a role in the inhibition process. This study is done in three steps.

IV-1. Determination of the Composition of LCFA in Different Microalgae

Different strains of algae were submitted to the BMP for a determination of their lipid profile. They were *Phaeodactylum tricornutum* and *Thalassiosira weissflogii* for the marine microalgae category and *Chlorella vulgaris* for the freshwater microalgae category. The preparation of the bottles was done as in Table VIII. Bottles of 500 ml capacity were used for a total liquid volume of 100ml. But in addition to the triplicate for the other BMP, 3 or 4 additional bottles were prepared and incubated for the analysis of the lipid profile. The same ratio ISR 2:1 was used; 2g TVS inoculum for 1 gTVS substrate or 20 g of inoculum for 4.9 g of *Phaeodactylum tricornutum*, 7.5 g of *Thalassiosira weissflogii* and 4.8 g of *Chlorella vulgaris*. We then added 3 ml of medium 5X, 4 ml of bicarbonate solution, 0.5 ml of sulfide solution and completed with dilution water at 100 ml. The bottles were incubated at 35°C at 100 rpm. At different time intervals, usually once a week, depending on the shape of the methane production curve (presence or absence of inhibition, a bottle was taken and a sample of 20 ml was collected and preserved at -20°C for the quantification of the LCFA. The rest was used for the different analyses (TVS, COD, VFA etc.). The identification and quantification of the LCFA was done by an external company called Exova, located in Portland, Oregon, USA.

IV-2. Choice of Algae and Pure LCFA

The choice of *Phaeodactylum tricornutum* and pure eicosapentaenoic acid (C 20:5) (EPA) were based on our lipid profile results (see results).

IV-3. BMP of Microalgae *Phaeodactyllum tricornutum* Rich in Lipid

In this last step, a series of defatted samples of a marine microalga, *Phaeodactylum tricornutum*, were enriched artificially in lipids at different concentrations of long chain fatty acids (10-25 and 50%). The enriched samples were then used as models for the study of the kinetic degradation of those fatty acids. The fatty acids chosen were palmitic acid (C16:0), palmitoleic acid (C16:1) and ecosapentaenoic acid (C 20:5).

Table XII shows the preparation of *Phaeodactylum tricornutum* enriched in lipids at differents concentrations. As in the other BMP experiments, we used an ISR 2:1 with 1 g TVS of inoculum for 500mg TVS of algae. 10 % of total fat corresponded to 50 mg TVS lipids and 450 mg TVS *Phaeodactylum tricornutum*; 25% corresponded to 125 mg TVS lipids and 375 mg TVS *Phaeodactylum tricornutum*; and 50% corresponded to 250 mg TVS lipids and 250 mg TVS *Phaeodactylum tricornutum*. In our sample of enriched *Phaeodactylum tricornutum*, the percentage of EPA is 20% of total lipids, 40% of total lipids for C16:0 and 40% of total lipids for C16:1, 40%

V. Activity Tests

Before submitting the enriched samples of *Phaeodactylum tricornutum* to the BMP, two controls tests were performed:

V-1. Hydrolysis Test on Oils

Hydrolysis tests were done on palm, macadamia and fish oils. Palm oil, rich in palmitic acid (35-48%), was purchased from Sigma-Aldrich; macadamia oil (18 -28% of palmitoleic acid) and fish oil (in the form of omega 3 capsules containing the eicosapentaenoic acid) were purchased at a natural food store: Aliments naturels Tau, Brossard, Quebec. The tests were performed in 160 ml bottle with a total volume of 100 ml in triplicates. For the amount of inoculum and substrate incorporated in each bottle, we used the same ISR of 2:1, i.e 1g TVS inoculum for 0.5 g TVS of oil. We would thus have 10 g of inoculum and 0.5g of oil, but because lipids are easily inhibitory above certain concentrations, we reduced to 0.2 g the quantity of each oil used. The palm oil was in solid form, so we weighed 0.2g. For the macadamia and fish oils which are in

liquid form, the quantity put in the bottles were 0.23 ml and 0.22 ml respectively, as determined through their density. We then added 2 ml of medium 5X, 2 ml of bicarbonate solution, 0.5 ml of sulfide solution and completed with dilution water at 100 ml. The bottles were incubated at 35°C at 100 rpm.

V-2. Activity Test on LCFA

The activity tests were performed individually on each LCFA. In this test, we used bottles of 60 ml capacity with a total liquid volume of 40 ml. For C16:0 and C16:1, we used an ISR of 2:1, 200 mg TVS of inoculum for 100 mg TVS of acids or 2 g of inoculum for 0.10g of acids (0.11 ml for C 16:1 which was in liquid form). The tests were done in triplicate. Because we were limited in the amount of EPA, we only did a duplicate, with a ratio ISR of 4:1, thus 200 mg TVS of inoculum for 50 mg TVS of EPA or 2 g of inoculum for 0.05g of EPA. We then added 2 ml of medium 5X, 2ml of bicarbonate solution, 0.5 ml of sulfide solution and completed with dilution water at 100 ml. The bottles were incubated at 35°C at 100 rpm.

Table XII: ***Preparation of Lipid - Enriched Phaeodactyllum tricornutum at 10, 25 and 50% of Total Lipids***

ID	#	*Phaeodactylum tricornutum*	C16:0	C16:1	C20:5	Inoculum	Medium 5X	Buffer	Na_2S	Dilution water
Assay BMP		g	g	ml	ml	g	ml	ml	ml	ml
Control	C1	0	0	0	0	10	2	2	0.5	85.50
	C2	0	0	0	0	10	2	2	0.5	85.50
	C3	0	0	0	0	10	2	2	0.5	85.50
Phaeodatylum tricornutum defatted with 10% total lipids	4	0.584	0.02	0.022	0.01060	10	2	2	0.5	84.85
	5	0.584	0.02	0.022	0.01060	10	2	2	0.5	84.85
	6	0.584	0.02	0.022	0.01060	10	2	2	0.5	84.85
		(584mg)	(20 mg)	(22 ul)	(11 ul)					
Phaeodatylum tricornutum defatted with 25%total lipids	7	0.486	0.05	0.0560	0.02651	10	2	2	0.5	84.84
	8	0.486	0.05	0.0560	0.02651	10	2	2	0.5	84.84
	9	0.486	0.05	0.0560	0.02651	10	2	2	0.5	84.84
		(586mg)	(50 mg)	(56 ul)	(27 ul)					
Phaeodatylum tricornutum defatted with 50% total lipids	10	0.324	0.10	0.112	0.05302	10	2	2	0.5	84.84
	10	0.324	0.10	0.112	0.05302	10	2	2	0.5	84.84
	11	0.324	0.10	0.112	0.05302	10	2	2	0.5	84.84
		(324mg)	(100 mg)	(112 ul)	(53 ul)					

Chapter Three: Results and Discussion

I. Objective 1: Macroalgae

I-1. Characterization of the Samples

A series of analyses: TVS, TS, COD, VFA, pH, anions, cations, total carbon, total Kjeldahl nitrogen, total phosphorus and total fats and oils were performed for the characterization of the two strains of macroalgae: *Ascophyllum nodosum* and *Fucus edentates*. The results of the characterization are presented in Tables XIII, XIV, XV.

A. nodosum has 20% of dry matter and an important organic fraction of 73% and *F. edentatus* has 13% of dry matter with an important organic fraction of 69%. The C/N ratio is the relationship between the mass of carbon and the mass of nitrogen present in organic materials. It is the balance of food a microbe requires for an optimal growth. This ratio was respectively 20 and 18 for *A. nodosum* and *F. edentatus,* which is quite balanced. Anaerobic digestion requires a C: N ratio between 10 and 30. According to Verma (2002) and Parkin and Owen (1986), this ratio should be between 20 and 30. A lower C/N ratio could result in high ammonia release, which would decrease methanogenic activity, resulting in high VFA accumulation and eventually leading to a failure of the anaerobic digestion. Another key consideration was the concentration of phosphorus although the consequences would not have been so important in case of excess. On the other side, a lack of it would limit the anabolic pathways and prevent the digestion process. The optimum carbon to phosphorus ratio is about 150 to 1 (Lucks, 2000) or less than 187 (Burke, 2001). The C: P ratios were, respectively, 158 and 147 for *A. nodosum* and *F. edentatus,* which was quite balanced. The ratio of total COD (tCOD) to TVS was 1.5 for *A. nodosum* and 1.42 for *F. edentatus*, close to a typical biomass ratio of 1.42 (Takacs and Vanrolleghen, 2006), indicating that those samples were either poor in proteins or lipids; this was confirmed by the low concentration of total fats and oils which was 11.2 g/kg dry or 1.12 % for *A. nodosum* and inferior to 5 g/kg dry (0.5 %) for *F. edentatus*. Those were very low percentages if we take into account that certain algae have an oil content of 50 % or more. The soluble fraction of the algae was important with a value of soluble COD of 49 g/*l* for *A. nodosum* and 35 g/*l* for *F. edentatus.*

Table XIII : *Characterization of Ascophyllum nodosum and Fucus edentatus*

Parameters	***Ascophyllum nodosum***	***Fucus edentatus***
TS (g/kg)	199 ± 8	133 ± 5
TVS (g/kg)	145 ± 5	91 ± 4
tCOD (g/kg)	214 ± 34	128 ± 23
pH	6.48	6.39
sCOD (mg/*l*)	48712 ± 1937	35019 ± 8383
Acetate (mg/*l*)	533 ± 239	304 ± 89
Propionate (mg/*l*)	174 ± 180	0
Butyrate (mg/*l*)	0	0
NH_4 (mg/*l*)	0	0
Na (mg/*l*)	4575 ± 316	4978 ± 1547
K (mg/*l*)	1661 ± 150	2057 ± 761
SO_4 (mg/*l*)	854 ± 84	969 ± 353
NO_3 (mg/*l*)	128 ± 8	311
Total carbon (% dry mass)	29.4	26.9
Total Kjeldahl nitrogen (mg/kg dry)	14500	14600
Total phosphorus (mg/kg dry)	1860	1830
Total oils and fats (mg/kg dry)	11200	< 5000

Table XIV : *t_0 Analysis for the BMP Assay for Ascophyllum nodosum and Fucus edentatus*

Parameters	***Ascophyllum nodosum***	***Fucus edentatus***
TVS (g/kg)	31	31
tCOD (g/kg)	263 ± 34	242 ± 15
sCOD (mg/*l*)	1674 ± 74	2065 ± 61
Acetate (mg/*l*)	82	148
Propionate (mg/*l*)	24	32
Butyrate (mg/*l*)	0	0
NH_4 (mg/*l*)	45	40
Na (mg/*l*)	1163	1291
K (mg/*l*)	1785	1885
SO_4 (mg/*l*)	57	107
NO_3 (mg/*l*)	n.a	n.a

n.a= below the detection limit

Table XV: ***Final Values of Methane and Different Parameters for the BMP Assay for Ascophyllum nodosum and Fucus edentatus***

Parameters	***Ascophyllum nodosum***	***Fucus edentatus***
TS (g/kg)	27 ± 4	34 ± 3
TVS (g/kg)	18 ± 4	23 ± 3
tCOD (g/kg)	36 ± 5	32 ± 1
pH	7.31	7.23
sCOD (mg/*l*)	1434 ± 183	1271 ± 47
Acetate (mg/*l*)	14 ± 3	29 ± 3
Propionate (mg/*l*)	0	0
Butyrate (mg/*l*)	0	0
NH_4(mg/*l*)	166 ± 7	188 ± 12
Na (mg/*l*)	1325 ± 35	1499 ± 109
K (mg/*l*)	1929 ± 88	2175 ± 140
SO_4 (mg/*l*)	41 ± 1	42 ± 1
NO_3 (mg/*l*)	3	4 ± 2
methane(ml/gTVS)	44	70

VFA are the most important intermediates in the anaerobic digestion and the compounds most easily degraded to methane; their concentrations were low for *A. nodosum:* 533 mg/*l* for acetate, 174 mg/*l* for propionate and 0 mg/*l* for butyrate and not present in *F. edentatus*. For the characterization of *A. nodosum* and *F. edentates*, the values of ammonium were 0 mg/*l*, 45 mg/*l* and 40 mg/*l* at t_o and 166 ±7 mg/*l* and 188 ± 12 mg/*l* for the final values, respectively (Table XIII, XXIV, XXV); these values were below the minimum inhibitory concentration of ammonium. The anaerobic digestion system can be inhibited by high concentrations of most chemical compounds, like heavy metals, cations (NH_4^+ , Na^+), sulfide, which are needed at lower concentration in order to have a beneficial effect. Ammonium and volatile fatty acid toxicity particularly have important consequences once they exceed a certain level. During anaerobic digestion, the protein content of the substrate gives a high content of nitrogen during the hydrolysis and the organic nitrogen is reduced to ammonium and ammonia. During anaerobic treatment, ammonium may be present in two forms: ammonium ions NH_4^+ or dissolved ammonia gas (NH_3). These two forms are in equilibrium with each other and their concentrations depend on the pH or hydrogen ion concentration, as indicated by the following equilibrium equation: $NH4^+ \leftrightarrow NH_3 + H^+$, which has an equilibrium constant (K_a of $10^{-9.23}$ for ammonium ion). This K_a or dissociation constant value is used to determine how much of the NH_4^+ is dissociated into its conjugate base NH_3. The pK_a value is defined from K_a, and can be calculated from the K_a value from the equation $pK_a = -\log_{10} (K_a)$ .The pK_a of ions ammonium/ammonia is 9.23. The Henderson-Hasselbach equation: $pH = pK_a + \log [A-]/ [HA]$ is the relationship between pH, pKa and the ratio of the concentration of the salt and

ionized forms. When the pH of the solution is equal to the pK_a: $pH = pK_a + \log 1$ or $pH = pK_a$, the concentration of the conjugate base and the undissociated acid are equal, [A-] = [HA]. This corresponds to 50% ionization. The concentrations of ammonia and ammonium are equal when the concentration of hydrogen ion is equal to the K_a or the pH is equal to the pK_a.

