Universidade do Minho
Escola de Engenharia

Ricardo Fontes Serpa

New strategies for improving the conversion of waste organic matter to methane

Outubro de 2016

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New strategies for improving the conversion of waste organic matter to methane

Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sob a orientação do
Doutor Gilberto Jorge da Silva Martins
e da **Doutora Andreia Filipa Ferreira Salvador**

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RESUMO

O biogás, que é composto maioritariamente por metano, é um componente gasoso produzido por microorganismos que usam como substrato moléculas simples, como o acetato, o hidrogénio ou o dióxido carbono, é uma conhecida alternativa aos combustíveis fósseis como fonte de energia. Em comunidades sintróficas, os organismos metanogénicos e as bactérias presentes trocam eletrões entre si com o objetivo de obtenção de energia para o seu crescimento, através de pequenos compostos químicos solúveis que servem de transporte, como o hidrogénio e o formato. Estudos recentes indicam que em alguns casos, estas trocas de eletrões podem ser realizadas diretamente ou por meio de materiais condutores, sendo esta uma abordagem que poderá ser mais favorável de um ponto de visto energético, aumentando assim a eficiência da metanogénese. Este projeto visou o estudo do efeito de nanotubos de carvão (NTC) nos mecanismos que envolvem a produção de metano em comunidades metanogénicas, através de dois diferentes estudos. No primeiro estudo, foram realizados ensaios em batch com culturas puras dos organismos metanogénicos hidrogenotróficos, *Methanobacterium formicicum* e *Methanospirillum hungatei*, e acetoclásticos, *Methanosaeta concilii* e *Methanosarcina mazei*, na presença de NTC. Além disso, também foram testadas culturas puras de *M. formicicum* na presença de NTC, mas sem adição de um agente redutor. Pelos resultados obtidos, verificou-se que os NTC provocaram um aumento na produção de metano e redução da fase de latência nas culturas puras dos metanogénicos hidrogenotróficos. Apesar de a presença dos NTC também ter acelerado a produção de metano nas culturas dos acetoclásticos com 1 g/L de NTC, numa maior concentração de 5 g/L de NTC, foi registada uma inibição na produção de metano e um aumento na duração das fases de latência. Num segundo estudo foram inoculadas culturas mistas contendo *Syntrophomonas wolfei*, uma bactéria que metaboliza butirato, e *M. hungatei*. Os resultados obtidos mostraram que a produção de metano também foi acelerada nas culturas mistas inoculadas com NTC, atingindo uma taxa de produção de metano de 1.03 ± 0.03 mM/d para as culturas incubadas com 5 g/L NTC, enquanto que na ausência de NTC, a taxa obtida foi de apenas 0.72 ± 0.01 mM/d. No âmbito deste trabalho, também foi realizada uma adaptação de biomassa em reatores anaeróbios, com o objetivo a longo prazo de adicionar os NTC para estudar o seu efeito em comunidades microbianas complexas. Por último, a análise dos resultados obtidos neste trabalho permitiu concluir que os NTC têm um efeito benéfico na conversão dos substratos a metano pelos microorganismos metanogénicos e pelas co-culturas sintróficas de *S. wolfei* e *M. hungatei*. Contudo, serão necessários mais estudos com os NTC para compreender melhor de que forma estes poderão estar envolvidos nos mecanismos de transferência de eletrões entre espécies, pois a sua influência neste processo continua por determinar.

Palavras-Chave: metano, nanotubos de carvão, culturas puras, sintrofia, transferência de eletrões entre espécies

ABSTRACT

Biogas is an alternative to substitute fossil fuels and is mostly composed by methane, a gas component that is produced by microorganisms called methanogens that use compounds such as acetate, hydrogen and carbon dioxide as substrates. In syntrophic communities, methanogens and bacteria exchange electrons for energetic purposes, normally through the use of soluble small chemical compounds that act as shuttles, such as hydrogen or formate. However, it has been recently suggested that in some cases this electron exchange can be performed directly or with the aid of conductive materials, which can potentially be a more energy conserving approach, thus improving the efficiency of methanogenesis. This project addressed the study of the effect of carbon nanotubes (CNT) in methane production by methanogenic communities, in two distinct studies. In the first study, assays were performed in batch with pure cultures of hydrogenotrophic, *Methanobacterium formicicum* and *Methanospirillum hungatei*, and acetoclastic methanogens, *Methanosaeta concilii* and *Methanosarcina mazei*, in the presence of CNT. Also, growth of pure cultures of *M. formicicum* was tested in the absence of a reducing agent. The results showed that CNT presence in pure cultures of the hydrogenotrophic methanogens resulted in an increased methane production and reduced lag phases. Although CNT also accelerated methane production by the acetoclastic pure cultures at 1 g/L CNT, a higher concentration of 5 g/L CNT, inhibited the methane production and induced longer lag phases. In the second study, co-cultures of butyrate-degrading bacteria *Syntrophomonas wolfei* and *M. hungatei* were grown with and without CNT, in order to determine the effect of CNT in butyrate-oxidizing syntrophic communities. The presence of CNT accelerated methane production by this co-culture, with methane production rate reaching a value of 1.03±0.03 mM/d for co-cultures incubated with 5 g/L CNT, while co-cultures in the absence of CNT only registered a rate of 0.72±0.01 mM/d. Adaptation of sludge in anaerobic bioreactors was also performed with the long-term objective of adding CNT, in order to assess its effect in complex microbial communities. By analysing the results obtained in this work, it was concluded that CNT improved hydrogen and acetate conversion to methane by pure cultures of methanogens and by syntrophic co-cultures of *S. wolfei* and *M. hungatei*. However, future studies with CNT are encouraged in order to understand the mechanisms by which CNT influences the methanogenic activity and in which cases it can be involved in interspecies electron transfer, since with the results obtained it remains unclear if they have a role in electron exchange between different species.

KEYWORDS: methane, carbon nanotubes, pure cultures, syntrophy, interspecies electron transfer

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List of Abbreviations

AD – Anaerobic digestion

AQDS – anthraquinone-2,6-disulphonate

CNT – Carbon nanotubes

COD – Chemical oxygen demand

DIET – Direct interspecies electron transfer

DNA – Deoxyribonucleic acid

EGSB – Expanded granular sludge bed

EPS – Extracellular polysaccharides

GAC – Granular activated carbon

GC – Gas chromatography

HPLC – High pressure liquid chromatography

MCR – Methyl-coenzyme M-reductase

MWCNT – Multi-walled carbon nanotubes

NTC – Nanotubos de carvão

OLR – Organic loading rate

ORP – Redox potential

RNA – Ribonucleic acid

SAA – Specific acetoclastic activity

SEM – Scanning electron microscopy

STP – Standard temperature and pressure

SWCNT – Single-walled carbon nanotubes

TSS – Total suspended solids

UASB – Up-flow anaerobic sludge blanket

VFA – Volatile fatty acids

VSS – Volatile suspended solids

1. State of the art

1.1 Anaerobic digestion

To use a reliable and renewable energy resource is a main societal goal and methane (CH_4) production from organic wastes is one of the most effective ways to accomplish that goal (Li et al. 2011). Large technological developments have been made in the field of anaerobic digestion (AD) in the last two decades, since this has proven to be a very promising approach to both waste management and renewable energy resources (Adekunle & Okolie 2015). AD process is one of the most economic ways to treat industrial wastewater and consists in the reduction of complex organic polymers through a series of biochemical reactions that degrade them into simpler molecules (i.e. CO_2 and CH_4) in the absence of oxygen (Stams et al. 2012).

Methane is the name given to the simplest existing alkane, composed by one atom of carbon and four atoms of hydrogen and it is the main component of natural gas. Methane is used as fuel for electrical generation, as vehicle fuel, and it can also be utilized in the production of biomethanol (Khirsariya & Mewada 2013; Düren et al. 2004). However, with a concentration increasing at a rate of about 1% per year, it is the second most abundant carbon-containing gas in the atmosphere (Cicerone & Oremland 1988), and is included in a group of gases called greenhouse gases.

With the very probable depletion of fossil fuels occurring in the not so distant future, comes the need to obtain alternative and more resourceful ways of generating energy (Merlin Christy et al. 2014), making methane an interesting option. Though it might be an environmental hazard when in abundance in the atmosphere, the controlled conversion of organic waste to methane in bioreactors can be a sustainable and efficient way of treating waste and generating bioenergy.

The following schematic drawing (Figure 1.1) represents the anaerobic food chain for the conversion of complex organic matter to methane in anaerobic conditions and shows the steps needed to convert complex organic molecules into the final products.