The normal pH of the anaerobic digestion is between 7 and 7.2. When the concentration of hydrogen ions is high (pH of 7.2 or lower) the equilibrium is shifted to the left and it is the NH_4+ ions that are present. When the pH is above 7.2, the equilibrium is shifted to the right and there is an accumulation of NH_3, which is responsible for inhibition. The ammonia gas is inhibitory at a much lower concentration than the ammonium ions. There is no precise value at which ammonia becomes inhibitory to methanogenesis. The ammonia nitrogen analysis gives some information about the total of ammonium ion and ammonia gas. The inhibition of ammonia occurs at a concentration of ammonia nitrogen ranging from 1500 mg/*l* to 3000 mg/*l* with a pH between 7.4 and 7.6. This state is characterized with an increase in VFA concentration which tends to decrease the pH, temporarily relieving the inhibitory condition. The VFA concentration will then remain high unless the pH is decreased between 7 and 7.2 by adding, for example, some hydrochloric acid. When the ammonia nitrogen concentration is above 3000 mg/*l*, NH_4^+ ions are toxic independently of the pH (Samson, 1995 and Mc Carty, 1964). According to Mata-Alvarez (2003), inhibition occurs at total ammonium (NH_4^+, NH_3) concentration of 1200 mg/*l* and above. The inhibition effect is mostly due to free ammonia at a concentration superior to 150 mg/*l* (Samson 1995, McCarty and McKinney 1961, Braun et al. 1981). A concentration of nitrate (NO_3^-) above 50 mg/*l* can lead to a proliferation of denitrifying bacteria that will keep the level of reduction too high to permit the methanogenesis (Samson, 1995). The reported concentration of NO_3 for both algae did not influence negatively the process of methanisation: the values at the beginning of the experiment at t_o were too low to be measured and by the end of the experiment, those values were respectively 3 and 4±2 mg/*l* for *A. nodosum* and *F. edentatus* (Tables XIV, XV). Cations like sodium and potassium can also inhibit the methanogenesis. Sodium and potassium values (at t_o) were 1163 mg/*l* and 1795 mg/*l* for *A. nodosum* and 1291 mg/*l* and 1885 mg/*l* for *F. edentatus* (Table XIV). At the end of the experiment, those values were 1325 ± 35 mg/*l* and 1929 ± 88 mg/*l* for *A. nodosum* and 1499 ± 109 mg/*l* and 2175 ± 140 mg/*l* for *F. edentatus* (Table XV). Those values were within the normal range. Lucks (2000) and Samson (1995) found that sodium and potassium exhibited strong inhibition at concentration levels of 8000 mg/*l* and 12000 mg/*l*, respectively, whereas a concentration between 3500 mg/*l* and 5500 mg/*l* for sodium and 2500 mg/*l* and 4500 mg/*l* for potassium led to a light

inhibition. The initial concentrations of sulfate were 854 ± 84 mg/*l* and 969 ± 353 mg/*l* (Table XIII), 57 mg/*l* and 107 mg/*l* at t_o (Table XIV), 41 ±1 mg/*l* and 42 ±1 mg/*l* at the end of the experiment (Table XV) for *A.nodosum* and *F. edentatus*, respectively. Those values were in a normal range, if compared to other studies. Isa et al. (1986) showed that sulfate levels up to 5000 mg/*l* had no significant effect on methane production from synthetic media containing acetate alone or acetate along with ethanol digested in high-rate anaerobic reactors. Szendrey (1983) also reported that 6000 mg/*l* of sulfate did not inhibit the methane production. According to Kroiss and Wabnegg (1983), the toxic or inhibitory form of sulfur in the anaerobic digestion is not sulfate but rather, soluble sulfide which is the product of sulfate reduction by sulfate reducing bacteria. A level of 200 mg/*l* of free sulfide concentration has been reported to severely inhibit methanogenic activity. Considering that 99% of sulfate was eliminated as sulfide, the concentration of soluble sulfide would be 60 mg/*l* for *A.nodosum* and 200 mg/*l* for *F. edentatus*, which may have had a negative impact on the degradation of *F. edentatus.*

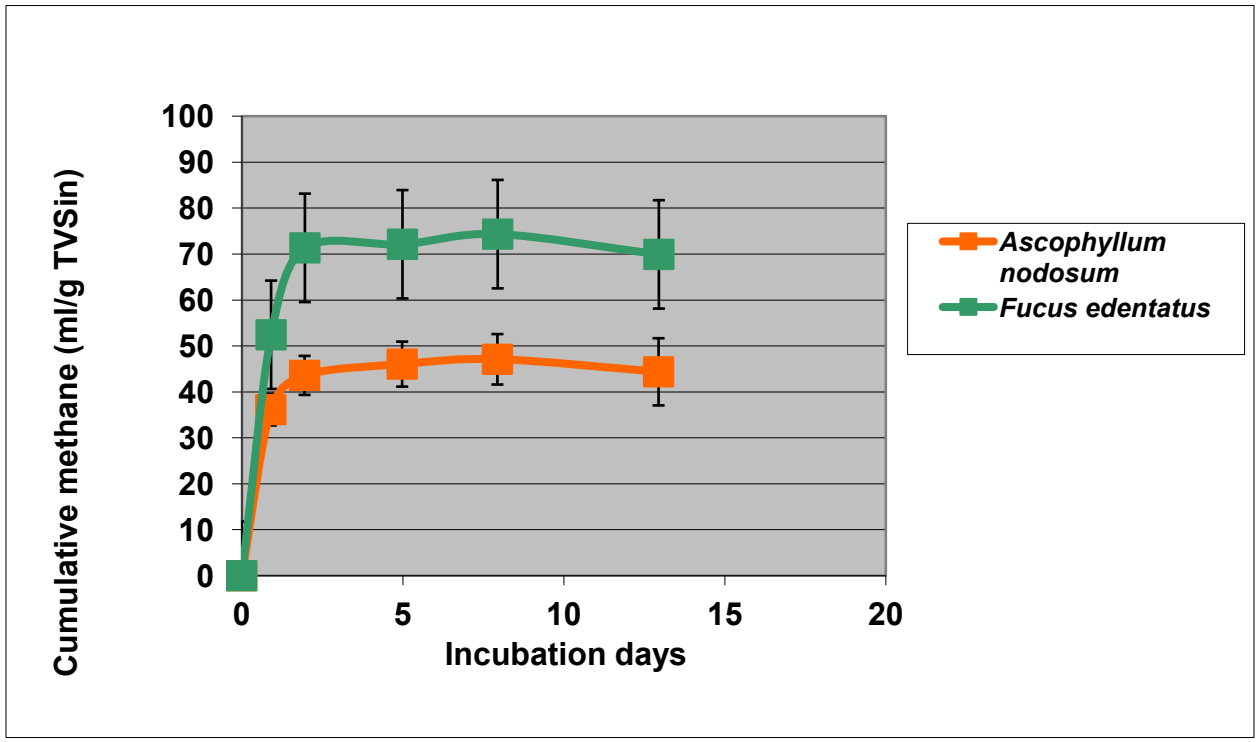
I-2. Methane Yield

Figure 8 shows the methane yield of *A. nodosum* and *F. edentatus* measured on days 1, 2, 5, 8 and 13. The maximum methane yield was reached after two days of incubation: 44 ml/gTVS and 70 ml/gTVS, respectively, for *A. nodosum* and *F. edentatus*. Hansen et al. (1987) found a superior value for *A. nodosum*: 110 ml/gVS. The difference may be due to the fact that these authors did their experiment in a semi-continuous culture setting with a loading rate of 1.75 gTVS l/d at 35°C and a retention time of 24 days.

It is possible that the small amount of degraded matter in the experiment was consumed quickly by the inoculum or that the production of methane was prevented by the inhibitory compounds presents in the algae or coming from their hydrolysis. The mineralization of the algae, which is the expected methane if 100% of the organic matter has been degraded, was very low for *A. nodosum* and *F. edentatus* (10 and 16% respectively). The values of methane were very low compared to the methane potential of the microalgae in our study (as much as 430 ml CH_4 /gTVS) and other studies (Samson and Leduy, 1986; Sanchez and Traviesco, 1993; Briand and Morand, 1997) which found values between 340 and 370 ml CH_4 /gTVS. There are several explanations for the low methane yield. First, the percentage of lipids was very low (0.5- 1.12%). According to Alves et al. (2009), lipids have a higher methane yield (0.99 *l* CH_4/g) than carbohydrates (0.42 *l* CH_4/g) and protein (0.63 *l* CH_4/g). Shay (1993), Huang et al. (2010) and Hossain et al. (2008) also found that

microalgae contain more oils than macroalgae. Another factor explaining the low methane yield is the presence of compounds associated with seaweed, such as salts, which can inhibit the fermentation process. A pretreatment involving washing is necessary to prevent inhibition (Roesijadi et al., 2010). A third factor is the fact that the macroalgae cell wall can be resistant to hydrolysis. In this case also, various pretreatments would be necessary for the breakdown of complex compounds of the algal biomass into biodegradable molecules and the release of inner cell components. Finally, the elevated concentration of free sulfide (200mg/*l*) in *F. edentatus* may inhibit methanogenic activity. Because of this, it was decided to focus on the anaerobic digestion of microalgae which seemed more promising.

Figure 8: ***Methane Potential of Ascophyllum nodosum and Fucus edentatus***



II. Objective 2: Screening of Different Strains of Microalgae

II-1 Characterization of Freshwater Algae

Tables XVI and XVII show the characterization of the 10 different species of freshwater microalgae: *Scenedesmus dimorphus, Neochloris oleoabundans, Chlorella vulgaris, Scenedesmus sp. PN2, Scenedesmus sp. AMDD, Chlorella sorokiniana, Chlorella sp. island R, Chlamydomonas debaryana ambi, Chlamydomonas sp. AMLS1b and Microactinium sp. Rb1.* The results of COD, VSS and the VFA showed that the degradation of the substrate has been efficient since the values

on the final test showed an important diminution compared to the t_o values. The pH which stayed around 7 proved that the process of the methanisation occurred in good condition and the VFAs have been well degraded. *S. dimorphus, N. oleoabundans, C. vulgaris, S. sp. PN2, S. sp. AMDD, C. Sorokiniana, Chlorella sp. island R and M. sp. Rb1b* had their content of TS superior to 20%: respectively 27%, 23%, 21%, 29%, 24%, 29%, 31% and 25% with an important volatile organic fraction of 90%, 84%, 93%, 80% , 87%, 87%, 93% and 87%, respectively, whereas *C. debaryana ambi* and *C. sp AMLS1b* had respectively, 15% and 16% with a volatile organic fraction of 91% and 88%. The tCOD to TVS ratio for *S. dimorphus*, *N. oleoabundans, C. sorokiniana, C. sp. island R* and *M. sp. Rb1b* were 1.92, 1.79, 1.91, 1.53 and 1.72, respectively, which was higher than 1.42, the ratio of a typical biomass. *C. debaryana ambi* and *C. sp AMLS1b* presented the highest ratio with 2.88 and 2.27, respectively, indicating that these samples may be rich in lipids.

The soluble fraction of the algae is important with a value of soluble COD between 25 and 116 g/*l*. The initial concentrations of VFA were between 211 and 3094 mg/*l* for acetate, 0 and 90 mg/*l* for propionate and 0 mg/*l* for butyrate, for all samples (Tables XVI, XVII). Those values fall in the normal range. According to Samson (1995), the concentration of acetic acid can go over 5000 mg/*l* and can even reach 10000 mg/*l*; and McCarty and McKinney (1961) found that a high concentration of acetic acid did not inhibit anaerobic digestion. In their studies, Hobson and Shaw (1976) also showed that concentrations of acetate and butyrate up to at least 10000 mg/*l* do not have an inhibitory effect on the bacteria *Methanobacterium formicium*, but above 1000 mg/*l*, propionate was inhibitory to *M. formicium*. The study of Andrews (1969) also suggested that propionic acid was inhibitory to the methanogenesis.

Table XVI: ***Characterization of Different Parameters of Scenedesmus dimorphus, Neochloris, oleoabundans, Chlorella vulgaris, Scenedesmus sp. PN2 and Scenedesmus sp. AMDD***

Parameters	***Scenedesmus dimorphus***	***Neochloris oleoabundans***	***Chlorella vulgaris***	***Scenedesmus sp. PN2***	***Scenedesmus sp. AMDD***
SS (g/kg)	239 ± 4	190 ± 15	n.a	n.a	n.a
VSS (g/kg)	218 ± 5	164 ± 12	n.a	n.a	n.a
TS (g/kg)	272 ± 6	225 ± 16	215 ± 5	292 ± 11	242 ± 2
TVS (g/kg)	246 ± 6	189 ± 14	200 ± 5	234 ± 2	210 ± 1
tCOD (g/kg)	472 ± 18	339 ± 14	n.a	n.a	n.a
pH	6.92	7.27	n.a	n.a	n.a
sCOD (mg/*l*)	78655 ± 3150	59113 ± 1465	115542	48677	54740
Acetate (mg/*l*)	3095	2195	1391	1162	2931
Propionate (mg/*l*)	15	90	0	90	251
Butyrate (mg/*l*)	10	0	0	0	
NH_4 (mg/*l*)		1100	81	427	n.a
Na (mg/*l*)	n.a	n.a	1181	375	n.a
K (mg/*l*)	n.a	n.a	5434	4967	n.a
SO_4 (mg/*l*)	n.a	n.a	137	n.a	n.a
NO_3 (mg/*l*)	n.a	n.a	209	55	n.a

n.a: not available or below the detection limit

Table XVII: ***Characterization of Different Parameters of Chlorella sorokiniana, Chlorella sp. Island R, Chlamydomonas debaryana ambi, Chlamydomonas sp. AMLS1b, Microactinium sp. Rb1***

Parameters	***Chlorella sorokiniana***	***Chlorella sp. island R***	***Chlamydomonas debaryana ambi***	***Chlamydomonas sp. AMLS1b***	***Microactinium sp. Rb1b***
TS (g/kg)	293	311	152	163	247
TVS (g/kg)	255	290	138	143	215
tCOD (g/kg)	486 ± 49	445 ± 28	398 ± 32	324 ± 14	369 ± 6
pH	6.74	5.71	6.09	5.74	6.21
sCOD (mg/*l*)	53253 ± 2701	5034 ± 614	25432 ± 614	31421 ± 2701	44528 ± 614
Acetate (mg/*l*)	440	253	211	215	244
Propionate (mg/*l*)	0	0	0	0	0
Butyrate (mg/*l*)	0	0	0	0	0
NH_4 (mg/*l*)	51	0	0	0	0
Na (mg/*l*)	752	555	886	746	887
K (mg/*l*)	3842	3386	988	842	3597
SO_4 (mg/*l*)	54	107	71	65	444
NO_3	414	57	584	406	191

Table XVIII: ***t_o and t_{final} Values of Methane and Different Parameters for the BMP Assay for Scenedesmus dimorphus, Neochloris oleoabundans, Chlorella vulgaris, Scenedesmus sp. PN2 and Scenedesmus sp. AMDD***