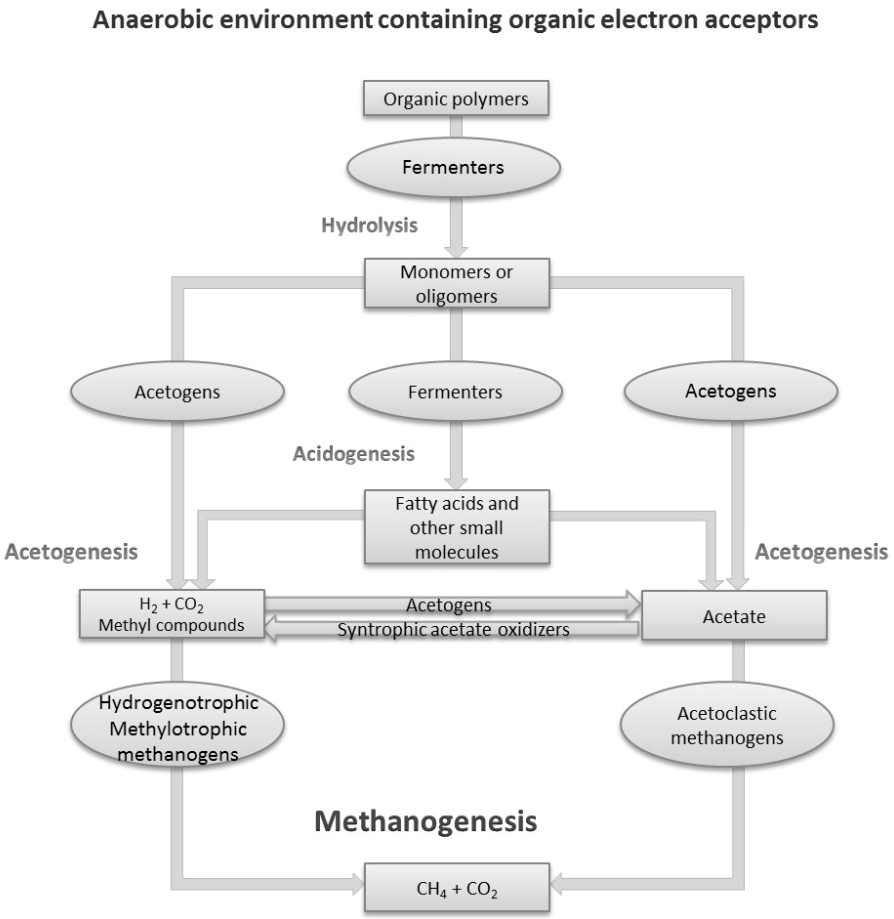


Figure 1.1 – Steps in the conversion of complex organic compounds to methane; adapted from Meslé et al. 2013.

The first step in anaerobic digestion is the hydrolysis, in which insoluble organic polymers such as proteins, polysaccharides and nucleic acids are hydrolysed by strict anaerobes, involving the production of several extracellular hydrolytic enzymes (Schink 1997). Different microorganisms produce different enzymes that are responsible for breaking down complex molecules into shorter chains. The complex biodegradable materials are transformed into simpler soluble organic compounds, such as monosaccharides, fatty acids, glycerol and amino acids, making this a fundamental step, since methanogens are unable to directly use such complex compounds as a substrate for methane production (Merlin Christy et al. 2014). The products of hydrolysis are further converted into smaller molecules such as organic acids, alcohols, carbon dioxide and hydrogen, in a process named acidogenesis. The intermediate products that are formed in this step can be reduced when in the presence of alternative electron acceptors, which is why these reactions can only be performed in anoxic conditions (McInerney et al. 2008). Hydrogen produced in this phase needs to be consumed by other microorganisms since the next phases depend on low hydrogen partial pressure to occur due to thermodynamic constraints (Adekunle &

Okolie 2015). Before they can be converted to methane, major products of acidogenesis, such as volatile fatty acids (VFA) and alcohols still need to go through one more process, named acetogenesis (Meslé et al. 2013). During this phase, hydrogen molecules are formed with protons serving as the final electron acceptors, leading to the formation of the methanogenic substrates, i.e. acetate, carbon dioxide and hydrogen. The final step of anaerobic digestion is named methanogenesis, where methane production takes place. This may be a slow reaction, depending on the substrate (i.e. hydrogenotrophic methanogens have a fast doubling time, while acetogenic methanogens perform this step slower) and can only be carried out under strict anaerobic conditions (Adekunle & Okolie 2015; Murphy & Thanasit Thamsiriroj 2013). Because acetogenesis can only occur in low hydrogen partial pressures, the activity of methanogens is crucial for the depletion of hydrogen, making both reactions a collaboration effort between the two different microorganisms. There is still much to know about methanogenesis, but it is currently accepted that it can be carried out in three distinct ways, depending on the source of carbon: hydrogenotrophic methanogenesis, methylotrophic methanogenesis and acetoclastic methanogenesis (Meslé et al. 2013). As their names suggest, these processes differ in the compound which acts as the electron donor, being hydrogen in hydrogenotrophic, methyl groups in methylotrophic and acetate in acetoclastic methanogenesis. All pathways have the final step in common, which is the demethylation of methyl-coenzyme M to methane and the reduction of the heterodisulfide of coenzyme M and coenzyme B (Peder E. Cedervall 2010; Thauer et al. 1993).
It is because of this ability to deplete resources such as carbon dioxide or smaller organics, that methanogenesis is considered to be a fundamental part of the carbon cycle (McInerney et al. 2009).

1.1.1 Microbiology and physiology of methanogens

Microorganisms capable of performing methanogenesis are called methanogens. All methanogens have a number of characteristics that set them apart from other microorganisms: they belong to the *Archaea* domain, phylum *Euryarchaeota*, obtain all their energy for growth by producing CH_4, are strict anaerobes, therefore unable to survive in oxic environments and, as mentioned before, capable of utilizing a limited number of substrates to produce methane (Liu et al. 2012). All known methanogens possess a core enzyme that catalyses the final reduction in methanogenesis, which is methyl-coenzyme M reductase (MCR), making it possible to detect

them in the environment by targeting the gene mcrA, which encodes the α-subunit of this enzyme (Denman et al. 2007; Juottonen et al. 2006; Luton et al. 2002)

Methanogens are most abundant in environments with the lack of alternative electron acceptors, such as Fe^{3+}, O_2, NO_3^- and SO_4^{2-}. They are most commonly found in places like deep subterranean habitats, flooded soils, gastrointestinal tracks of humans and animals and oil reservoirs (Whitman et al. 2006). Also, methanogens have been reported to be able to survive in a wide range of temperatures (15 ºC to 100 ºC) and pH (3 to 10) (Zinder 1993). Currently, there are fourteen known families of methanogenic archaea and some characteristics of a few of these families are listed in the Table 1.1.

Table 1.1 – General characteristics of some methanogenic archaea families

	Characteristics				
Family	**Shape**	**Substrates for methanogenesis**	**Gram stain**	**Motility**	**GC content (mol %)**
Methanobateriaceae	Long or short rods	$H_2 + CO_2$, formate, alcohols	Mostly positive	Non motile	23-61
Methanothermaceae	Rods	$H_2 + CO_2$	Positive	Non motile	33-34
Methanococcaceae	Irregular cocci	$H_2 + CO_2$ and formate	Negative	Motile	29-34
Methanomicrobiaceae	Rods, spirals, plates or irregular cocci	$H_2 + CO_2$, formate and alcohols	Negative	Motile or non motile	39-61
Methanocorpusculaceae	Small irregular cocci	$H_2 + CO_2$, formate and alcohols	Negative	Motile or non motile	48-52
Methanospirillaceae	Curved rods	$H_2 + CO_2$, formate	Mostly negative	Motile	40-45
Methanosarcinaceae	Pseudosarcina, irregular cocci, sheathed rods	$H_2 + CO_2$, acetate and methyl compounds	Positive or negative	Frequently non motile	36-52
Methanosaetaceae	Sheathed rods	Acetate	Negative	Non motile	49-57

Adapted from: Whitman et al. 2006; Zinder 1993; Barber et al. 2011; Mori et al. 2012; Iino et al. 2010; Zhou et al. 2014; Parshina et al. 2014.

Each of these families is divided in genera: in the *Methanobacteriaceae* family, there are *Methanobacterium*, *Methanobrevibacter* and *Methanosphaera* and *Methanothermobacter* genera; *Methanosarcinaceae* includes the *Methanosarcina, Methanosalsum, Methanomethylovorans, Methanolobus, Methanohalophilus, Methanolabium, Methanococcoides* and

Methanomicrococcus genera; *Methanosaetaceae* contains the *Methanosaeta* genus; and *Methanospirillaceae* contains the *Methanospirillum* genus. There are also other methanogens that are unaffiliated to any of these families as they require a more thorough analysis to define their taxonomic classification (Whitman et al. 2006).

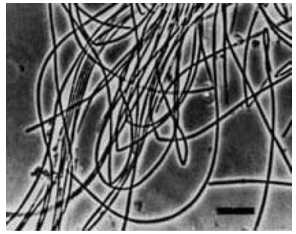
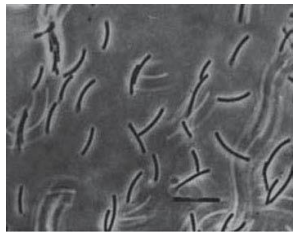
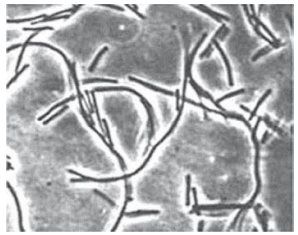
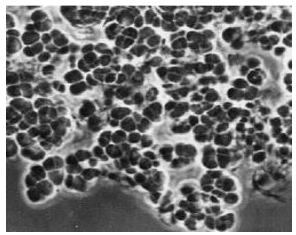
The most abundant species of methanogens found in anaerobic reactors include *Methanospirillum hungatei, Methanosarcina mazei*, *Methanosaeta concilii* and *Methanobacterium formicicum* (Tale et al. 2015; Silva et al. 2016; Chen, Rotaru, Liu, et al. 2014; Leclerc et al. 2004; Kato et al. 2012; Salvador et al. 2013). In Table 1.2, some of the main characteristics of these four different species are represented.

1.2 Syntrophy and interspecies electron transfer in microbial anaerobic communities

Methanogens are unable to use complex organic molecules as substrates, thus requiring them to be degraded into more simple compounds before they can be used for methanogenesis. The key for making this happen lies in syntrophic interactions between different species belonging to distinct taxonomic and metabolic groups (McInerney et al. 2008). Syntrophy was first noticed in 1967 by Marvin P. Bryant and his colleagues when they were conducting a study with a *Methanobacillus omelianskii* culture that lost its ability to use ethanol as a substrate. It was at that time that they were able to identify two distinct microorganisms in the culture, instead of only one: the S-organism, which fermented ethanol to acetate and hydrogen and *Methanobacterium bryantii* strain MoH that used the hydrogen, creating favourable conditions for the growth of the S-organism (Stams & Plugge 2009). The culture of *Methanobacillus omelianskii* was then found to be in fact a syntrophic co-culture.

According to Schink et al. (1997), syntrophic interactions can be defined as "cooperations in which both partners depend on each other to perform the metabolic activity observed and in which the mutual dependence cannot be overcome by simply adding a co-substrate or any type of nutrient". So as H_2 and formate are the major electron carriers, methanogens become crucial to syntrophy because they can efficiently remove either hydrogen or formate from the environment. Bacteria are crucial as well due to their role in degrading more complex molecules and providing the substrates for methanogenesis.

Table 1.2 – Characteristics of mesophilic methanogens *M. concilii, M. hungatei, M. formicicum, M. mazei*

Methanogenic species	Microscopic images	Substrate	Cell envelope structure	References
M. concilii		Acetate	S-layered sheath overlying an amorphous granular layer; spacer plugs subunits form concentric rings	(Albers & Meyer 2011; Whitman et al. 2006)
M. hungatei		H_2/CO_2; formate	S-layered sheath overlying a single S-layered wall; multilayered spacer plugs between cells; intermediate amounts of tetraether lipids in plasma membrane	(Migas et al. 1989; Whitman et al. 2006; Beveridge & Schultze-Lam 1996)
M. formicicum		H_2/CO_2; formate	Amorphous cell wall of pseudomurein; plasma membrane with intermediate amounts of tetraether lipids	(Battumur et al. 2016; Beveridge & Schultze-Lam 1996; Whitman et al. 2006)
M. mazei		Acetate; H_2/CO_2 (poor growth)	Amorphous cell wall of methanochondroitin; over top of a single S-layer; few tetraether lipids in plasma membrane	(Osumi et al. 2008; Beveridge & Schultze-Lam 1996; Rivkina et al. 2007)

Anaerobic growth on propionate is an example of a syntrophic interaction, in which methanogens live off the products of proton-reducing bacteria that metabolize propionate, producing acetate and H_2 that are then used by methanogens for methane production. These reactions and others that occur in obligate methanogenic syntrophic cultures are shown in Table 1.3.

Syntrophic microorganisms can be either facultative or obligate, depending whether or not they can survive in environments with high H_2 partial pressure. For example, during glycolysis, glucose is converted to acetate, releasing CO_2, H_2 and H^+ as a reduced product (Equation 1.1)

$$C_6H_{12}O_6 \rightarrow 2CH_3COO^- + 2CO_2 + 4H_2 + 4H^+ \quad (\Delta G = -216\ kJ/mol) \quad \text{Eq. 1.1}$$

In the midst of this process, NADH is produced by oxidation of glyceraldehyde-3-phosphate to 3-phosphoglycerate. However, in the presence of high amounts of H_2, this oxidation is no longer thermodynamically possible (Stams & Plugge 2009). Some facultative bacteria are able to oxidize NADH by reducing intercellular metabolites in energetic favourable reactions, compensating the lack of H_2 depletion by methanogens. In the downside of this ability to oxidize NADH on their own, these bacteria end up losing some energy potential, as their ATP gain would be higher when H_2 utilizing anaerobes are present (Stams & Plugge 2009).

Stams & Plugge (2009) defined obligate syntrophic consortia as the following:

- Microorganisms are capable of degrading fatty acids while working together, but individually neither the methanogens nor the bacteria can;
- They grow in conditions close to the thermodynamic equilibrium;
- Due to the influence of the inter-microbial distances in biodegradation and specific growth rates, both microorganisms form aggregates;
- Sharing of chemical energy is possible due to evolved biochemical mechanisms.

Apart from hydrogen, the acetate that is formed during syntrophic reactions listed in Table 1.3 can also be used by acetoclastic methanogens for methane production according to the following reaction:

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad (\Delta G = -36\ kJ/mol) \quad \text{Eq. 1.2}$$

A good example of a microorganism capable of establishing syntrophic interactions with methanogens is *Syntrophomonas wolfei*. This species is considered a metabolic specialist that metabolizes saturated

short-chain fatty acids in syntrophic association with hydrogen and formate users, and can only grow alone in crotonate, an unsaturated fatty acid (Sieber et al. 2010). Other species such as *Syntrophomonas zehnderi* and *Pelotomaculum schinkii* are obligate syntrophs, thus unable to grow alone in any substrate (McInerney et al 2008).

One common group in methanogenic aggregates present in wastewater digesters treating wastewater from brewery industry (therefore, in the presence of ethanol) is *Geobacter* (Rotaru et al. 2014a). *Geobacter sulfurreducens* and *Geobacter metallireducens* can grow together in anaerobic medium containing fumarate as the electron acceptor and ethanol as the electron donor. This was the first documented case of direct interspecies electron transfer (DIET) (Summers et al. 2010). This syntrophic relationship is described in detail in Section 1.2.4.

Electron exchange between species in microbial communities is still a mechanism not completely understood, but it is a fundamental process in methanogenic environments (Shrestha & Rotaru 2014). Interspecies electron transfer is the way that microorganisms transfer energy between each other and can be conducted in four different ways as shown in the following diagram (Figure 1.2).