Parameters	***Scenedesmus dimorphus***		***Neochloris oleoabundans***		***Chlorella vulgaris***		***Scenedesmus sp. PN2***		***Scenedesmus sp. AMDD***	
	t_0	t_{final}	t_0	t_{final}	t_0	t_{final}	t_0	t_{final}	t_0	t_{final}
SS (g/kg)	33	27 ± 1	34	28 ± 2	n.a	22 ± 1	n.a	28 ± 0.98	n.a	24 ± 1
VSS (g/kg)	29	22 ± 1	29	22 ± 2	n.a	19 ± 1	n.a	24 ± 0.79	n.a	20 ± 1
TS (g/kg)	n.a	n.a	n.a	n.a	34	30 ± 2	36	31 ± 0.37	35	30 ± 1
TVS (g/kg)	n.a	n.a	n.a	n.a	31	22 ± 2	31	24	31	23 ± 1
tCOD (g/kg)	n.a	n.a	n.a	n.a	n.a	48 ± 1	n.a	47 ± 4	n.a	50 ± 4
pH	n.a	7.15	n.a	7.15	n.a	7.52	n.a	7.36	n.a	7.34
sCOD (mg/*l*)	3937	643 ± 73.76	3922	931 ± 171.21	5863	1245 ± 270	2313	641 ± 13	2835	518 ± 30
Acetate (mg/*l*)	142	3	134	5	67	6 ± 1	48	7 ± 1	135	5 ± 1
Propionate (mg/*l*)	1	0	5	0	n.a	0	4	0	12	0
Butyrate (mg/*l*)	n.a	0	n.a	0	n.a	0	n.a	0	n.a	0
NH_4 (mg/*l*)	148	761	180	826	117	1052 ± 3	131	820 ± 19	n.a	992 ± 59
Na (mg/*l*)	n.a	n.a	n.a	n.a	574	887 ± 19	532	850 ± 13	n.a	869 ± 21
K (mg/*l*)	n.a	n.a	n.a	n.a	1614	1931 ± 54	1557	1896 ± 37	n.a	1914 ± 66
Cl (mg/*l*)	n.a	n.a	n.a	n.a		415 ± 141	-	346 ± 25	n.a	310 ± 9
SO_4 (mg/*l*)	n.a	n.a	n.a	n.a	7	n.a	n.a	n.a	n.a	n.a
NO_3 (mg/L)	n.a	n.a	n.a	n.a	14	n.a	14	9 ± 15	2 ± 1	n.a
Methane (ml/gTVS)	430		340		361		258		306	
Theorical methane (ml/gTVS)	759		725		476		476		480	
Percentage of degradation	67		55		76		54		64	

x/y: x= values at t_0 and y = final values; n.a: not available or below the detection limit

The initial values of potassium in *C. vulgaris* and *S. sp. PN2* (respectively 5334 mg/*l* and 4967 mg/*l*) (Table XVI) were higher than the medium inhibition limit which is 4500 mg/*l*, but did not exceed the 12000 mg/*l* which indicates a strong inhibition. The concentration of NO_3 of *C. vulgaris* and *S. sp. PN2, C. sorokiniana, C. sp. island R, C. debaryana ambi, C. sp AMLS1b and M. sp. Rb1b* (respectively 209, 55, 414, 57, 584, 406, 191 mg/*l*) (Tables XVI, XVII) were also greater than the required 50 mg/*l*; however, those were the values of the initial analysis; the values at t_o

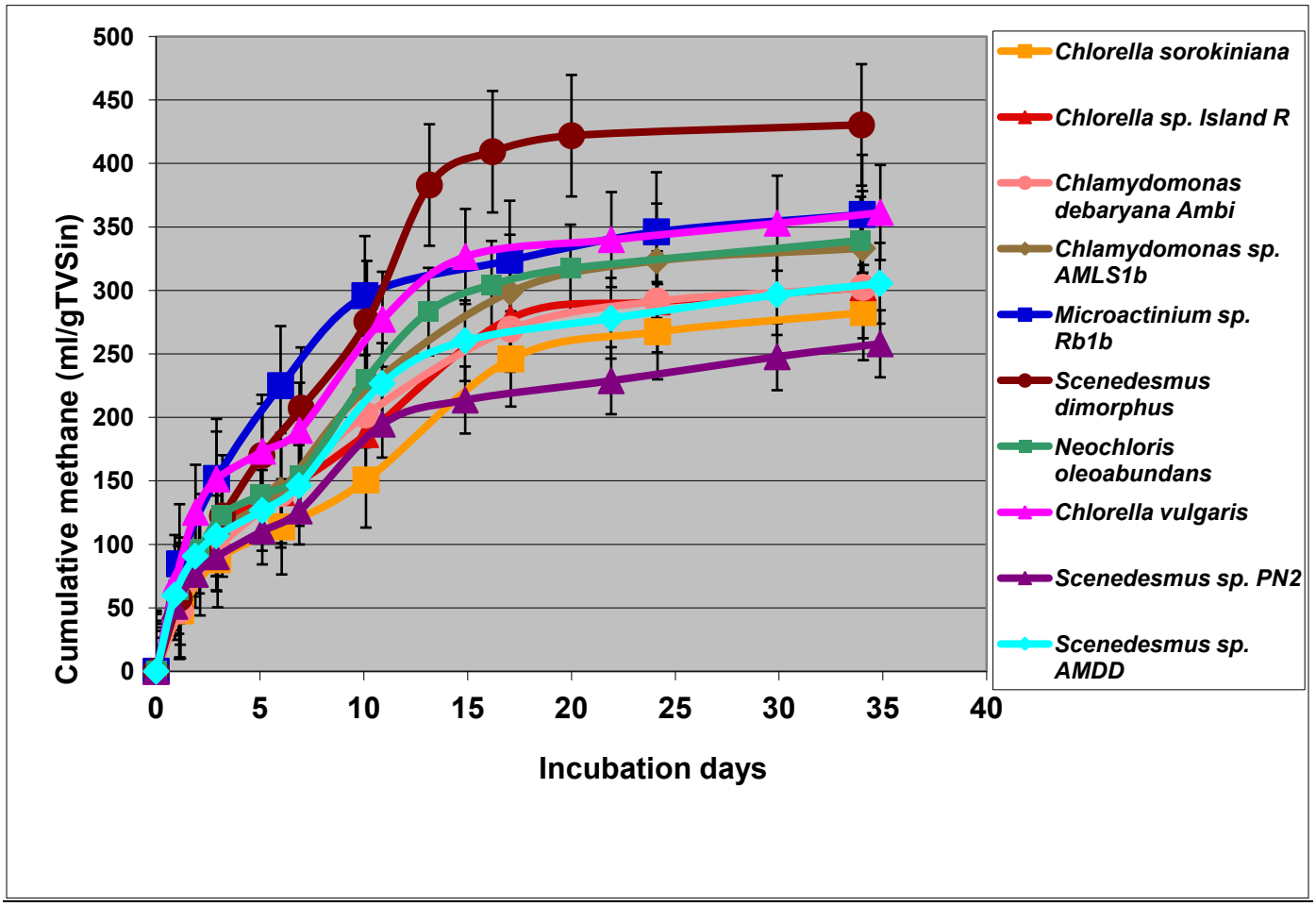
Table XIX: ***to and tfinal Values of Different Parameters for the BMP Assay for Chlorella sorokiniana, Chlorella sp. island R, Chlamydomonas debaryana Ambi, Chlamydomonas sp. AMLS1b, Microactinium sp. Rb***

Parameters	***Chlorella sorokiniana***		***Chorella sp. island R***		***Chlamydomonas debaryana ambi***		***Chlamydomonas sp. AMLS1b***		***Microactinium sp. Rb1b***	
	t_0	**t_{final}**	**t_0**	**t_{final}**	**t_0**	**t_{final}**	**t_0**	**t_{final}**	**t_0**	**t_{final}**
TS (g/kg)	38 ± 2	28 ± 2	35 ± 2	26 ± 1	31 ± 0	23 ± 1	29	23 ± 2	29 ±0	25 ± 2
TVS (g/kg)	31 ±1	21 ± 1	29 ± 2	20 ±1	25 ± 0	19 ± 1	23	17 ± 2	24 ± 0	21 ± 2
tCOD (g/kg)	108 ±5	57 ± 16	108 ±3	54±20	102 ± 4	64 ± 3	113 ± 16	61 ± 9	14 ± 9	73 ± 1
pH	7.12	7.28	7.29	7.44	7.26	7.33	7.35	7.31	7.6	7.31
sCOD (mg/*l*)	3029 ± 37	839 ± 43	3229 ± 417	686 ± 105	3159 ± 246	1839 ± 144	3906 ± 98	1971 ± 59	4088 ± 430	1044 ± 47
Acetate (mg/*l*)	26 ± 2	0	50 ± 2	0	24 ± 1	0	25 ± 1	0	68 ± 10	0
Propionate (mg/*l*)	41 ± 2	0	86 ± 6	0	13 ± 3	0	20 ± 1	0	67 ± 6	0
Butyrate (mg/*l*)	0	0	0	o	0	0	0	0	0	0
NH_4 (mg/*l*)	203 ± 3	788 ± 16	198 ± 1	863 ± 29	183 ± 4	943 ± 25	200 ± 1	1031 ± 53	222 ± 4	973 ± 42
Na (mg/*l*)	850 ± 12	778 ± 31	809 ± 8	808 ± 27	796 ± 9	861 ± 42	857 ± 2	809 ± 27	867 ± 12	797 ± 39
K(mg/*l*)	1464 ± 22	1455 ± 72	1315 ± 12	1488 ± 57	1247 ± 14	1444 ± 133	1357 ± 6	1390 ± 98	1594 ± 27	1425 ± 54
SO_4 (mg/*l*)	179 ± 1	n.a	183 ± 4	n.a	184 ± 2	n.a	188 ± 6	n.a	207 ± 7	n.a
NO_3(mg/*l*)	n..a	n.a	n..a	n.a	33 ± 1	n.a	26 ±1	n.a	14 ± 2	n.a
Methane (ml/gTVS)	283		302		302		333		360	
Theorical methane (ml/gTVS)	333		269		506		396		300	
Percentage of degradation	48		63		34		47		68	

n.a= below the detection limit

(Table XIX) which were more important because they represented the concentration in the bottle at the beginning of the experiment, were inferior to 50 mg/*l* for *C. sorokiniana, C. sp. island R, C. debaryana ambi, C. sp AMLS1b and M. sp. Rb1b, C. vulgaris* and *S. sp. PN2*

Figure 9: ***Methane Yield of Scenedesmus dimorphus, Neochloris oleoabundans, Chlorella vulgaris, Scenedesmus sp. PN2, Scenedesmus sp. AMDD, Chlorella sorokiniana, Chlorella sp. island R, Chlamydomonas debaryana ambi, Chlamydomonas sp. AMLS1b and Microactinium sp. Rb1***



The methane yield of these algae will be discussed in page 50.

II-2. Characterization of the Marine Algae

Tables XX and XXI show the characterization of the marine microalgae: *Nannochloropsis gaditana, Phorphyridium aeruginosa, Phaeodactylum tricornutum and Thalassiosira weissflogii*. The results of COD, VSS and the VFA showed that the degradation of the substrate has been efficient, since the values on the final test showed an important diminution of the initial values. The pH stayed around 7, proving that the process of the methanisation occurred in good condition and the VFAs have been well degraded.

Table XX: ***Characterization of Different Parameters for the BMP Assay for Phorphyridium aeruginosa, Phaeodactylum tricornutum, Thalassiosira weissflogii and Nannochloropsis gaditana***

Parameters	***Phorphyridium aeruginosa***	***Phaeodactylum tricornutum***	***Thalassiosira weissflogii***	***Nannochloropsis gaditana***
TS (g/kg)	201 ± 8	238 ± 1	168 ± 9	287 ± 8
TVS (g/kg)	184 ± 7	205 ± 1	133 ± 7	263 ± 9
tCOD (g/kg)	262 ± 15	439 ± 13	370 ± 15	493 ± 6
pH	n.a	6.47	6.3	6.95
sCOD (mg/*l*)	115166	94372 ± 2515	108490 ± 4702	72744 ± 1029
Acetate (mg/*l*)	1074	1135	1065	2715
Propionate (mg/*l*)	0	875	20	0
Butyrate (mg/*l*)	0	0	40	0
NH_4 (mg/*l*)	37	800	2500	650

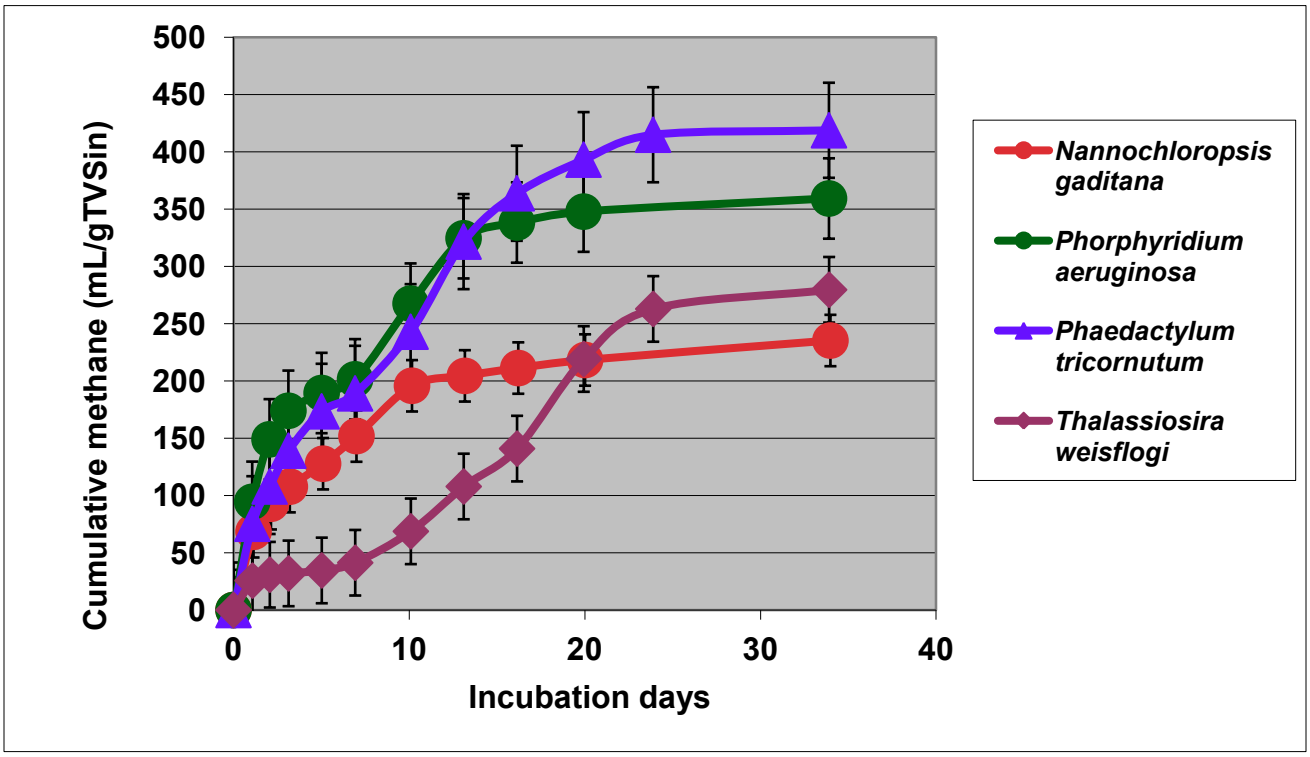
n.a= not available

Table* XXI:** ***t_o and t_{final} Values of Methane and Different Parameters for the BMP Assay for Phorphyridium aeruginosa, Phaeodactylum tricornutum, Thalassiosira weissflogii and Nannochloropsis gaditana.