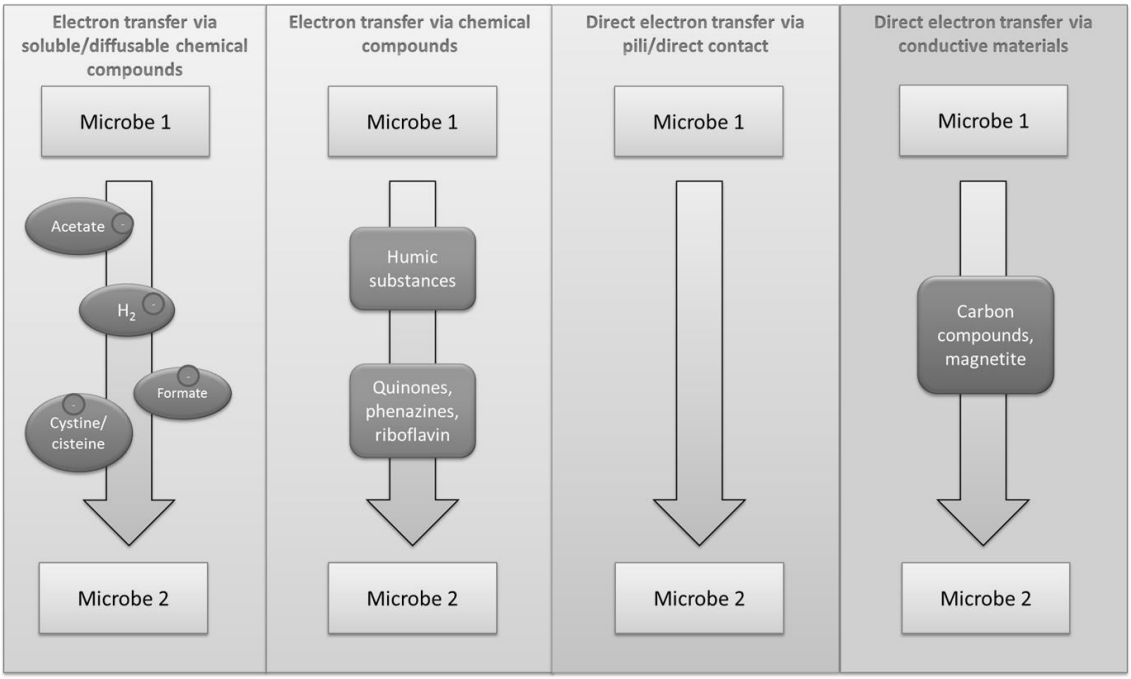


Figure 1.2 – Different mechanisms for interspecies electron transfer; adapted from Morris et al. 2013.

1.2.1 Indirect electron transfer via H_2/formate

The most commonly observed mechanism of electron transfer in methanogenic communities is the indirect electron transfer via H_2/formate (Sieber et al. 2014). Hydrogen acts as a shuttle in many syntrophic interactions because it is a small molecule and can easily diffuse in the medium and it is a powerful electron donor under anaerobic conditions. The task of keeping hydrogen at low concentrations is attributed to the hydrogenotrophic methanogens and their ability of removing the hydrogen in metabolic processes (Sieber et al. 2012). The acetogens' metabolism is inhibited when hydrogen concentration rises and the methanogens are stimulated, consuming the hydrogen formed which will then in turn stimulate the acetogenic activity (Sieber et al. 2012)

The following chain of reactions is an example of the chemical reaction that occurs when a bacterium (ethanol consumer) and a hydrogenotrophic methanogen are in syntrophy and interspecies hydrogen transfer takes place:

First, the metabolism of ethanol produces acetate with the release of electrons (Equation 1.3):

$$CH_3CH_2OH + H_2O \rightarrow CH_3COOH + 4H^+ + 4e^- \quad (\Delta G = 9.6 \text{ kJ/mol}) \qquad \text{Eq. 1.3}$$

Then, the bacteria use these electrons to produce hydrogen, through different reactions (Equation 1.4 to Equation 1.7):

$$2Fd_{(red)} + 2H^+ \rightarrow 2Fd_{(ox)} + H_2 \quad (\Delta G = 3 \text{ kJ/mol}) \qquad \text{Eq.1.4}$$

$$Formate + H^+ \rightarrow CO_2 + H_2 \quad (\Delta G = -3 \text{ kJ/mol}) \qquad \text{Eq.1.5}$$

$$NADH + H^+ \rightarrow NAD^+ + H_2 \quad (\Delta G = 18 \text{ kJ/mol}) \qquad \text{Eq.1.6}$$

$$FADH_2 \rightarrow FAD + H_2 \quad (\Delta G = 37 \text{ kJ/mol}) \qquad \text{Eq.1.7}$$

Finally, H_2 serves as an electron donor for methane production, according to Equation 1.8:

$$4H_2 + CO_2 \rightarrow 2H_2O + CH_4 \quad (\Delta G = -130.6 \text{ kJ/mol}) \qquad \text{Eq.1.8}$$

For indirect electron transfer via formate, the global reaction is as follows (Equation 1.9):

$$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O \quad (\Delta G = -144.543 \text{ kJ/mol}) \qquad \text{Eq.1.9}$$

Table 1.3 - Reactions occurring in obligate methanogenic syntrophic cultures; adapted from (Zinder 1993)

Substrate	Examples of organisms	Reaction	Total $\Delta G^{\circ\prime}$ (kJ/mol CH_4)	T_d (h)
Ethanol	*Geobacter metallireducens*	$2EtOH + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 4H_2$	-116.3	<24
	Hydrogenotrophic methanogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$		
	Overall reaction	$2EtOH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$		
Butyrate	*Syntrophomonas wolfei*	$2Butyrate + 4H_2O \rightarrow 4CH_3COO^- + H^+ + 4H_2$	-39.4	84
	Hydrogenotrophic methanogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$		
	Overall reaction	$2Butyrate + HCO_3^- + H_2O \rightarrow 4CH_3COO^- + H^+ + CH_4$		
Propionate	*Syntrophobacter wolinii*	$4Propionate + 12H_2O \rightarrow 4CH_3COO^- + 4HCO_3^- + 4H^+ + 12H_2$	-34	168
	Hydrogenotrophic methanogen	$12H_2 + 3HCO_3^- + 3H^+ \rightarrow 3CH_4 + 9H_2O$		
	Overall reaction	$4Propionate + 3H_2O \rightarrow 4CH_3COO^- + HCO_3^- + H^+ + 3CH_4$		
Benzoate	*Syntrophus buswelii*	$4Benzoate + 28H_2O \rightarrow 12CH_3COO^- + 4HCO_3^- + 12H^+ + 12H_2$	-15.8	168
	Hydrogenotrophic methanogen	$12H_2 + 3HCO_3^- + 3H^+ \rightarrow 3CH_4 + 9H_2O$		
	Overall reaction	$4Benzoate + 19H_2O \rightarrow 12CH_3COO^- + HCO_3^- + 9H^+ + 3CH_4$		
Acetate	*Acetate oxidizing bacteria*	$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2$	-31	36
	Methanogen	$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$		
	Overall reaction	$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4$		

1.2.2 Indirect electron transfer via chemical compounds

Chemical compounds such as humic substances can also act as electron shuttles that allow interspecies electron transfer. Lovley et al. (1999) demonstrated that quinone structures present in humic substances, can mediate electron transfer between humics-reducing and humics-oxidizing microorganisms. Electron transfer between *Geobacter metallireducens* and *Wolinella succinogenes* could be mediated by anthraquinone-2,6-disulphonate (AQDS), a humic analogue (Lovley et al. 1999). In this specific case, *G. metallireducens* oxidizes acetate and reduces AQDS, which is further oxidized by *W. succinogenes* with reduction of fumarate. Oxidation of acetate without AQDS could not happen since *G. metallireducens* does not have the ability to reduce fumarate and *W. succinogenes* is unable to oxidize acetate. These quinone structures are reduced to a hydroquinone form that can either react with Fe(III) or act as an electron donor for anaerobic microbial respiration. Therefore, humic substances and soluble quinones may play an important environmental role as mediators of exocellular electron transfer, since they can also act in other reduction processes, like azo-dye cleavage, reduction of nitroaromatics and reduction of uranium (Stams et al. 2006), as well as act as a terminal electron acceptor in the anaerobic oxidation of aromatic hydrocarbons such as toluene and benzene (Lovley et al. 1996; Cervantes et al. 2001). Aside from humic substances, other soluble chemical compounds, such as phenazines, melanin and riboflavin can also play a similar role in the mediation of interspecies electron transfer, with riboflavin being a particularly efficient mediator in azo-dye reduction (dos Santos et al. 2004).

1.2.3 Direct interspecies electron transfer (DIET)

An alternative mechanism for electron transfer in methanogenic environments is DIET, in which electrons are transferred from one species to another directly, without the need for electron exchange through soluble molecules (Rotaru et al. 2014a) or mediated by other compounds, as discussed in previous sections. DIET was first reported by Summers et al. (2010) in defined co-cultures of *Geobacter sulfurreducens* and *G. metallireducens* grown in a medium with ethanol as the electron donor and fumarate as the electron acceptor. In these co-cultures, it was known that *G. metallireducens* could metabolize ethanol, but was unable to use fumarate as an electron acceptor, while *G. sulfurreducens* could reduce fumarate, but was unable to metabolize ethanol (Summers et al. 2010). Co-cultures were grown with a strain of *G. sulfurreducens* in which the *hyb* gene that encodes a hydrogenase subunit was deleted. Previous studies had shown that the *G. sulfurreducens* strain in which the *hyb* gene was

deleted was unable to use hydrogen. Therefore, it was suggested that both *Geobacter* were most likely exchanging electrons directly by forming large electrically conductive aggregates, since the acetate produced by *G. metallireducens* could not be used as an electron donor either, because it was consumed by *G. sulfurreducens* (Summers et al. 2010). Both species are capable of establishing electrical connections through their pili, which makes this direct electron exchange possible (Rotaru et al. 2014a). Knowledge of this process is still very limited and further studies are required to understand and evaluate the impact of DIET on methane producing environments.