Parameters	***Porphyridium aeruginosa***		***Phaeodactylum tricornutum***		***Thalassiosira weissflogii***		***Nannochloropsis gaditana***	
	t_o	t_{final}	t_o	t_{final}	t_o	t_{final}	t_o	t_{final}
SS (g/kg)	33	20 ±2	33	26 ± 2	33	31 ± 2	33	28 ± 5
VSS (g/kg)	29	17 ±1	29	22 ± 1	29	25 ± 2	29	24 ± 4
pH	n.a	7.22	n.a	7.25	n.a /	7.33	n.a	7.08
sCOD (mg/*l*)	6997	n.a	5885	1976± 167	9322	2768± 133	3300	518± 105
Acetate (mg/*l*)	62	n.a	67	7	88	7	1114	0
Propionate (mg/*l*)	n.a	n.a	52	0	2	0	0	0
Butyrate (mg/*l*)	n.a	n.a	0	0	3	0	0	0
NH4 (mg/*l*)	134	n.a	160	974	321	1019	140	716
CH_4 (ml/gTVS)	359		419		280		235	
Theorical methane (ml/gTVS)	532		906		1075		707	
Percentage of degradation	80		55		31		39	

n.a=not available or below the detection limit

Figure 10: ***Methane Yield of Nannochloropsis gaditana, Phorphyridium aeruginosa, Phaeodactylum tricornutum and Thalassiosira weissflogii***



N. gaditana and *P. tricornutum* had respectively, 29% and 24% of TS and an important volatile organic fraction of 92 and 86 %, respectively; whereas *P. aeruginosa* and *T. weissflogii* had respectively 20% and 17% of TS, with a volatile organic fraction of 92 and 79%.

The tCOD to TVS ratio for *P. aeruginosa* is 1.42, the same as the ratio of a typical biomass. *N. gaditana, P. tricornutum, T. weissflogii* had the highest ratio: 1.87, 2.14, 2.80, respectively, indicating that these samples may be rich in lipids. The soluble fraction of the algae was important with a value of COD soluble between 73 and 115 g/*l*. The initial concentrations of VFA were between 1065 mg/*l* and 2715 mg/*l* for acetate, 0 and 875 mg/*l* for propionate and 0 and 40 mg/*l* for butyrate, which are normal (Table XXI). *T. weissflogii* had the highest value of ammonium initially: 2500 mg/*l* which is a little high, but the concentration in the bottle at t_0 (321 mg/*l*) was below the mininum inhibitory concentration of ammonium.

II-3. Methane Yield of Freshwater and Marine Algae

Figures 9 and 10 showed the methane yield of the different freshwater and marine microalgae. Among the freshwater algae, *Scenedesmus dimorphus* produced more methane (430 ml/gTVS). One possible explanation is the high percentage (90%) of his organic fraction. The tCOD to TVS

ratio was 1.92, indicating that the sample may be rich in lipids or proteins. The degradation efficiency of *S. dimorphus* in methane is 67%. *M. sp. Rb1b, C. sp. AMLS1b, N. oleoabundans* and *C. vulgaris* had a methane yield inferior to *S. dimorphus,* with 360 ml/gTVS, 333 ml/gTVS, 340 ml/gTVS and 361 ml/gTVS, respectively (Figure 9). The last group of samples, *S. sp. PN2*, *C. sorokiniana, C. sp. island R, C. debaryana ambi* and *S. sp. AMDD* had the lowest methane values with, respectively, 258 ml/gTVS, 282 ml/gTVS, 302 ml/gTVS, 302 ml/gTVS, 306 ml/gTVS. The tCOD/TVS ratio is 1.53 for *C. sp. island R*, 1.72 for *M. sp. Rb1b* and 1.79 *for N. oleoabundans,* with a percentage of degradation of 63%, 68% and 55%, respectively, 54 % for *S. sp. PN2* and 64% for *S. sp. AMDD*. The high tCOD/TVS ratio of *C. sorokiniana* (1.91*)*, *C. sp AMLS1b* (2.27) and *C. debaryana Ambi* (2.88) in spite of a low gas production and low percentages of degradation (48%, 47%, and 34%, respectively) may indicate some disturbances in the anaerobic digestion process, possibly the formation of inhibitory compounds. Another explanation of the low methane yield may be the conditions of the algal culture which may change the composition. For example, a low nitrogen level enhances the percentage of lipid and increases the methane yield. *C. vulgaris* had the highest percentage of degradation of all the freshwater algae, with 75%. We can assume that much of its degradable material has been transformed to biogas.

Among the marine algae, *P. tricornutum* held the highest yield of methane with 419 ml/gTVS, compared to *N. gaditana, T. weissflogii*, *P. aeruginosa,* with 235 ml/gTVS, 280 ml/gTVS and 359 ml/gTVS, respectively (Figure 10). The lag phase observed during the first 7 days of incubation of *T.* weissflogii (Figure 10) may be due to the presence of inhibitory compounds initially present in the algae or generated during their hydrolysis. The tCOD/TVS ratio was 1.42, just as the ratio for a typical biomass, but the degradation was high since it detained the highest percentage of degradation of 80% of the marine microalgae. *N. gaditana, P. tricornutum* and *T. weissflogii* had higher tCOD to TVS ratio*,* with 1.87, 2 and 2.8 respectively, indicating that these sample may be rich in lipids, but their percentage of degradation was low: 39 % 55% and 31 % respectively, due maybe to the presence of inhibitory compounds.

The average methane yield for freshwater algae was 327 ± 49 ml/gTVS and 323 ± 82 ml/gTVS for marine algae. According to Jerger and Tsao (1987), marine algae are considered ideal substrates for anaerobic fermentation, because of their high content of easily degradable polysaccharides, such as alginate, laminaran, and the sugar-alcohol mannitol. Methane yields of 200-450 ml/gVS added have been reported for various marine microalgae (Asinari Di San Marzano, 1982; Sialve et al., 2009; Ghosh et al., 1981; Hanssen et al, 1987; Jerger and Tsao, 1987) and 260-350 ml/gVS added

for the freshwater microalgae. From these results, we can conclude that the anaerobic digestion does not show a big difference between freshwater and marine microalgae in term of methane yield. However, from our first two objectives we can see that microalgae are slightly better than macroalgae for methane production.

III. Objective 3: Pretreatment

III-1. Characterization of the Samples

Scenedesmus dimorphus and *Neochoris oleoabundans* were subjected to two kinds of pretreatment: a thermal one, with the microwave and an alkaline one, with sodium hydroxide. As shown in Table XXII and XXIII, the values of COD, VSS and VFA showed that there was a good hydrolysis and solubilization of the substrate.

Table XXII : *Characterization of Different Parameters of Scenedesmus dimorphus and Neochloris oleoabundans with Pretreatment*

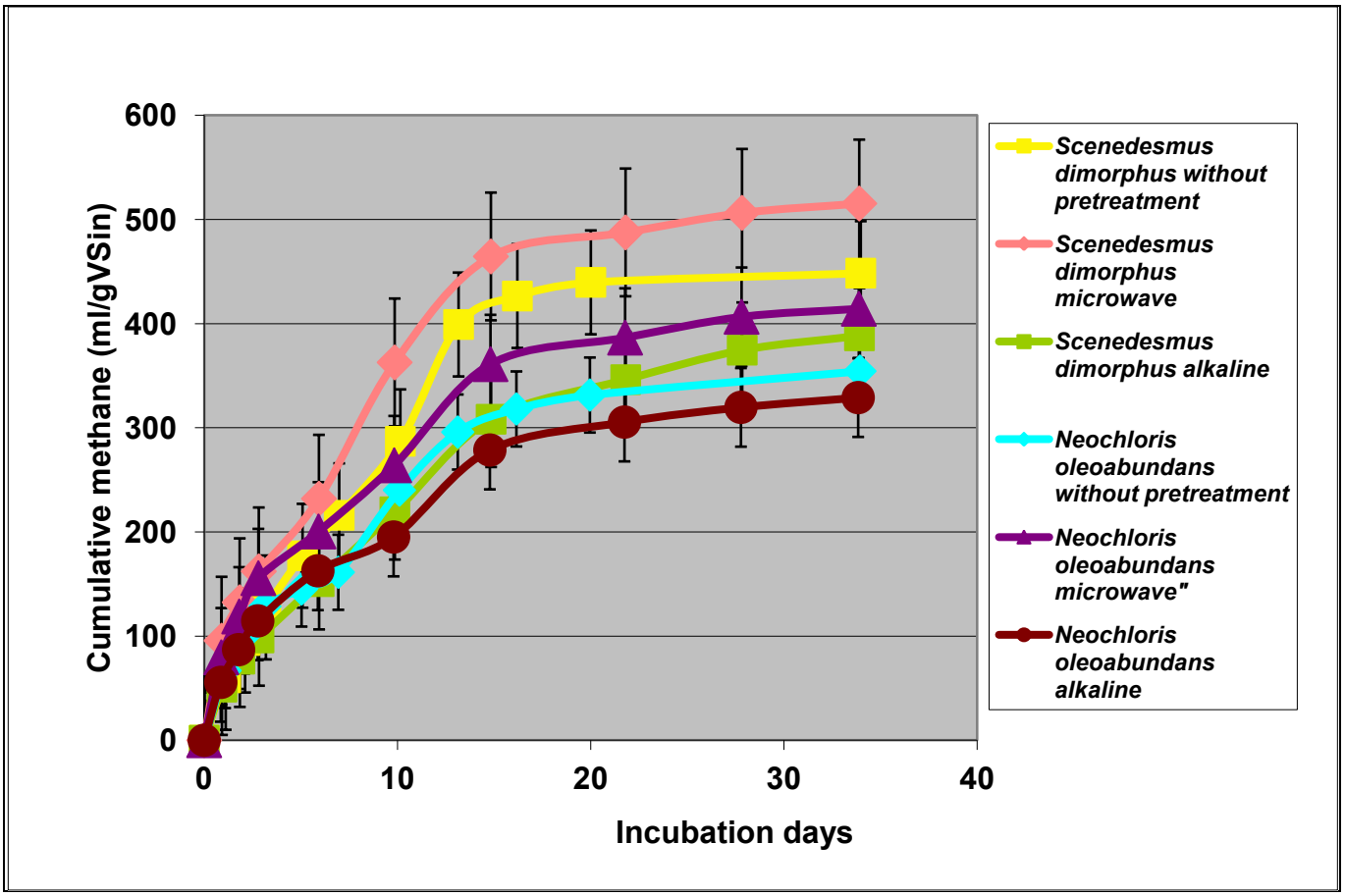
Parameters	***Scenedesmus dimorphus***			**Neochloris *oleoabundans***		
Pretreatment	Before pretreatment	microwave	Alkaline	Before pretreatment	microwave	Alkaline
		After pretreatment			After pretreatment	
SS (g/kg)	239 ± 4	191	225 ± 14	190 ± 15	159	192 ± 1
VSS (g/kg)	218 ± 5	173	205 ± 128	164 ± 12	133	164 ±5
pH	6.92	7	7	7.27	7	7
sCOD (mg/*l*)	78655 ± 3150	83542± 5496	66542± 4505	59113 ± 1465	79776± 1967	53344 ± 473
Acetate (mg/*l*)	3095	832	667	2195	82	599
Propionate (mg/*l*)	15	22	15	90	9	20
Butyrate(mg/*l*)	10	0	0	0	0	0
NH_4 (mg/*l*)	750	300	492	1100	158	156

Table XXIII: ***Final Values of Methane and Different Parameters of Scenedesmus dimorphus and Neochloris oleoabundans with Pretreatment***

Parameters	***Scenedesmus dimorphus* microwave**	***Scenedesmus dimorphus* alkaline**	***Neochloris oleoabundans* microwave**	***Neochloris oleoabundans* alkaline**
SS (g/kg)	145 ± 5	181 ± 2	112 ± 2	144 ± 2
VSS (g/kg)	118 ± 36	143 ± 13	88 ± 16	109 ± 11
TS (g/kg)	n.a	265 ± 8	147 ± 5	242 ± 5
TVS (g/kg)	145 ± 99	171 69	86 ± 31	157 ± 31
pH	7.44	7.41	7.41	7.46
sCOD (mg/*l*)	1194 ± 170	819 ± 266	1419 ± 385	2593 ± 126
Acetate (mg/*l*)	6	2	8	10
Propionate (mg/*l*)	0	0	1	1
Butyrate (mg/*l*)	0	0	0	0
NH_4 (mg/*l*)	740	750	920	1030
SO_4 (mg/*l*)	0	0	0	0
NO_3 (mg/*l*)	0	0	0	0
Methane ml/gVSS)	515	388	414	329

n.a: not available

Figure 11: ***Methane Yield of Scenedesmus dimorphus and Neochloris oleoabundans with Pretreatment***



We observed a diminution of the values of VSS for the microwave pretreatment especially for *S. dimorphus*: 173 g/kg compared to 218 g/kg for the untreated sample, and 205 g/kg for the alkaline pretreatment (Table XXII). This shows that we had more solubilized materials than in the samples without pretreatment. This is confirmed by the higher values of sCOD after the microwave pretreatment: 84 g/*l* for *S. dimorphus* and 80 g/*l* for *N. oleoabundans* compared to the samples without pretreatment (79 g/*l* and 59 g/*l* respectively for *S. dimorphus* and *N. oleoabundans*). For the alkaline pretreatment the values of sCOD (67 g/*l* for *S. dimorphus* and 53 g/*l* for *N. oleoabundans*) were lower compared to the untreated samples, which mean that the untreated samples were more solubilized. However, the concentration of volatile fatty acids is modest when compared to the experiment without pretreatment. For the acetate, the values were between 599 mg/*l* and 832 mg/*l* for *S. dimorphus* pretreated by microwave, *S. dimorphus* and *N. oleoabundans* after alkaline pretreatment. Microwave pretreatment of *N. oleoabundans* had the lowest value of acetate: 82 mg/*l*. The concentration of ammonium, which was between 156 and 492 mg/*l* for the initial analysis and 740 and 1030 mg/*l* for the final analysis, were below the inhibitory level.

III-2. Methane Yield

Figure 11 shows the measurements of methane production yield after pretreatment of *S. dimorphus* and *N. oleoabundans* on days 1, 2, 3, 6, 10, 15, 22, 28 and 34. There was no lag phase at the beginning of the test. The microwave pretreatment of *S. dimorphus* gave a methane yield of 515 ml/gVS, compared to 449 ml/gVS for the untreated biomass (15% increase) and for *N. oleoabundans,* we had a methane yield of 414 ml/gVS compared to 354 ml/gVS for the untreated material (17% increase) (Table XXIII). One explanation may be the high mineralization and COD solubilization obtained with this pretreatment. Also, recent studies show that irradiation at 2450 MHz can effectively break down exocellular polymeric substances (EPS) and microbial cells that are resistant to anaerobic digestion due to a slow and incomplete hydrolysis (Eskicioglu et al., 2007). In addition to the thermal effect, the microwave pretreatment can also cause an athermal effect by polarizing macromolecules that may cause the possible breakage of hydrogen bonds (Eskicioglu et al., 2008). The sludge disintegration and hydrolysis lead to an increase of the anaerobic digestion rate and the improvement of dewaterability (Eskicioglu et al., 2007). On the other hand, the alkaline pretreatment did not give better results when compared to the untreated sample: we had 388 ml/gVS for alkaline-pretreated *S. dimorphus* compared to 449 ml/gVS for the untreated biomass and 329 ml/gVS for alkaline-pretreated *N. oleoabundans* compared to 354

ml/gVS for the untreated biomass (Table XXIII). This is supported by the results of sCOD and VS which showed that these samples had not been more solubilized than the samples without pretreatment. In their study on different pretreatments (microwave, chemical and ultrasonic) of wastewater treatment sludge, Saha et al. (2011) also found that the microwave pretreatment proved to be more effective than the chemical one. The microwave pretreatment could then be considered as a mean of increasing the methane yield.