Recent studies have showed that the acetoclastic specialist *Methanosaeta harundinacea* possesses the ability to accept electrons via DIET and that DIET can possibly predominate over interspecies H_2/formate transfer during anaerobic digestion (Rotaru et al. 2014a). However, the electron accepting components that allow *Methanosaeta* to participate in DIET are not known, although a mechanism has been hypothesized by Rotaru et al (2014). In this study, co-cultures of ethanol-metabolizing *G. metallireducens* and strictly acetoclastic methanogen *M. harundinacea* were grown. As depicted in Equation 1.3, ethanol oxidation results in the formation of acetate and the release of electrons and, in order for the ethanol present in these cultures to be completely metabolized, it was necessary for *M. harundinacea* to utilize the electrons released, as well as the acetate formed. The results showed that ethanol was completely consumed, indicating that the methanogen was able to utilize the electrons released when ethanol was consumed by the bacteria and, since *M. harundinacea* is unable to use hydrongen as an electron acceptor, it was suggested that they were exchanging electrons directly (Rotaru et al. 2014a). Furthermore, some methanogens, namely *M. hungatei* and *M. formicicum* are unable to form co-cultures with *G. metallireducens* (Rotaru et al. 2014a), suggesting that not all methanogenic archaea have the ability of exchanging electrons directly.

DIET can be a more efficient way of interspecies electron transfer when compared to indirect electron transfer if we take into account that this mechanism has a greater potential for energy conservation, since no electron carriers are involved (Lovley 2011), so it could represent a very interesting alternative for improving anaerobic degradation and methane production rates.

1.2.4 Direct electron transfer via conductive materials

Many studies have pointed that a variety of conductive materials can promote interspecies electron transfer (Lovley 2011; Sieber et al. 2012; Shrestha & Rotaru 2014). Methanogens could be enriched from rice paddy field soil samples incubated with acetate or ethanol and in the presence of semiconductive minerals, such as magnetite and haematite (Kato et al. 2012). These materials could

accelerate methanogenesis in terms of onset time and production rate, and caused a significant enrichment of *Geobacter* and *Methanosarcina* species (Kato et al. 2012). Moreover, another recent study performed with magnetite suggested that magnetite could compensate for the lack of pilin-associated cytochrome OmcS of *G. sulfurreducens* to mediate electron transfer between *G. sulfurreducens* and extracellular electron donors or acceptors (Liu et al. 2014). It was also verified in the same study that OmcS gene expression by *G. sulfurreducens* decreased in the presence of magnetite which could mean that the bacteria adapted to the conductive materials, spending less energy in the cytochrome biosynthesis to facilitate electron transfer.

In anaerobic digesters, granular activated carbon (GAC) has been used to stimulate the metabolism in reactors' start-up, as well as to deal with reactor instabilities (Rotaru et al. 2014; Liu et al. 2012). Therefore, GAC was evaluated in its ability to promote DIET and it was found that in co-cultures with *G. metallireducens* and *Methanosarcina barkeri*, methane was produced in large amounts and with a very small or non-existent lag phase, when compared to the co-cultures with the same microorganisms in the absence of GAC (Liu et al. 2012).

Biochar, which is a charcoal-like product of the incomplete combustion of biomass in a limited supply of oxygen, used mostly as soil amendment, is a conductive material and has been shown to promote DIET between syntrophic co-cultures in a study performed with co-cultures of *G. metallireducens* and either *G. sulfurreducens* or *M. barkeri* with ethanol as an electron donor (Chen et al. 2014b). The methanogenic co-cultures (with *G. metallireducens* and *M. barkeri*) in the presence of biochar were able to utilize 86% of the electrons that resulted from ethanol metabolism for methane production, while the co-cultures grown in the same conditions but without biochar did not register any methane production or ethanol consumption at all. Also, *G. metallireducens* and *M. barkeri* did not form aggregates, but attached to the biochar, which suggests that the electrons were exchanged via electrical connections formed by the biochar, rather than biological connections (Chen et al. 2014b).

Another example of a conductive material used in the study of DIET is carbon cloth. In a similar study as the one performed with biochar, carbon cloth was also added to co-cultures of *G. metallireducens* and *G. sulfurreducens* or *M. barkeri* (Chen et al. 2014a). It was showed that this carbon material accelerated the ethanol consumption and methane production by the co-cultures. While co-cultures not amended with carbon cloth took 40 days to start metabolizing ethanol, comparing to the 10 days necessary to start this process in the presence of carbon cloth (Chen et al. 2014a).

All these materials have one thing in common: they are all highly conductive and can influence interspecies electron exchange, even though the mechanisms of action are not always well understood.

1.3 Carbon nanotubes (CNT)

Due to their properties, conductive materials in a nanoscale can favour microbial extracellular electron transfer, which can play an important role in biogeochemical cycles, bioremediation and several bioenergy strategies (Liu et al. 2014). CNT are long cylindrical hollow structures, with a diameter rounding the order of a nanometer, whose walls are formed by a single layer of carbon atoms bonded in an hexagonal lattice, named graphene (Javey & Kong 2009). They are known for their unique physical properties, such as their large surface area and strength despite their small dimensions, and can be excellent semiconductors (Javey & Kong 2009). They also have been reported to improve settleability and dewaterability in activated sludge under aerobic conditions, producing no negative effects (L. L. Li et al. 2015). In addition, the conductive properties of carbon nanotubes make them function as great redox mediators, accelerating electron transfer from a primary electron donor to a terminal electron acceptor (Pereira et al. 2014), and can possibly facilitate reactions in methanogenic communities. There are two types of carbon nanotubes in terms of structure: single-walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). While SWCNT can be thought of as a graphene sheet rolled up to form a seamless cylindrical structure, MWCNT are arranged by multiple sheets as shown in Figure 1.3 (Vidu et al. 2014; Zhang et al. 2013).

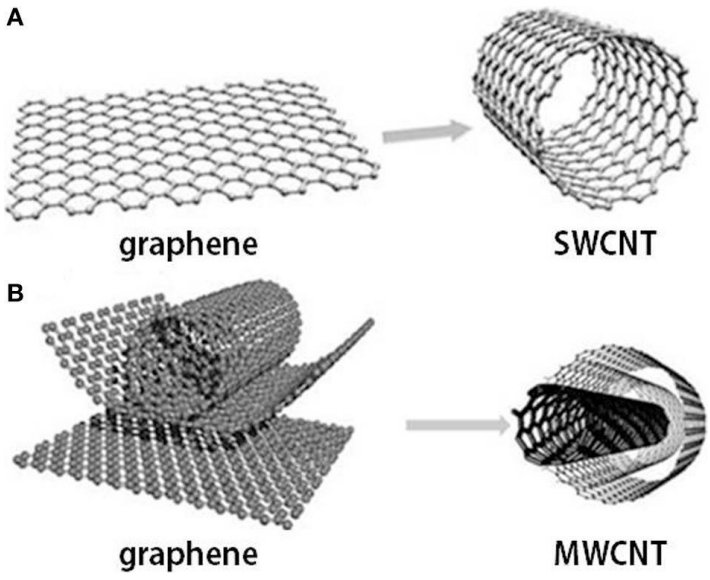


Figure 1.3 – Graphene sheets folded up as A) SWCNT; and B) MWCNT.

Besides the number of walls, SWCNT and MWCNT also have other characteristics that distinguish them. Both types of CNT have a high specific surface area that depends on a number of properties, such as the number of walls but ,theoretically the specific surface area of a single SWCNT exceeds that

of the MWCNT (Peigney et al. 2001). However, when CNT form aggregates, the surface area decreases significantly in the case of the SWCNT (Zhang et al. 2009). Also, the SWCNT have a diameter of about 1 nm, while the diameter of MWCNT has a wider range that depends on the number of walls (Peigney et al. 2001).

CNT influence on anaerobic communities (complex cultures and co-cultures) still needs investigation. Recent studies have demonstrated that CNT can improve acetate consumption and methane production in anaerobic granular sludge containing a great amount of the acetotrophic methanogen *Methanosaeta concilii* (L. L. Li et al. 2015). This fact allied to the much higher conductivity verified in anaerobic granular sludge, due to the exposure to CNT, suggests that these materials may simulate methane production by *M. concilii*. CNT also have the ability to alter electron flow routes of microbial communities (Yan et al. 2013). In this case, *Shewanella oneidensis*, a metal-reducing bacterium that relies on extracellular electron transfer for its respiration, was studied for its ability to reduce nitrobenzene in the presence of CNT. It was shown that the presence of CNT led to a significantly faster reduction of nitrobenzene by immobilized cells, which suggests that they were involved in the extracellular electron transfer process (Yan et al. 2013).

CNT may however be toxic to microorganisms and can affect their growth. Kang et al (2007) demonstrated that SWCNT can have a strong antimicrobial activity in a study performed with *Escherichia coli*. In addition, MWCNT were studied for their effects in the microbial activity of upflow anaerobic sludge blanket reactors (UASB) and it was found that their presence resulted in a reduced microbial viability, as well a reduction in VFA and biogas production (Yadav et al. 2016). Another study performed by Luongo & Zhang (2010) showed that MWCNT caused inhibition in respiration by aerobic sludge, having a toxic effect that was intensified with higher concentrations.

Cytotoxicity of these CNT seems to be related to the damage of cell walls when direct contact is established and this effect seems to be more evident with SWCNT than with MWCNT (Pasquini et al. 2012; Vecitis et al. 2010; Kang et al. 2008). Kang et al. (2008) performed a study with *E. coli* in which was verified that the diameter of CNT was an important factor in their cytotoxicity towards bacteria. A concentration of 0.3 g/L of CNT was enough to cause a major loss in cell viability that was over 80% in the SWCNT case, while it was of over 20% in the case of MWCNT (Kang et al. 2008). Some authors have hypothesized that both aggregate morphology and dispersitivity of the CNT are the main factors affecting cytotoxicity (more than the physiochemical properties of the functional groups present in CNT), meaning that they can be manipulated to be less harmful to the microorganisms (Pasquini et al. 2012).

2. AIMS AND MOTIVATION

Electrons are exchanged between anaerobic microorganisms when they are performing AD. This is a fundamental process in methane production and it can be aided by the presence of conductive materials, in order to improve its efficiency in energy conservation. Also, there is still much room for comprehension of the mechanisms involved in interspecies electron transfer by methanogenic communities. Therefore, the research done in the framework of this thesis aimed to study the effect of conductive materials in methanogenic cultures and how we can improve methane production in a larger scale.

The mechanisms involved in interspecies electron transfer in a methanogenic consortium were studied, so that they can be better understood. In order to achieve this, the three main goals were:

1) to assess the influence of CNT on CH_4 production rate by different methanogenic archaea, both hydrogenotrophic and acetoclastic methanogens;
2) to assess the effect of CNT on the syntrophic conversion of butyrate to CH_4;
3) to adapt syntrophic consortia to certain substrates in anaerobic reactors and the posterior application of CNT, in order to determine their effect on methane production.

3. EFFECT OF CNT IN METHANOGENIC PURE CULTURES

3.1 Introduction

Carbon materials have been reported to influence methane production by methanogenic aggregates and anaerobic granular sludge (Liu et al. 2012; Chen et al. 2014b; L. L. Li et al. 2015). It has been reported that materials such as CNT, graphite, biochar, carbon cloth and GAC improved methane production by the methanogenic communities (Liu et al. 2012; Chen et al. 2014a; Chen et al. 2014b; L. L. Li et al. 2015; Zhao et al. 2015). Some authors suggested that carbon materials participate in interspecies electron transfer, between *Geobacter* species and acetoclastic methanogens from genera *Methanosaeta* (Rotaru et al. 2014a) and *Methanosarcina* (Rotaru et al. 2014b). However, the effect of multi wall carbon nanotubes directly on pure cultures of methanogens was never studied before. Therefore, in this Chapter, pure cultures of hydrogenotrophic and acetoclastic methanogens were incubated with CNT in order to investigate its influence on methanogenesis directly and also to provide a better comprehension on the processes in which methane production by microbial communities, such as anaerobic sludge or defined co-cultures, is improved when carbon materials are present. The methanogenic species selected for conducting these studies are commonly found in anaerobic sludge (Leclerc et al. 2004; Kato et al. 2012; Salvador et al. 2013; Chen, Rotaru, Liu, et al. 2014; Tale et al. 2015; Silva et al. 2016): *Methanobacterium formicicum* and *Methanospirillum hungatei* as hydrogenotrophic methanogens, meaning that they convert hydrogen and carbon dioxide to methane (Equation 3.1), and *Methanosaeta concilii* and *Methanosarcina mazei* as acetoclasts, which produce methane and carbon dioxide from acetate (Equation 3.2).

Methane production from hydrogen and CO_2 by hydrogenotrophic methanogens:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \text{Equation 3.1}$$

Methane production from acetate by acetoclastic methanogens:

$$CH_3COOH^- \rightarrow CH_4 + CO_2 \qquad \text{Equation 3.2}$$

3.2 Materials and methods

3.2.1 Preparation and incubation of the pure cultures

Pure cultures of four different methanogens namely, *Methanobacterium formicicum* (DSM 1535), *Methanosaeta concilii* (DSM 3671), *Methanosarcina mazei* (DSM 2053) and *Methanospirillum hungatei* (DSM 864) were grown in anaerobic medium with and without CNT, in order to evaluate the effect of CNT on methane production rate. For each pure culture, different concentrations of CNT were tested; 0 g/L, 1 g/L and 5 g/L, and all assays were carried out in triplicate.

Commercial carbon nanotubes (CNT, Nanocyl 3100) were used for this purpose. CNT are characterized by having a homogeneous size and morphology with aligned walls parallel to the main axis of the nanotubes (Tessonnier et al. 2009). They are highly conductive, have an extremely porous surface with a high area and a narrow tubular morphology with outer and inner tube diameters at an average of 10 nm and 4 nm, respectively (Tessonnier et al. 2009). The Nanocyl 3100 CNTs also have small amounts of debris in their interior as well as on the exterior when compared to other MWCNTs available in the market (Tessonnier et al. 2009).

The experimental assays were conducted in 120 mL serum bottles containing 45 mL of basal medium and 5 mL of inoculum (10 % v/v). Anaerobic basal medium was prepared according to Stams et al., (1993), and its detailed composition can be found in appendix 1. Resazurine (0.5 mg/L) was added as a redox indicator. After distributing the medium, serum bottles were closed with black butyl rubber stoppers and aluminum caps, before being pressurized (by using a manifold) with a gas mixture of 80% H_2 and 20% of CO_2 for growing the hydrogenotrophic cultures and 80% N_2 and 20% CO_2 mixture for growing acetoclastic methanogens. The medium was sterilized by autoclaving at 120 °C, at 1 Bar, during 20 min.

Active methanogenic cultures were maintained for several weeks. The inoculation of test vials was done by using the cultures during their exponential phase. Before inoculation the following solutions were added: sodium bicarbonate (4 g/L) for buffering the medium; salts and vitamins, required for growth (detailed composition is displayed in Appendix I; and sodium sulfide (240 mg/L) to reduce the medium. The bicarbonate solution used for the hydrogenotrophic cultures contained 0.33 g of acetate (approximately 0.17 g/L) which was added to serum bottles containing H_2/CO_2 for growing hydrogenotrophic methanogens, serving as an additional carbon source required for growth (Ekiel et al. 1983). For growing *M. concilii* and *M. mazei*, acetate was supplemented in a concentration of 20 mM and 10 mM,

respectively. The addition of solutions and inoculation of pure cultures were done under strict anaerobic and sterile conditions. Cultures were then incubated in the dark at 37 °C. For growing hydrogenotrophic cultures, serum bottles were incubated with agitation (120 rpm) in order to improve gas dissolution.
Abiotic control assays were prepared as described above (with H_2/CO_2 and with N_2/CO_2 + acetate), but without inoculation with methanogenic archaea.

3.2.2 Analytical methods

Methane and hydrogen accumulated in the bottles headspace were sampled with a gas tight syringe and quantified by gas chromatography (GC) by using a GC BRUKER SCION 456 connected to a TCD detector (MOLSIEVE/PLOT).
Acetate concentrations were determined by high performance liquid chromatography (HPLC) using an HPLC (Jasco, Tokyo, Japan) with a Chrompack column (67H) coupled to a UV detector at 210 nm. For that purpose, liquid samples were taken at the time of inoculation, during exponential phase and during stationary phase, centrifuged and the supernatant filtered by using a syringe filter (0.20 μm). Samples were stored at -20 °C until analysis by HPLC. Redox potential and pH were measured with a mV meter (Consort 533 VWR: 662-1409) and a pH/mV meter (WTW pH7110 VWR: 662-1767), respectively.