IV. Lipid Profile of selected algae, and limits in their biodegradation

Another mean of increasing the methane productivity is to choose a substrate rich in lipids, which produced more methane than the other organic substrates (carbohydrates and proteins) (Alves et al., 2009). Algae are an important source for methane production, due to their high content of lipids, which varies between 1 and 70%. The degradation efficiency can reach 90% under certain conditions (Mata et al, 2010).

This section determine the limits of biodegradation of lipids in the anaerobic digestion and is divided in many sections: 1) choice of our substrate (algae); 2) controls tests: 2-1: hydrolysis test on oils that contains the main LCFA of our selected algae to verify that the hydrolysis is not the limited step in the anaerobic digestion; 2-2: activity test on those LCFA to see the performance of our inoculum on our biomass; 3) a BMP to evaluate the methane yield of our algae and identify the limits of biodegradation of algal lipids.

IV-1. Choice of Algae, Pure Acid and Enrichment Preparation

We chose *Phaeodactylum tricornutum* because: 1) it contains more lipid: 2.08% compared to *Thalassiosira weissflogii* (1.75%) and *Chlorella vulgaris* (1.53%); 2) it contains more eicosapentaenoic acid (EPA) 0.43g/100 g than *Thalassiosira weissflogii* (0.12g/100) and *Chlorella vulgaris* (10 mg/100g) (Table XXIV).

The marine microalga *P. tricornutum* is a substrate rich in oil with a high proportion of EPA, which can represent 20-40% of the total fatty acids (Molina Grima et al., 1999; Ibánez González et al, 1998). *P. tricornutum* is a potential source of EPA because it is fast growing. Molina Gima et al. (1994a) obtained an outdoor production of EPA of 47.8 mg d^{-1} $^{-1}$. As pure acid, we chose eicosapentaenoic acid C20:5 (EPA) because it constitutes alone 51% of the polyunsaturated total fatty acids and 20% of the total lipid in *P. tricornutum* and to date, no study has been done on its anaerobic degradation.

From a defatted sample of *P. tricornutum* three samples of *P. tricornutum* were created by an enrichment in LCFA, with three different concentrations: 10%, 25% and 50%. The choice of individual LCFA was done according to the results of lipid content of *P. tricornutum* (Table XXIV). Three fatty acids were predominant, because they alone constituted 69% of the total lipid content in the sample. They are: palmitic acid C16:0, palmitoleic acid C16:1 and eicosapentaenoic acid (EPA) C20:5. Table XXV and XXVI show the composition of *P. tricornutum* at different concentration of LCFA and the amount of each LCFA put in the defatted *P. tricornutum*. The quantity of palmitoleic acid and eicosapentaenoic acid in volume were calculated from their density (respectively 0.895 g/ml and 0.943 g/ml).

IV-2. Fatty acid composition of the selected algae

Table XXIV shows the composition of fatty acid in *Phaeodactylum tricornutum, Thalassiosira weissflogii* and *Chlorella vulgaris.* Those low values maybe due to a culture medium that did not enhance the percentage of lipids, such as a low nitrogen level. The BMP of the new samples of *Phaeodactylum tricornutum* presented many advantages, including:

1- The comparison of the methane potential of the sample defatted with the samples rich in acids at different concentration, so as to quantify the role of lipids in the production of methane.

2- The study of the effect of synergy between the lipids and other components of the algae: carbohydrates, proteins.

3- The interest of Eicosapentaenoic acid (EPA) as bioproducts and its impact on the anaerobic digestion. EPA is an omega-3 fatty acid and a polyunsaturated fatty acid (PUFA). In the human diet, it is provided by fish oil; however fish do not produce EPA, but obtain it from the algae they consume. EPA is an essential fatty acid which therapeutic value has been shown 1) in reducing blood cholesterol and degree of platelet aggregation; 2) in protecting against blood cardiovascular, coronary heart diseases, hyperlipidemy, hypercholesterolemy, hypertriglyceridemy and chronic inflammation processes (Simopoulos, 1991 and Rambjor and al., 1996).

Table XXIV: ***Initial Composition of Fatty Acid for Phaeodactylum tricornutum, Thalassiosira weissflogii and Chlorella vulgaris***

	C16:0 (g/100g)	C16:1 (g/100g)	C 20:5 (g/100g)	Other Acids (g/100g)	Saturated Fat (g/100g)	Mono Unsaturated Fat (g/100g)	Poly Unsaturated Fat (g/100g)	Total Fat (g/100g)
Phaeodactylum tricornutum	0.42	0.58	0.43	0.65	0.58	0.65	0.85	2.08
Thalassiosira Weissflogii	0.54	0.51	0.12	0.58	0.70	0.66	0.39	1.75
Chlorella vulgaris	0.37	<0.01	<0.01	-	0.38	0.14	1.02	1.53

Table XXV: ***Composition of Phaeodactylum tricornutum at Different Concentrations of LCFA***

	% total lipids	% total saturated fatty acids	% total monounsaturated fatty acids	% total polyunsaturated fatty acids
C16:0	20	72		
C16:1	27		89	
C20:5	20			51

Table XXVI: ***Different Concentrations of C16:0, C16:1, C20:5 at 10, 20 and 50% in Phaeodactylum tricornutum Defatted***

	C16:0 (40% of total lipids = 2/5) (mg)	C16 :1 (40% of total lipids = 2/5) (mg)	C20 :5 (20% of total lipids = 1/5) (mg)	*Phaeodactylum tricornutum* (mg)	Concentration of acids in the bottle (total volume: 100ml)
Phaeodactylum tricornutum: 10% or 50 mg of total lipids	20	20	10	599	0.5g/*l* (50mg/100ml)
Phaeodactylum tricornutum: 25% or 125 mg of total lipids	50	50	25	524	1.25 g/*l*
Phaeodactylum tricornutum : 50 % or 250 mg of total lipids	100	100	50	399	2.5g/*l*

IV- 3. Hydrolysis Test on Palm Oil, Macadamia Oil and Fish Oil

A BMP control test was done on the three types of oil: palm (rich in palmitic acid), macadamia (rich in palmitoleic acid) and fish oil (rich in EPA) to see if the hydrolysis of those oils was not the limiting step. Before the BMP, a characterization of the oils (TVS, TS, tCOD) was done. The results are in Table XXVII.The initial total solids of all three oils, approximately 1 kg of TS for 1 kg of oil showed that our lipids were composed of 100% organic matter. The ratio total COD/ TVS were very low for the three oils (less than 1, Table XXVII) and did not reflect the normal ratio tCOD/TVS of a lipid which is between 2 and 3. Those low values were due to the low values of tCOD obtained during our initial analysis. For palm oil, the tCOD was 14817 ± 3455 mg/*l*. This applied also to the t_o analysis. The reason is that those oils, especially palm oil, do not dissolve well in water. Thus, it was very difficult to collect an homogeneous sample. At t_o, the oils were not yet hydrolyzed in LCFA; this was confirmed by the results of VFA analysis (acetate, propionate and butyrate, isovalerate, valerate and caproate) which showed zero concentration (Table XXVIII). The curve of the methane yield (Figure 12) showed no lag phase. At day 9, the methane yield was 24 *l*/kg TVS for palm oil, 63 *l*/kg TVS for macadamia oil and 83 *l*/kg TVS for fish oil. The values of acetate, propionate, butyrate, isovalerate, valerate and caproate at day 9 (Table XXIX) were at or near zero, indicating that there was hydrolysis of oils and no accumulation of VFAs. This means that neither the hydrolysis nor the methanogenesis were the limiting steps. Therefore it is likely that acetogenesis LCFAs was limited and that LCFAs were accumulating. Ortega et al., 2008 in their study on mesophilic activity on olive oil, also found that olive oil was degraded in methane without lag phase and the limiting step in the degradation of olive oil was not related to the hydrolysis of the triglyceride molecule but, rather, was from the inhibitory effect of the long chain volatile fatty acids on acetoclastic methanogens.

Table XXVII: ***Initial Values of Different Parameters of Palm, Macadamia and Fish Oils***

Parameters	**Palm oil**	**Macadamia oil**	**Fish oil**
TS (g/kg)	1000	998	999
TVS (g/kg)	1001	999	1000
tCOD (mg/*l*)	14817 ± 3455	156429 ± 8462	151300 ± 6044
tCOD/TVS	0.02	0.16	0.15

Table XXVIII: ***Values of VFA, Isovalerate, Valerate and Caproate of Palm, Macadamia and Fish Oils at Day 9***

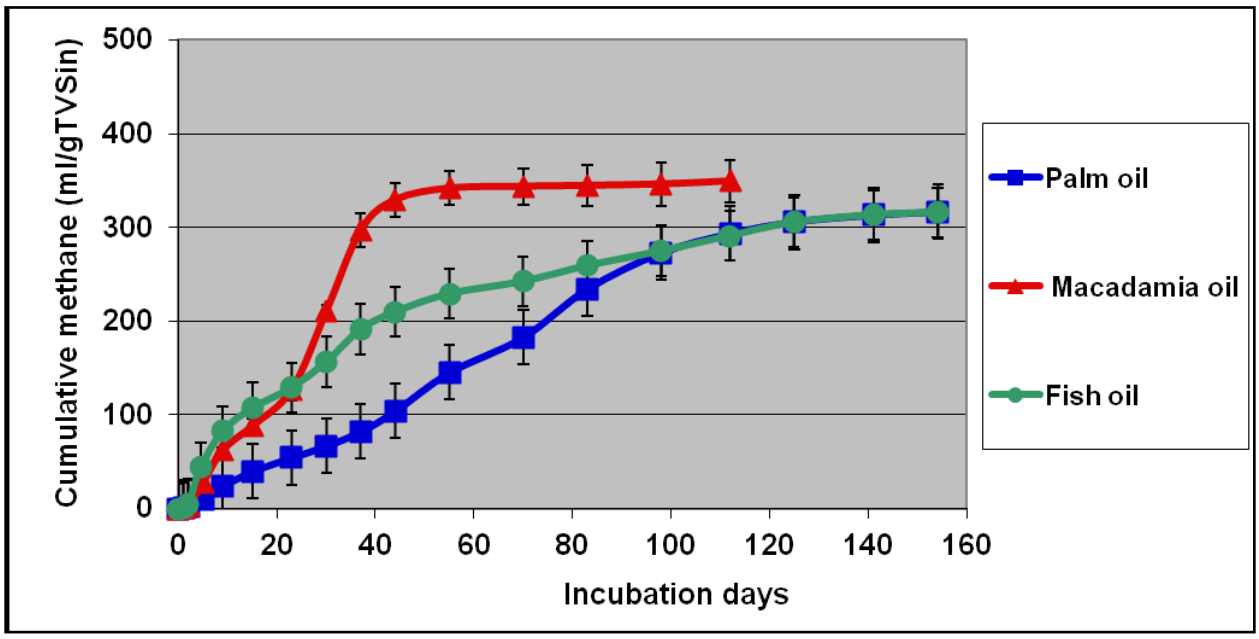
Parameters	**Palm oil**	**Macadamia oil**	**Fish oil**
Acetate (mg/*l*)	9 ± 0.57	0	18 ± 1.27
Propionate (mg/*l*)	0	0	0
Butyrate (mg/*l*)	0	0	0
Isovalerate (mg/*l*)	0	0	0
Valerate (mg/*l*)	0	0	0
Caproate(mg/*l*)	0	0	0

Table XXIX: ***t_o and t_{Final} Values of Different Parameters of Palm, Macadamia and Fish Oils***

Parameters	**Palm oil**		**Macadamia oil**		**Fish oil**	
	t_o	**T_{final}**	**t_o**	**T_{final}**	**t_o**	**T_{final}**
TS (g/kg)	17 ± 0.37	11 ± 0.59	20 ± 1	13 ± 2	16 ± 1/	11 ±1
TVS (g/kg)	14 ± 0	8 ± 1	16 ± 1	10 ± 2	13 ± 1	8 ± 1
tCOD (mg/*l*)	14389± 2689	14070 ± 1619	19019 ± 3917	15777 ± 1736	16099 ± 3114	13808 ± 3089
pH	8.53	7.21	7.83	6.87	8.05	7.29
Acetate (mg/*l*)	0	0	0	0	0	0
Propionate (mg/*l*)	0	0	0	0	0	0
Butyrate (mg/*l*)	0	0	0	0	0	0
Iso-valerate (mg/*l*)	0	0	0	0	0	0
Valerate (mg/*l*)	0	0	0	0	0	0
Caproate (mg/*l*)	0	0	0	0	0	0
NH4 (mg/*l*)	89 ± 2	413 ± 9	90 ± 1	313 ± 5	92 ± 1	404 ± 19
Na (mg/*l*)	421 ± 10	537 ± 12	409 ± 12	432 16	435 ± 9	540 ± 21
K (mg/*l*)	703 ±1	857 ±13	677 ± 11	785 ± 51	760 ± 21	859 ± 1
C l (mg/*l*)	191 ± 4	220 ± 6	189 ± 1	224 ± 2	196 ± 14	220 ± 2
SO4 (mg/*l*)	4 ± 1	n.a	5 ± 0	22 ± 1	5 ± 1	n.a
NO3 (mg/*l*)	n.a	n.a	n.a	n.a	n.a	n.a
Methane (ml/gTVS)	317		350		316	

n.a: below the detection limit

Figure 12: ***Methane Yield of Palm, Macadamia and Fish Oils***



The lower methane yield of palm oil during the first days may be due to the texture of palm oil explained above. The bacteria might not have had good accessibility to the substrate which floats as a mixture; however, Angelika and Ahring (1992) suggested that the response to the addition of neutral lipids may depend on the degree of biomass adaptation. The last option seemed to be the case, because by week 14, palm oil produced as much methane as fish oil, respectively 271 ml/gTVS and 273 ml/gTVS. Macadamia oil produced more methane and reached a plateau on week 16 with 350 ml CH_4/gTVS. At the end of the experiment which lasted 22 weeks, palm oil and fish oil produced, respectively, 317 ml CH_4/gTVS and 316 ml CH_4/gTVS. Those values are far from the theoretical value of methane for lipids which is 1000 ml/gTVS. The presence of LCFA coming from the degradation of the lipids may have an inhibitory impact on the acetoclastic methanogens; however, this theorical value is not often achieved. Fountoulakis et al. (2008) found a methane yield of 110 ml/g COD added in their studies on palm oil mill wastewater. Faisal and Unno (2001), and Najafpour et al. (2006) also found respectively 320-420 ml/g COD and 310-350 ml/g COD. Those values are inferior to the values of palm oil we found in our studies: 317 ml CH_4/gTVS or 634 ml CH_4/g COD (if we considered that 1g of lipid=2g COD).

IV-4. Activity Test on Palmitic Acid (C16:0), Palmitoleic Acid (C16:1) and Eicosapentaenoic Acid (EPA).