3.2.3 Scanning Electron Microscopy (SEM)

At the end of each experiment, samples were prepared for SEM analysis by filtering 1 mL through a 0.20 μm filter. Fixation of the samples was performed with a 2.5% (v/v) glutaraldehyde solution, made in PBS 1x buffer, for 2 hours, in order to stabilize and cross-link organic molecules within the cellular material, thus preserving the morphology and making the cells more resistant to subsequent preparation steps. After washing 3 times with PBS 1x buffer for 15 minutes each, samples were dehydrated through a series of ethanol baths of increasing concentrations (10, 25, 50, 75, 90 and 100% (v/v)) for the duration of 20 minutes each. Samples were then washed with 100% ethanol one last time for 30 minutes. Finally, samples were dried in a desiccator and after sputter coated with Au/Pt to increase conductivity. The electronic microscopic images were obtained with a Leica Cambridge S360 microscope (SEMAT/UM).

3.3 Results

Methane production by acetoclastic and hydrogenotrophic methanogens in the presence of CNT was followed in batch experiments. The obtained results are exposed in the following sections.

3.3.1 Methane production by pure cultures of *M. concilii*

The results obtained for *M. concilii* are depicted on Figure 3.1. When *M. concilii* cultures were incubated with 1 g/L and 5 g/L CNT, the methane production rate was affected as can be observed in Figure 3.1.

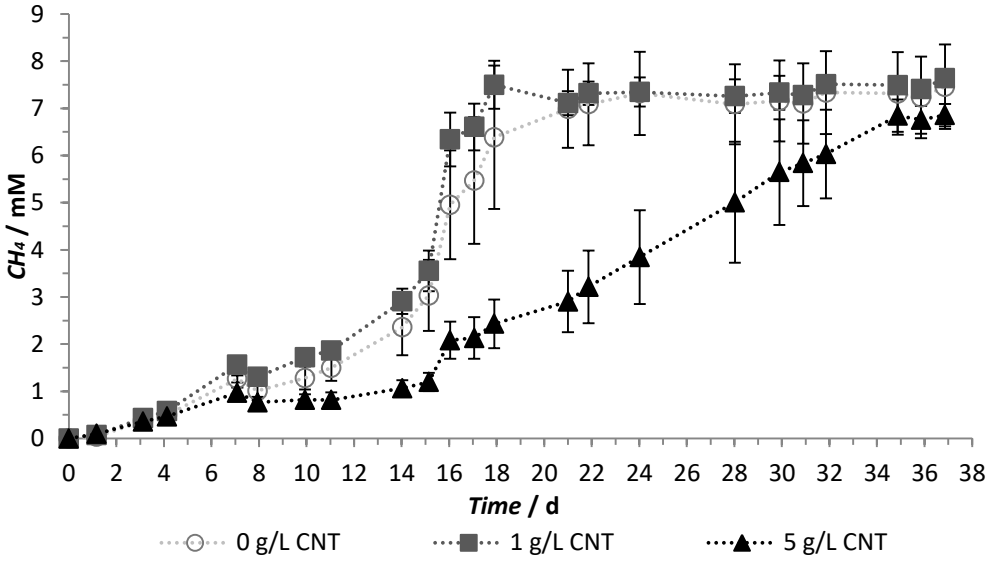


Figure 3.1 – Cumulative methane production by the acetoclastic methanogen *M. concilii* in the presence of different CNT concentrations.

The evolution of lag and exponential phases were similar in the control assay and in the assay containing 1 g/L CNT, while for 5 g/L CNT methane was produced at a slower rate, resulting in a less perceptive exponential phase. Maximum methane yield was reached after 18 days of incubation when *M. concilii* was exposed to 1 g/L of CNT, while in the presence of 5 g/L about 35 days were necessary for the complete conversion of acetate to methane.

Methane produced by *M. concilii* after 3, 7, 18 and 32 days of incubation, with and without CNT, is shown in Table 3.1. Three points of interest were chosen for the evaluation of the methane produced by the cultures with and without CNT in order to compare the results obtained. The amount of methane started off similarly within 3 days past the beginning of the assay and after that, results varied according to the concentration of CNT supplied to the cultures. At day 7, the control assay and 1 g/L

CNT cultures produced 2.36±0.08 mM and 2.87±0.22 mM respectively, while cultures with 5 g/L CNT produced only 1.06±0.17 mM. By the time the 0 g/L and 1 g/L CNT assays reached plateau phase at day 18, they had produced 6.39±1.52 mM and 7.19±0.49 mM respectively, having the 5 g/L CNT cultures produced only 2.43±0.513 mM. This means that the cultures supplied with 1 g/L CNT produced more methane and 5 g/L CNT cultures produced far less, when compared to control cultures.

Table 3.1 – Methane produced by *M. concilii* when incubated with different CNT concentrations

Incubation time (d)	*CNT* (g/L)	*Methane produced* (mM)	*CNT/Ct ratio* [a]	*Methane produced* (%) [b]
3	0	0.50 ± 0.03	—	7 ± 1
	1	0.57 ± 0.07	1.1 ± 0.1	7 ± 1
	5	0.47 ± 0.06	0.9 ± 0.1	6 ± 1
7	0	2.36 ± 0.08	—	33 ± 8
	1	2.89 ± 0.21	1.2 ± 0.1	37 ± 3
	5	1.06 ± 0.17	0.5 ± 0.1	15 ± 2
18	0	6.39 ± 1.52	—	88 ± 21
	1	7.45 ± 0.47	1.2 ± 0.1	97 ± 6
	5	2.43 ± 0.51	0.4 ± 0.1	34 ± 7
32	0	7.34 ± 0.85	—	100 ± 12
	1	7.56 ± 0.40	1.0 ± 0.1	100 ± 5
	5	6.03 ± 0.94	0.8 ± 0.1	88 ± 13

[a]CNT/Ct ratio represents the number of times the production of methane increased relatively to the control assay.

[b]The percentage of methane produced was calculated relatively to the maximum methane produced during the experiment, which correspond to the theoretical methane production expected assuming total conversion of the acetate provided at the beginning of the incubation.

Acetate was converted to methane by all cultures. The initial and final concentrations of acetate are presented in Table 3.2.

Table 3.2 – Initial and final acetate concentrations detected in *M. concilii* incubations and final methane concentration obtained

CNT (g/L)	*[acetate]i* (mM)	*[acetate]f* (mM)	*[CH_4]f* (mM)
0	7.23 ± 1.32	not detected	7.35 ± 0.88
1	7.71 ± 0.44	not detected	7.51 ± 0.35
5	7.24 ± 0.35	not detected	6.82 ± 0.29

The pH was monitored throughout the assay and showed a tendency to slightly increase from the beginning to the end of the incubation in all assays, with small differences between concentrations of CNT used. The results displayed in Table 3.3 show that redox potential (ORP) values of the growth medium were more negative for higher concentrations of CNT and decreased throughout the experiment.

The results of SEM analysis for each concentration of CNT studied are depicted in Figure 3.2. In the absence of CNT, cells appear to present more extracellular polymeric substances (EPS), than when CNT are present. In Figure 3.2D, the spacer plug that these cells form between them when they connect with each other can be seen. The CNTs have the same aspect in the abiotic assay as in the biotic assays.