The three acids (palmitic, palmitoleic and EPA) were submitted individually to the BMP tests to study their degradation. Figure 13 shows the production of methane from the three acids. Palmitic acid showed a lag phase during the first two weeks of the experiment, probably due to the adaptation of the inoculum. From day 17, we observed an increase in the methane production. EPA produced more methane with 472 ml/gTVS, compared to 423 ml/gTVS for palmitic acid, over a period of 11 weeks. At the end of the experiment which lasted 22 weeks, the methane yield of palmitic acid was 799 ml/gTVS versus 453 ml/gTVS for EPA. Palmitoleic acid produced methane the first 10 days, up to 42 ml/gTVS, after which an inhibition started to occur. This inhibition may be due to the presence of inhibitory compounds from the hydrolysis of palmitoleic acids or may be concentration dependent. Angelidaki and Ahring (1992) and Rinzema and al. (1994) suggest that the addition of free LCFA above a certain concentration may directly results in process failure due to a permanent toxic effect of these compounds towards acetogenic bacteria and methanogenic Archaea. The concentration of palmitoleic acid in our bottle was 2.5g/*l*. This value was much higher than the minimum inhibitory concentration (0.6 g/*l*) for different LCFA (C 8:0, C 14:0, C16:0, C18:1, C18:2, C 18:3) in other studies (Angelidaki and Ahring, 1992; Salvador et al., 2007; Galbraith et al., 1971; Koster and Cramer, 1987; Salvador et al., 2007). A second experiment was done on palmitoleic acid in the same conditions as the first BMP to assess the fatty acids profile at the moment of the inhibition. A sample was taken on days 7, 14, 20 and at the end of the experiment and analyzed in duplicates (Figure 14 and Table XXX). The predominant LCFAs found were C14:0, C16:0, C16:1, C18:0 C18:1 and C18:2. During the first week, all substrates were degraded. From day 7, the process of inhibition started for all the acids, as we noted that the methane production of palmitoleic acid was inferior to the methane production of the control. Between day 7 and 14, there was a partial degradation of 65% for saturated fat and 67% for monounsaturated fat.

Figure 13: ***Methane Yield of Palmitic Acid, Palmitoleic and Eicosapentaenoic Acids***

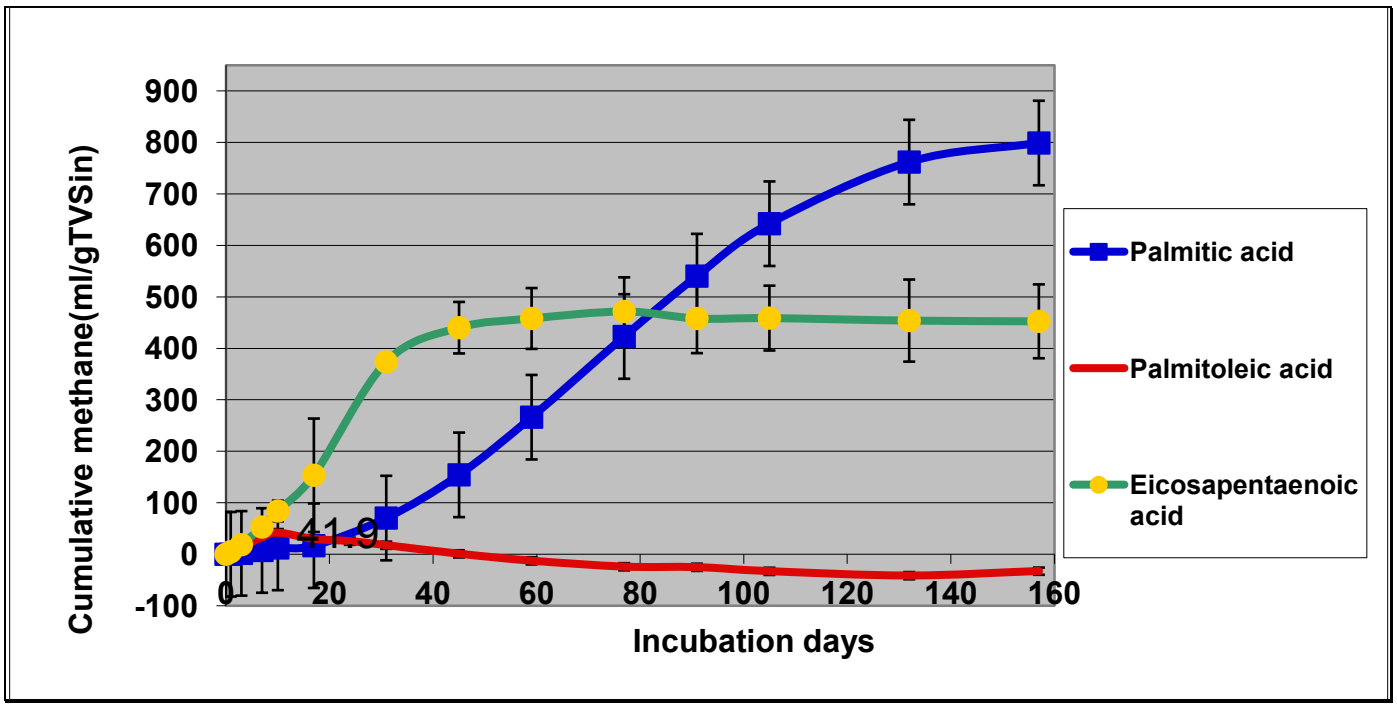


Figure 14: ***Methane Yield of Palmitoleic Acid***

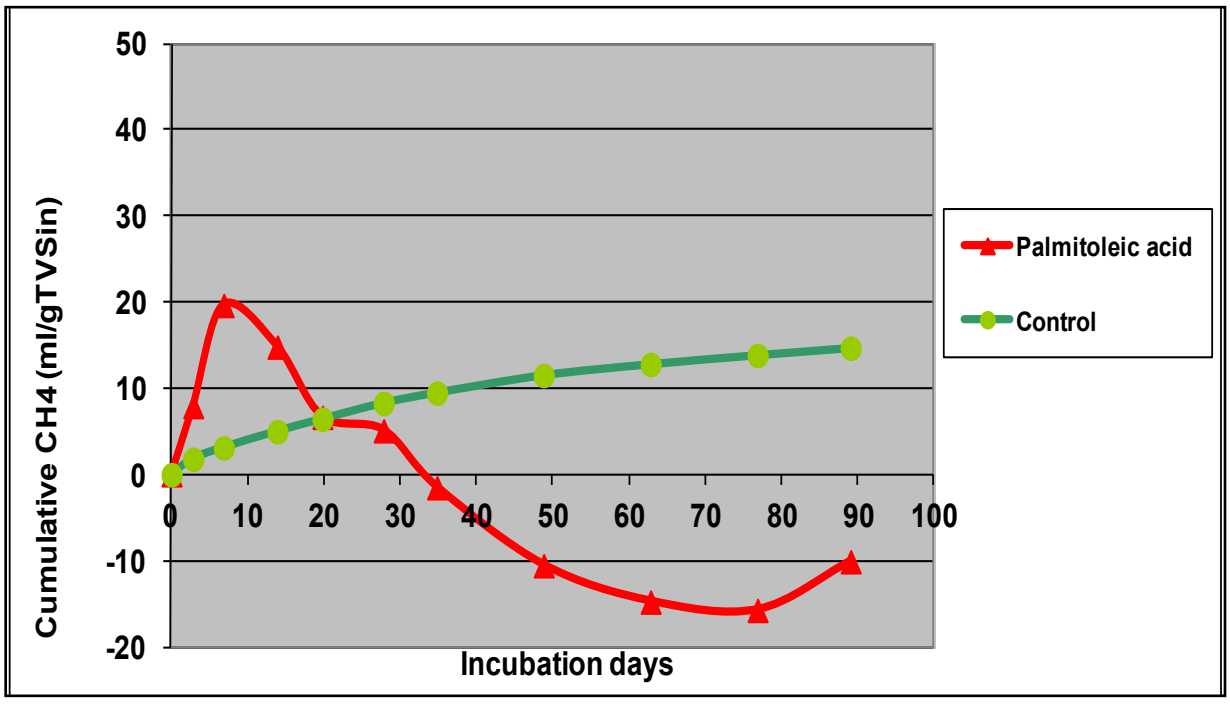


Figure 15: Curves of Methane Yield Showing the Process of Inhibition and Degradation for the two bottles (duplicate) of Palmitoleic Acid

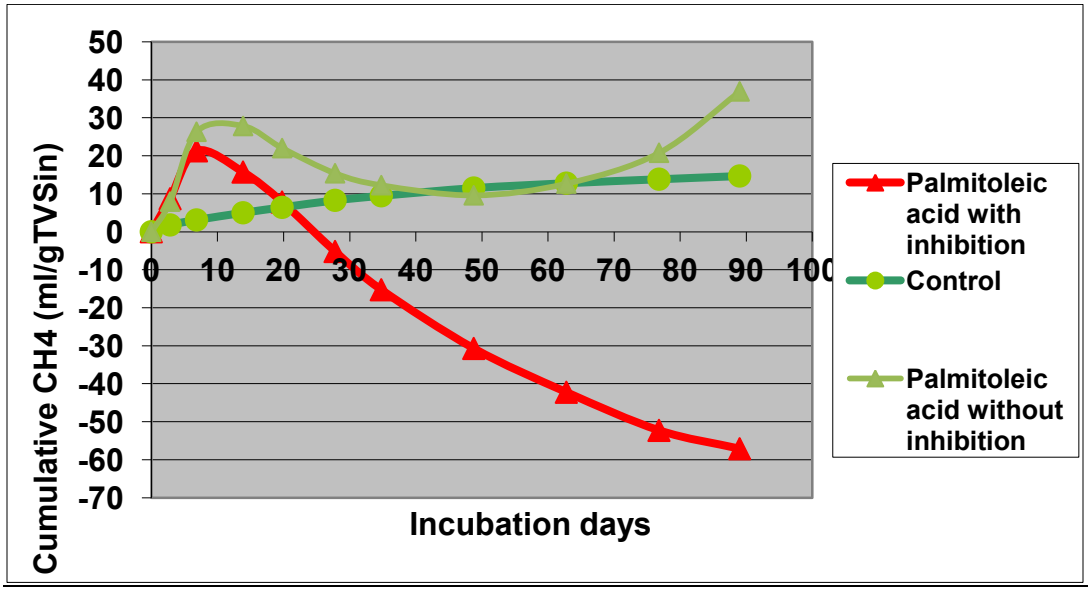


Table XXX: *Percentage of Degradation or Accumulation* in Fatty Acid Profile of Palmitoleic Acid*

Parameters	Day 7- day 14	Day 14- day 20	Day 20 – final day (Duplicata 1)	Day 20 – final day (Duplicata 2)
C14:0	67	*50	*200	67
C16:0	64	*25	*80	20
C16:1	33	50		
C18:0				
C18:1	83			
C18:2				
Total saturated fatty acids	65	* 14	*125	38
Total monounsaturated fatty acids	67	33		

*: percentage of accumulation

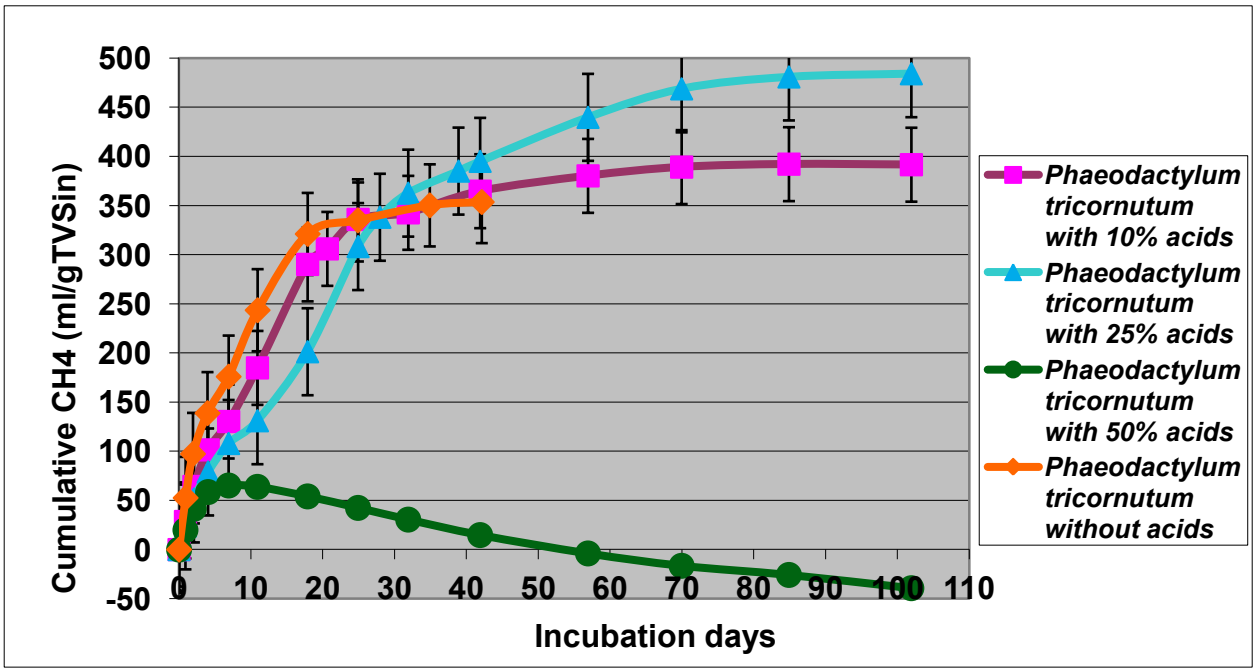
As the inhibition continued from day 14 to day 20, the monounsaturated fatty acid C16:1 was again partially degraded by 50%, but we observed an accumulation of the saturated LCFA (C14:0 and C16:0) which increased by 50 and 25% respectively. At the end of the experiment, an analysis was done on the last two bottles (Figure 15). We observed that the inhibition happened in the first bottle, as the methane production of palmitoleic acid decreased and was inferior to that of the control. The results of the analyses showed an increase of myristic acid C14:0 and palmitic acid C16:0 by 200 and 80% respectively. In the second bottle, there was no inhibition of the process. At the end of the experience, the methane production of palmitoleic acid was superior of the methane production of the control. The results of the analyses showed a degradation of C14:0 and C16:0 by 67 and 20% respectively. The slight diminution of the methane production may be due to an accumulation of C14:0 and C16:0 followed by their degradation. Those results showed a correlation between the accumulation of C14:0 and C16:0 and the inhibition and that the accumulation of the saturated fatty acids C14:0 and C 16:0 may play a role in the processes of inhibition. Other authors had drawn the same conclusions: according to Grossi et al. (2001), polyunsaturated fatty acids were degraded much faster than monounsaturated fatty acids which in turn were degraded faster than saturated acids. Lalman and Bagley (2000) found that during degradation of linoleic acid, unsaturated C16 byproducts form but do not accumulate significantly while saturated C16 and C14 byproducts accumulate and inhibit their own subsequent degradation. In our experiment, the inhibition of palmitoleic acid seemed permanent as there was no recovery from the process of methanogenesis until we stopped the experiment.

IV-5. BMP of *Phaeodactyllum tricornutum* Rich in Palmitic, Palmitoleic and Eicosapentaeonoic Acids at 10%, 25% and 50%.

The necessity of artificially enriching some samples of *Phaeodactylum tricornutum* with lipids came from the fact that the substrates we tested were very poor in total lipids: 2.08% for *Phaeodactylum tricornutum*, 1.75% for *Thalassiosira wiessflogii* and 1.53% for *Chlorella vulgaris*. The time profiles of the methane produced from the *P. tricornutum* samples enriched in palmitic, palmitoleic and eicosapentaeonoic acids are shown in Figure 16. All three samples were degraded without a lag phase, although the methane production was lower during the first week, when compared to that of defatted *P. tricornutum.* On day 7, we obtained 130 *l*/kg TVS for *P. tricornutum,* with 10% of LCFA, 108 *l*/kg TVS for *P. tricornutum* with 25% of LCFA and 65 *l*/kg

TVS for *P. tricornutum* with 50% while defatted *P. tricornutum* gave 176 *l*/kg TVS. This confirmed that LCFAs are difficult to degrade and that the microbial population needs to adapt to that substrate. The higher the LCFA concentration, the slower is the degradation; however from day 11, methane production increased significantly for *P. tricornutum* with 10% and 25% LCFA, showing that LCFA are degraded whereas for *P. tricornutum* with 50% LCFA, there was a beginning of inhibition. As the experiment continued, the inhibition of the acetoclastic methanogens became stronger since initial rates of methane production decreased significantly and even showed negative values. The ß-oxidation process did not seem to be the limiting step in the degradation of *P. tricornutum* with 50% LCFA since we had an initial degradation. The inhibition may be due to the elevated concentration of acid (2.5g/*l*) present in the sample, which was superior to the minimum inhibitory concentration of 0.6g/*l* in certain studies (Angelidaki and Ahring, 1992; Salvador et al., 2007; Galbraith et al., 1971; Koster and Cramer, 1987; Salvador et al., 2007). It is not clear if the inhibition is due to the concentration of a particular LCFA or to the total concentration of the mixture. In the experiment with palmitoleic acid, the inhibition seemed permanent as there was no recovery from the process of methanisation until the end of the experiment (Figure 13, 14). By day 32, *P. tricornutum* with 25% showed higher methane yield with 385 *l*/kg TVS versus 365 *l*/kg TVS for *P. tricornutum* with 10% LCFA. At week 6, the methane production of defatted *P. tricornutum* reached a plateau at 354 *l*/kg TVS, whereas the methane yield were respectively 365 *l*/kg TVS and 395 *l*/kg TVS for *P. tricornutum* with 10% and 25% LCFA respectively. At the end of the test (week 15) we had a maximum methane yield of 392 *l*/kg TVS for *P. tricornutum* with 10% LCFA and 484 *l*/kg TVS for *P. tricornutum* with 25% LCFA. The values were different of those of Zamalloa and al. (2011) who studied the anaerobic digestibility of *P. tricornutum* in mesophilic condition for 30 days. The maximum methane yield obtained in their study was 360 ± 0.03 *l*/ kgVS. The maximum substrate utilization occurred during the first six days of digestion (about 250 *l*/kgVS), contrary to our experiment where the maximum degradation occurred after the first week. The difference may be due 1) to the types of fatty acids in the sample: our sample may have contained more LCFA which could have delayed the degradation process, 2) to the rapid adaptation of their inoculum to the substrate. Although the quantity of methane in our experiment was not quite different for *P. tricornutum* defatted and *P. tricornutum* with 10% LCFA because of the lower percentage of LCFA, our results showed that the addition of fat in the substrates increased the methane yield and this augmentation is proportional to the quantity of fat added. The lipids are then good substrates for the anaerobic digestion up to a certain limit.

Figure 16: ***Methane Yield of Defatted Phaeodactyllum tricornutum Added with 10%, 25% and 50% of EPA, Palmitic, Palmitoleic Acids***



Chapter Four - Conclusion and Perspectives

I. Conclusion

Anaerobic digestion is a process for treating organic wastes and sewage sludge. It reduces the emission of greenhouse gas into the atmosphere and is widely used as a source of renewable energy. The process produces a biogas consisting of methane, carbon dioxide and other impurities. Studies have shown that among the organic substrates, lipids are the most productive compounds of methane compared to other compounds (carbohydrates and protein), however because they are hardly hydrolysable compounds, the acetogenesis of LCFA may be the limiting step in the production of energy.

The interest of this study was:

- To screen different macro and microalgae using the Biochemical methane Potential (BMP) technique. The test proved to be effective for the comparison of the methane yield of different freshwater and marine microalgae;
- To identify the limit of biodegradation of lipid in the anaerobic digestion.

The experimental results showed that:

1- The degradation of macroalgae *Ascophyllum nodosum* and *Fucus edentatus* gives low values of methane: 44 ml/gTVS and 70 ml/gTVS added, respectively, compared to the microalgae which can produce up to 370 ml CH_4 /gTVS added. The small quantities of methane produced in the case of macroalgae may be due to several reasons:
   - the inhibitory compounds presents in the algae or coming from their hydrolysis;
   - the low percentages of lipids (0.5- 1.12%);
   - the presence of compounds associated with seaweed, such as salts which can inhibit the fermentation process;
   - the resistance of macroalgae cell wall to hydrolysis;
   - the elevated concentration of free sulfide (200 mg/*l*) in *Fucus edentatus*.

2- In terms of methane yield, the anaerobic digestion of freshwater algae did not show a big difference, when compared to that of the marine microalgae. The average methane yield for freshwater algae was 327 ± 48.89 ml/gTVS versus 323 ± 81.83 ml/gTVS for marine algae.

3- The microwave pretreatment proved to be more effective for improving the methane yield than the untreated biomass and the alkaline pretreatment. For *Scenedesmus*

dimorphus microwave pretreatment, we have an amelioration of 15% compared to the untreated biomass and for *Neochloris oleoabundans*, this percentage is 17.

4- Lipids are the best substrates for anaerobic digestion in term of methane potential. They can be converted to methane by acetogenic bacteria and methanogenic Archea, but with a risk of inhibition.

4-1. The hydrolysis of palm oil, macadamia oil and fish oil in glycerol and LCFA is not the limiting step.

4-2. Palmitic and eicosapentaenoic acids are easily degraded and converted into methane. The incomplete degradation of palmitoleic acid is due to the accumulation of its own degradation products: saturated fatty acid C14:0 and C16:0 which in turn may inhibit their own subsequent degradation.

4-3. Artificial enrichment of defatted *Phaeodactylum tricornutum* with LCFA improved the methane yield in proportion to the quantity of LCFA added.

II. Contribution to Knowledge

This research contributes by providing additional information about the anaerobic digestion of freshwater and marine micro and macroalgae and also about the impact of a mild pretreatment on the potential of methane.

It also shows that hydrolysis of lipids in long chain fatty acids and glycerol is not the limiting step of the anaerobic degradation process and that lipids are good substrates in the production of methane, although their degradation may have certain limits related to their concentration or release of inhibitory compounds.

Additionally, this research reports in the first time in the literature the anaerobic digestion of eicosapentaenoic acid and shows that EPA may be a good substrate for anaerobic digestion, in terms of methane yield.

III. Future works

Anaerobic digestion is a remarkable process for renewable energy production and its efficiency depends on the choice of substrate. It would be interesting in some future studies to explore the different steps of degradation of the eicosapentaenoic and to find out more about its inhibitory potential.

It would also be interesting to study the inhibition of *Phaeodactylum tricornutum* rich in lipid at 50% to see if the inhibition is due to the concentration of a particular LCFA or to the total concentration of the mixture (palmitic, palmitoleic and EPA).

References

Alvarez R., Lidén G. 2008. Anaerobic co-digestion of aquatic flora and quinoa with manures from Bolivian Altiplano. Waste Manage., 28(10), 1933-1940.

Alves M.M., Mota Vieira J.A., Alvares Pereira R.M., Mota M. 2001a. Effect of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part I: biofilm growth and activity. Water Res., 35(1), 255-263.

Alves M.M., Mota Vieira J.A., Alvares Pereira R.M., Mota M. 2001 b. Effect of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part II: Oleic acid toxicity and biodegradability. Water Res., 35(1), 264-270.

Alves M.M., Pereira M. A., Sousa D. Z., Cavaleiro A. J., Picavet M., Smidt H., Stams A. J. M. 2009. Waste lipids to energy: how to optimize methane production from long-chain fatty acids (LCFA). Microbial Biotechnol., 2(5) 538-550.

Andrews J. F. 1969. Dynamic model of the anaerobic digestion process. J. Sanit. Engineer. Div. 95(SA1), 95-116.

Angelidaki I., Alves M., Bolzonella D., Borzacconi L., Campos J.L., Guwy A.J., Kalyuzhnyi S., Jenicek P., Van Lier J.B. 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. Water Sci. Technol., 59(5) 927-934

Angelidaki I., Petersen S.P., Ahring B.K.1990. Effect of lipids on thermophilic anaerobic digestion and reduction of lipid inhibition upon addition of bentonite. Appl. Microbiol. Biotechnol., 33(4), 469–472.

Angelidaki I., Ahring B.K. 1992. Effects of free long-chain fatty acids on thermophilic anaerobic digestion. Appl. Microbiol. Biotechnol., 37(6), 808-812.

Apt K.E., Behrens P.W. 1999. Commercial developments in microalgal biotechnology. J. Phycol., 35(2), 215–226.

Asinari Di San Marzano C.M., Legro A., Naveau H.P., Nyns E.J. 1982. Biomethanisation of the marine algae Tetraselmis. Int. J. Sustain. Energy, 1(4), 263-272 .

Becker W. 2004. Microalgae in human and animal nutrition. In Richmond A. (ed.), Handbook of microalgal culture. Blackwell, Oxford, p. 312– 351.

Becker, W. 1994. Microalgae: Biotechnology and Microbiology.In Illustrated reprint(ed) Cambridge University Press. 394 pages.

Borowitzka M. A. 1997. Microalgae for aquaculture: opportunities and constraints. J. Appl. Phycol., 9(5), 393–401.

Bozbas K. 2008. Biodiesel as an alternative motor fuel: production and policies in the European Union. Renew. Sust. Energ. Rev., 12(2), 542–552.

Braun R., Huber P., Meyrath J. 1981. Ammonia toxicity in liquid piggery manure digestion. Biotechnol. Lett., 3(4), 159-164.

Briand X., Morand P. 1997. Anaerobic digestion of Ulva sp. 1. Relationship between Ulva composition and methanisation. J. Appl. Phycol., 9(6), 511-524.

Burke D. A. 2001. Dairy waste anaerobic digestion handbook. Options for recovering beneficial products from dairy manure. Olympia, W.A Environmental Energy Company. p 1-54

Cavaleiro A.J., Pereira M.A., Alves M. 2008. Enhancement of methane production from long chain fatty acid based effluents. Biores. Technol., 99 (10), 4086-4096.

Cecchi F., Pavan P., Mata-Alvarez J. 1996. Anaerobic co-digestion of sewage sludge: application to the macroalgae from the Venice Lagoon. Resour. Conserv. Recy., 17(1), 57-66.

Chen P.H. 1987. Factors influencing methane fermentation of microalgae. PhD thesis, University of California, Berkeley, CA, USA, 1987.

Chisti Y. 2008. Biodiesel from microalgae beats bioethanol . Trends in Biotechnol., 26(3), 126-131.

Chynoweth D.P. 2002. Review of biomethane from marine biomass. Department of Agricultural and Biological Engineering, University of Florida. Gainesville, Florida, USA, p 1-98

Cornacchio L., Hall E. R., Trevors J. T. 1986. Modified serum bottle testing procedures for industrial wastewaters. Technology Transfer Workshop On Laboratory Scale Anaerobic Treatability Testing Technique. Environment Canada - Wastewater Technology Centre, Downsview ON, Canada.

Cornet J. F. 1998. Le technoscope: les photobioréacteurs. Biofutur, 176, 1–10.

Danielo O. 2005. Un carburant à base d'huile d'algue . Biofutur, Lavoisier, Cachan, France, 255, 33-36.

Demirel B., Scherer P. 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Environ. Sci. Biotechnol., 7(2), 173-190.

Eskicioglu C., Kennedy K. J., Droste R. L. 2007. Enhancement of Batch Waste Activated Sludge Digestion by Microwave Pretreatment. Water Environ. Res., 79(11), 2304-2317.

Eskicioglu C., Prorot A., Marin J., Droste R.L., Kennedy K.J. 2008. Synergetic pretreatment of sewage sludge by microwave irradiation in presence of H_2O_2 for enhanced anaerobic digestion. Water Res., 42(18), 4674-4682.

Faisal M., Unno H. 2001. Kinetic analysis of palm oil mill wastewater treatment by a modified anaerobic baffled reactor. Biochem. Eng. J., 9 (1), 25-31.

Fernandez A., Sanchez A., Font X. 2005. Anaerobic co-digestion of simulated organic fraction of municipal solid waste and fats of animal and vegetable origin. Biochem. Eng., 26(1), 22–28.

Fountoulakis M.S., Drakopoulou S., Terzakis S., Georgaki E., Manios, T. 2008. Potential for methane production from typical Mediterranean agro-industrial by-products. Biomass and Bioenergy**,** 32(2), 155-161.

Galbraith H., Miller T.B., Paton A.M., Thompson J.K. 1971. Antibacterial Activity of Long Chain Fatty Acids and the Reversal with Calcium, Magnesium, Ergocalciferol and Cholesterol. J. Appl. Bacteriol., 34(4), 803-813.

Gavrilescu M., Chisti Y. 2005. Biotechnology - a sustainable alternative for chemical industry. Biotechnol. Adv., 23(7-8), 471-99.

Ghirardi M.L., Zhang J.P., Lee J.W., Flynn T., Seibert M., Greenbaum E., Melis A. 2000. Microalgae : a green source of renewable H_2. Trends Biotechnol., 18(12), 506-11.

Ghosh S., Klass D. L., Chynoweth D. P. 1981. Bioconversion of *Macrocystis pyrifera* to methane. *J.* Chem.Tech. Biotechnol., 31(1), 791-807.

Goldman J.C. 1979. Outdoor algal mass cultures - I. Application. Water Res., 13(1)**,** 1–19.

Golueke C.G., Oswald W.J, Cotaas H.B. 1957. Anaerobic digestion of algae. Appl. Microbiol., 5(1), 47–55

Gouveia L., Veloso V., Reis A., Fernandes H., Novais J., Empis J. 1996. Evolution of pigment composition in *Chlorella vulgaris*. Bioresour. Technol., 57(2), 157–159.

Grossi V., Blokker P., Sinninghe Damsté J.P. 2001. Anaerobic degradation of lipids of the marine microalaga *Nannochloropsis salina*. Org. Geochem., 32(6), 795-808.

Guil-Guerrero J. L., Navarro-Juárez R., López-Martínez J. C., Campra-Madrid P., Rebolloso- Fuentes M.M. 2004. Functional properties of the biomass of three microalgal species. J. Food Eng., 65(4), 511–517.

Hanaki K. 1981. Mechanism of inhibition caused by long-chain fatty acids in anaerobic digestion process. Biotechnol. Bioeng., 23(7), 1591-1610.

Hanssen J.F., Indergaard M., Ostgaard K., Baevre O.A., Pedersen T.A., Jensen A. 1987. Anaerobic digestion of *Laminaria* spp. and *Ascophyllum nodosum* and application of end products. *Biomass*, 14(1), 1–13.

Hobson P. N., Shaw B. G. 1976. Inhibition of methane production by Methanobacterium formicicum. Water Res., 10(10), 849-852.

Hossain A.B.M.S., Salleh A., Boyce A.N., Chowdhury P., Naqiuddin M. 2008. Biodiesel fuel production from algae as renewable energy, Am. J. Biochem. Biotech., 4(3), 250–254.

Hu Z.-H., Yu H.-Q. 2006. Anaerobic digestion of cattail by rumen cultures. Waste Manage., 26(11), 1222-1226.

Hu Z.-H., Yu H.-Q., Zheng J.-C. 2006. Application of response surface methodology for optimization of acidogenesis of cattail by rumen cultures. Biores. Technol., 97(16), 2103-2109.

Huang G., Chen F., Wei D., Zhang X., Chen G. 2010. Biodiesel production by microalgal biotechnology. Appl Energy, 87(1), 38–46.

Hwu C.-S., Van Beek B., Van Lier J.B., Lettinga G. 1997. Thermophilic high-rate anaerobic treatment of wastewater containing long-chain fatty acids: effect of washed out biomass recirculation. Biotechnol. Lett., 19(5), 453–456.

Hwu S.H., Tseng S.K., Yuan C.Y., Kulik Z., Lettinga, G. 1998. Biosorption of long chain fatty acids in UASB treatment process. Water Res., 32(5), 1571–1579.

Ibáñez González M.J., Robles Medina A., Molina Grima E., Giménez Giménez A., Cartens M., Esteban Cerdán L. 1998. Optimization of fatty acid extraction from *Phaeodactylum tricornutum* UTEX 640 biomass. J. Am. Oil Chem. Soc., 75(12), 1735–1740.

Ihrig D., Heise H.M, Brunert U., Poschmann M., Kuckuk R., Stadtlander K. 2008. Combination of Biological Processes and Fuel Cells to Harvest Solar Energy. J. Fuel Cell Sci. Technol., 5(3), 31001-31005.

Isa, Z., Grusenmeyer S., Verstraete W. 1986. Sulfate reduction relative to methane production in high-rate anaerobic digestion: technical aspects. Appl. Environ. Microbiol., 51(3), 572-579.

Jerger D. E., Tsao, G. T. 1987. Feed composition. In Anaerobic Digestion of Biomass, ed. D. P. Chynoweth and R. Isaacson. Elsevier Applied Science, p. 65-90.

Kangmin L., Ho M.-W., 2006 Le biogaz en Chine. Communiqué de presse de l'Institut ISIS, 1-23.

Kerner K.N., Hanssen J.F., Pedersen T.A. 1991. Anaerobic digestion of waste sludges from the alginate extraction process. Biores. Technol., 37(1), 17-24.

Kim S.H., Han S.K., Shin H.S. 2004. Two-phase anaerobic treatment system for fat-containing wastewater. J. Chem. Tehnol. Biotechnol., 79(1), 63-71.

Koster I.W., Cramer A. 1987. Inhibition of Methanogenesis from Acetate in Granular Sludge by Long-Chain Fatty Acids. Appl. Environ. Microbiol., 53(2), 403–409.

Kroiss H., Wabnegg P.F. 1983. Sulfide toxicity with anaerobic wastewater treatment. In Proceedings of the European Symposium on Anaerobic Waste-Water Treatment (AWWT). AWWT Symposium Secretariat, TNO Corporate Communications Department, The Hague, The Netherlands, p. 72-85.

Kuang Y., Pullammanappallil P., Lepesteur M., Ho G.-E. 2006. Recovery of oleate inhibited anaerobic digestion by addition of simple substrates. J. Chem. Technol. Biotechnol., 81(6), 1057–1063.

Lalman J. A., Bagley D.M. 2000. Anaerobic degradation and inhibitory effects of linoleic acid. Water Res., 34(17), 4220-4228.

Lalman J. A, Bagley D.M. 2002. Effects of C_{18} long chain fatty acids on glucose, butyrate and hydrogen degradation. Water Res., 36(13), 3307–3313.

Li Y, Horsman M., Wu N, Lan CQ, Dubois-Calero N. 2008. Biofuels from microalgae. Biotechnol. Progr., 24(4), 815–820.

Li Y.Y., Sasaki H., Yamashita K., Seki K., Kamigochi I. 2002. High-rate of methane fermentation of lipid-rich food wastes by a high-solids co- digestion process. Water Sci. Technol., 45(12), 143-150

Lucks S. 2000. Anaerobic digestion: the process. Solar Engineering Services. p 1-17

Mata T.M., Martins A.A., Caetano N.S. 2010. Microalgae for biodiesel production and other applications: A review. Renew. Sust. Energ. Rev., 14(1), 217–232.

Mata-Alvarez J. 2003. Biomethanisation of the oganic Fraction of Municipal Solids Wastes. IWA Publishing, London, UK. p 141-176

McCarty P. L., McKinney R. E. 1961. Volatile acid toxicity in anaerobic digestion. J. Water Pollut. Control. Fed., 33(3), 223-232.

McCarty P.L., McKinney R.E. 1961. Salt toxicity in anaerobic digestion. J Water Pollut Control Fed., 33(4), 399-415.

McCarty P.L. 1964. Anaerobic waste treatment fundamentals III. Public Works 95, 91-94.

Minowa T., Sanayama S. 1999. A novel microalgal system for energy production with nitrogen cycling. Fuel, 78 (10), 1213-1215.

Moletta R. 2008. La méthanisation. Lavoisier: Paris, France, 532 pages

Moletta, R. 1993. La digestion anaérobie: du plus petit au plus grand. Biofutur, 119, p 16-25.

Molina Grima E., Camacho F.G., Perez J.A.S., Cardona J.U., Fernadez F.G.A., Sevilla J.M.F. 1994a. Outdoor Chemostat culture of *Phaeodactylum tricornutum* UTEX 640 in a tubular photobioreactor for the production of eicosapentaenoic acid. Biotechnol. Appl. Biochem., 20(2), 279-290.

Molina Grima E., Robles Medina A., Giménez Giménez A., Ibáñez González M.J. 1999. Gram-scale purification of eicosapentaenoic acid (EPA, 20:5*n*3) from the microalga *Phaeodactylum tricornutum* UTEX 640 biomass. J. Appl. Phycol., 8(4-5), 359–367.

Morgan J. W., Evison L.M., Forster C.F. 1991. Changes to the Microbial Ecology in Anaerobic Digesters Treating Ice-Cream Wastewater during Start-up. Water Res., 25(6), 639-653.

Muller-Feuga A. 2000. The role of microalgae in aquaculture: situation and trends. J. Appl. Phycol., 12(3-5), 527–534.

Nagamani B., Ramasamy K. 1999. Biogas production technology: an Indian perspective, Curr. Sci. India*,* 77(1), 44–55.

Najafpour G.D., Zinatizadeh A.A.L., Mohamed A.R. Isa M.H., Nasrollahzadeh H. 2006. High-rate anaerobic digestion of palm oil mill effluent in an upflow anaerobic sludge-fixed film bioreactor. Process Biochem.**,** 41(2), 370-379.

Neves L., Pereira M.A., Mota M., Alves M.M. 2009. Detection and quantification of long chain fatty acids in liquid and solid samples and its relevance to understand anaerobic digestion of lipids. Biores. Technol., 100(1), 91-96

Ortega L., Husser C., Barrington S. and Guiot S.R. 2008. Evaluating limiting steps of anaerobic degradation of food waste based on methane production tests. Water Sci. Technol., 57(3), 419-422.

Owen W. F., Stuckey D. C., Healy J. B., Young Y., McCarty P. L. 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res., 13(6), 485–492.

Palatsi J., Laureni M., Andres M.V., Flotats X., Nielsen H.B., Angelidaki I. 2009. Strategies for recovering inhibition caused by long chain fatty acids on anaerobic thermophilic biogas reactors. Biores. Technol., 100(20), 4588-4596.

Parkin G. F., Owen W. F. 1986. Fundamentals of anaerobic digestion of wastewater sludges. J. Environ. Eng., 112, 867–920.

Pereira M.A., Pires O.C., Mota M., Alves M.M. 2005. Anaerobic biodegradation of oleic and palmitic acids: evidence of mass transfer limitation caused by long chain fatty acid accumulation onto anaerobic sludge. Biotechnol. Bioeng., 92(1), 15–23.

Pereira M.A., Cavaleiro A.J., Mota M., Alves M.M. 2003. Accumulation of long chain fatty acids onto anaerobic sludge under steady state and shock loading conditions: effect on acetogenic and methanogenic activity. Water Sci. Technol., 48 (6), 33–40.

Piorreck M., Baasch K.H., Pohl P. 1984. Biomass production, total protein, Chlorophylles, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Photochemistry, 23(2), 207-216.

Rajeshwari K.V., Balakrishman M., Kansal A., Lata K., Kishore V.V.N. 2000. State- of-the- art of anaerobic digestion technology for industrial wastewater treatment. Renew. Sust. Energ. Rev., 4(2), 135-156.

Rambjor G.S., Walen A.I., Windsor S.L., Harris W.S. 1996. Eicosapentaenoic acid is primarily responsible for hypotriglyceridemic effect of fish oil in humans. Lipids, 31(1), S45–S59.

Richmond A. 2004. Handbook of microalgal culture: Biotechnol. Appl. Phycol. Ed (A.Richmond) Blackwell Publishing Lt, Oxford,UK. 584 pages.

Rigoni-Stern S., Rismondo R., Szpyrkowicz L., Zilio-Grandi F., Vigato P.A. 1990. Anaerobic digestion of nitrophilic algal biomass from the Venice lagoon. Biomass, 23(3), 179-99.

Rinzema A., Boone M., Van Knippenberg, K and Lettinga G. 1994. Bactericidal effect of long chain fatty acids in anaerobic digestion. Water Environ. Res., 66**(**1), 40–49.

Roesijad G., Jones S.B., Snowden-Swan L.J., Zhu Y. 2010 Macroalgae as a biomass feedstock: a preliminary analysis, PNNL 19944. Pacific Northwest National Laboratory, Richland, Washington, USA. .p 1-50

Saha M., Eskicioglu C., Marin J. 2011. Microwave, ultrasonic and chemo-mechanical pretreatments for enhancing methane potential of pulp mill wastewater treatment sludge. Biores. Technol., 102(17), 7815-7826

Salvador A.F., Pereira M.A., M. M. Alves M.M. 2007. Anaerobic biodegradation of oleate by a highly loaded biomass before and after degrading the associated substrate. 11th IWA World Congress on Anaerobic Digestion, Brisbane, Australia, p 1-6.

Samson R., Leduy A. 1982. Biogas production from anaerobic digestion of Spirulina maxima algal biomass. Biotechnol. Bioeng., 24(8), 1919-1924.

Samson R., Leduy A. 1986**.** Detailed study of anaerobic digestion of Spirulina maxima algal biomass. Biotechnol. Bioeng., 28(7), 1014-1023.

Samson R. 1995. Les développements technologiques récents dans l'opération des sites d'enfouissement sanitaire des déchets solides. Mémoire, Ecole polytechnique de Montreal, Canada. p 1-32

Sanchez Hernandez E.P., Traviesco Cordoba L. 1993. Anaerobic digestion of *Chlorella vulgaris* for energy production. Resour. Conserv. Recy., 9(1-2), 127-132

Shay E.G. 1993. Diesel fuel from vegetable oils: Status and Opportunities. Biomass Bioenerg., 4(4), 227-242.

Sheehan J., Dunahay T., Benemann J., Roessler P. 1998. A look back at the U.S. Department of Energy's aquatic species program: Biodiesel from algae. NREL/TP-580-24190. U.S. Department of Energy's Office of Fuels Development.

Shelton D. R., Tiedje J. M. 1984. General method for determining anaerobic biodegradation potential. Appl. Environ. Microbiol., 47(4), 850–857.

Sialve B., Bernet N., Bernard 0. 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol. Advances, 27(4), 409-416.

Simopoulos A.P. 1991. Omega-3 fatty acids in health and disease and in growth and developments. Am. J. Clin. Nutr., 54(3), 438–463.

Soletto D., Binaghi L., Lodi A., Carvalho J. C. M., Converti, A. 2005. Batch and fed-batch cultivations of Spirulina platensis using ammonium sulphate and urea as nitrogen sources. Aquaculture, 243(1-4), 217–224.

Spoehr H.A., Milner H.W. 1949. The chemical composition of *Chlorella*. Effect of environmental conditions. Plant Physiol., 24(1), 120-149 .

Spolaore P., Joannis-Cassan C., Duran E., Isambert A. 2006. Commercial Applications of Microalgae. J. Biosci. Bioeng.**,** 101(2), 87-96.

Szendrey L.M. 1983. Start-up and operation of the Bacardi Corporation anaerobic filter. In: Proceedings of the Third International Symposium on Anaerobic Digestion (p 365–377). Evans and Faulkner Inc. Watertown, Massachusetts.

Takacs I., Vanrolleghem P.A. 2006. Elemental balances in activated sludge modelling. In: Proceedings of IWA World Water Congress, Beijing, China, 10-14 September 2006.

Tholen A., Brune A. 1999. Localisation and in situ activities of homoacetogenic bacteria in the highly compartmentalized hindgut of soil-feeding higher termites (Cubitermes spp.). Appl. Environ. Microbiol., 65(10), 4497-4505

Van-Harmelen T., Oonk H. 2006. Microalgae Biofixation Processes: Application and Potential Contributions to Greenhouse Gas Mitigation Options. Report, International Network on Biofixation of CO_2 and Greenhouse Gas Abatement with Microalgae, The Netherlands, p 1-47.

Vergara-Fernández A.,Vargas G., Alarcón N., Velasco A. 2008. Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. Biomass Bioenerg., 32(4), 338-344.

Verma S. 2002. Anaerobic Digestion of Biodegradable Organics in Municipal Solid Wastes. MSc Dissertation. Columbia University <http://www.seas.columbia.edu/earth/vermathesis.pdf>. Accessed on May 5th, 2011

Yamaguchi K. 1996. Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. J. Appl. Phycol., 8(6), 487–502.

Yang S., Li J., Zheng Z., Men Z. 2009. Lignocellulosic structural changes of *Spartina alterniflora* after anaerobic mono- and co-digestion. Int. Biodeter. Biodegr., 63(5), 569-575.

Yen H.-W. and Brune, D.E. 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. Biores. Technol. 98(1), 130-134.

Zheng-Bo Y., Yua, H.-Q., Harada, H., Li, Y.-Y. 2007. Optimization of anaerobic acidogenesis of an aquatic plant, *Canna indica* L., by rumen cultures. Water Res., 41(11), 2361-237

Zamalloa C., De Vrieze J., Verstraete W. 2011. Anaerobic digestibility of marine microalgae *Phaeodactylum tricornutum* in a lab-scale anaerobic membrane bioreactor . App. Microbiol. Biotechnol., 93(2), 859-869