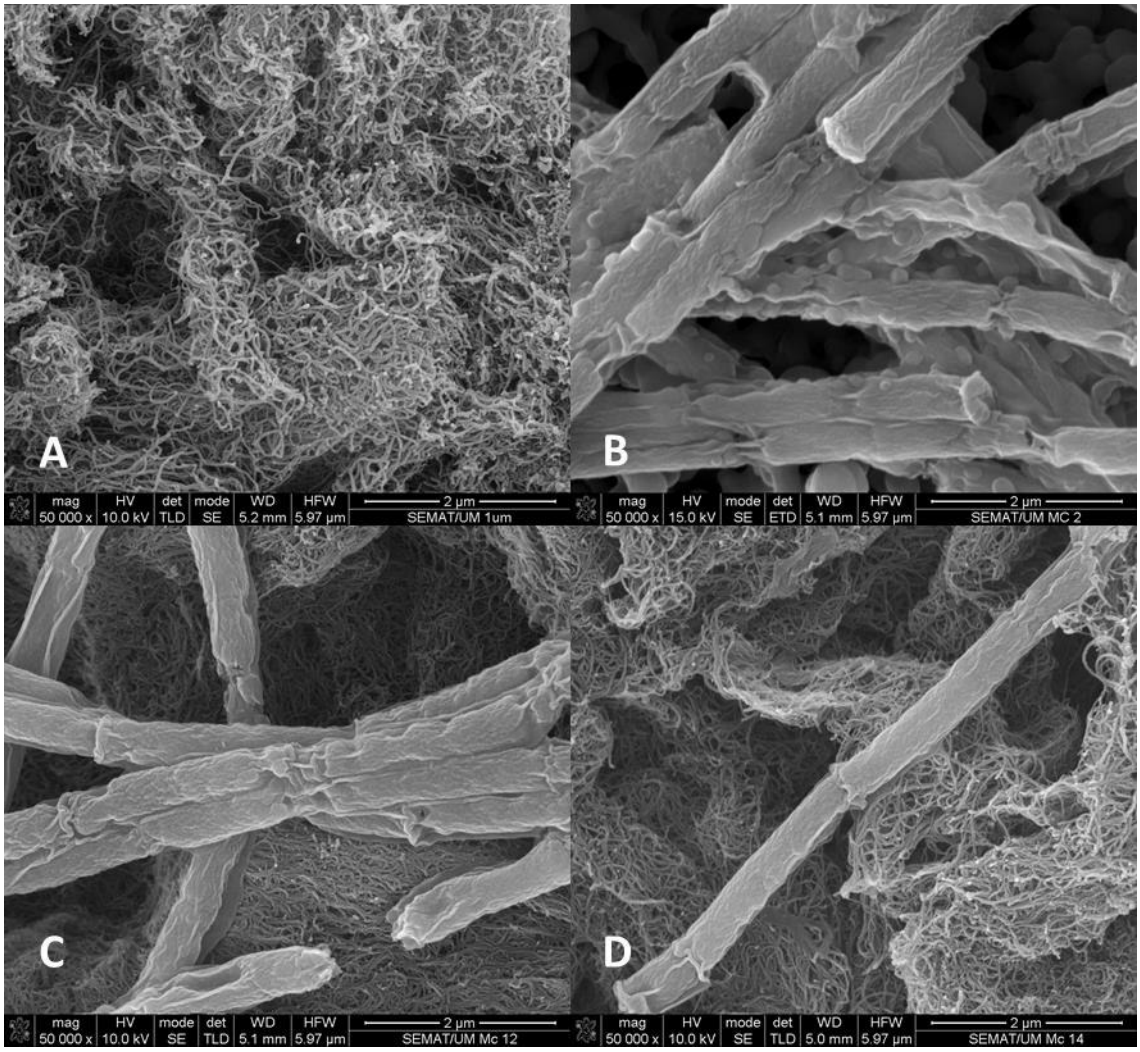


Figure 3.2 – SEM analysis of *M. concilii* with and without CNT. A) abiotic assay with only CNTs displayed with a concentration of 0.5 g/L; B) *M. concilii* cultures in the absence of CNT; C) *M. concilii* cultures with 1 g/L of CNT; D) *M. concilii* cultures with 5 g/L of CNT.

Table 3.3 – pH and ORP values measured in pure cultures containing *M. concilii* supplied with CNT in different concentrations

CNT (g/L)	*Sampling time*	*pH*	*ORP* (mV)
0	Beggining of incubation	7.23 ± 0.11	-278 ± 10
	End of incubation	7.38 ± 0.06	-307 ± 6
1	Beggining of incubation	7.18 ± 0.04	-293 ± 2
	End of incubation	7.29 ± 0.03	-326 ± 3
5	Beggining of incubation	7.11 ± 0.04	-334 ± 17
	End of incubation	7.23 ± 0.01	-345 ± 6

3.3.2 Methane production by pure cultures of *M. mazei*

Pure cultures of *M. mazei* grown with acetate as the substrate behaved differently to the presence of CNT when compared to *M. concilii*, the other acetoclastic methanogen tested. Methane production by *M. mazei* is illustrated in Figure 3.3.

This methanogen had a distinct answer when incubated with different concentrations of CNT. While the presence of CNT at a concentration of 1 g/L benefited the overall methane production, the cultures containing 5 g/L of CNT showed a lower methane production rate, when compared to the control cultures growing without CNT. As it can be seen in Figure 3.3, the control assay's curve of cumulative methane production remained in between both curves of cultures containing CNT, whereas the curves obtained for the cultures incubated with 1 g/L CNT and 5 g/L CNT were higher and lower respectively, for all points measured during incubation time.

Maximum methane yield was achieved earlier in the vials with 1 g/L of CNT, when compared to the others. The cultures supplied with 5 g/L of CNT were the ones that produced methane at a slower rate.

Methane produced after 8, 13 and 21 days of incubation are presented in Table 3.4. In an earlier phase (until 8 days of incubation), the amount of methane produced (approximately 2.3 mM) by the cultures incubated with 1 g/L CNT was higher than the amount in control cultures (approximately 1.5 mM), while in the cultures supplied with 5 g/L CNT methane production was lower (approximately 0.9 mM). As the experiment proceeded, the cultures supplied with 1 g/L CNT produced methane always at a higher rate, when compared to the cultures without CNT. However, with 5 g/L of CNT, the pure cultures grew at a slower rate, although in all conditions cultures ended up reaching a similar maximum methane yield by the end of incubation time, after 21 days (Figure 3.3). Nonetheless, cultures with 0 g/L and 1 g/L of CNT reached the maximum methane concentration faster than cultures incubated with 5 g/L of CNT.

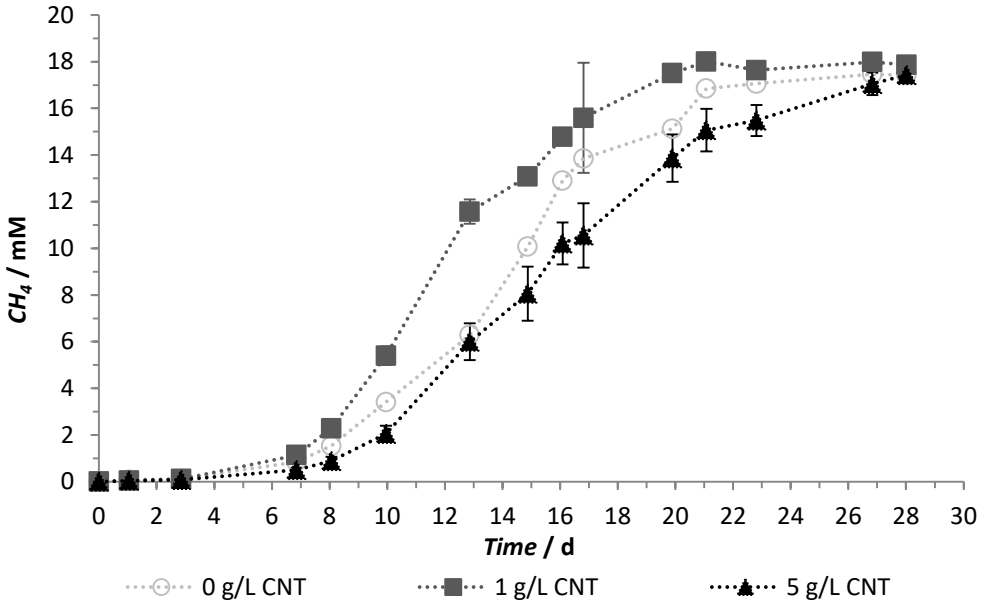


Figure 3.3 – Cumulative methane production by pure cultures of *M. mazei* in the presence of different CNT concentrations

Acetate was not completely consumed in any of the conditions (Table 3.5). Conversion of acetate to methane was similar for all CNT concentrations, although the cultures supplied with 5 g/L took longer to produce the same amount of methane.

Table 3.4 – Methane production by pure cultures of *M. mazei* with and without CNT at different time points of the assay

Incubation time (d)	*CNT* (g/L)	*Methane produced* (mM)	*CNT/Ct ratio* [a]	*Methane produced (%)* [b]
8	0	1.53 ± 0.38	—	7 ± 2
	1	2.28 ± 0.14	1.5 ± 0.1	10 ± 1
	5	0.89 ± 0.18	0.6 ± 0.1	4 ± 1
13	0	6.28 ± 1.10	—	29 ± 5
	1	11.57 ± 0.52	1.8 ± 0.1	51 ± 2
	5	6.00 ± 0.79	1.0 ± 0.1	28 ± 4
21	0	17.06 ± 0.27	—	77 ± 1
	1	17.64 ± 0.13	1.0 ± 0.0	80 ± 1
	5	15.48 ± 0.66	0.9 ± 0.0	70 ± 4

[a]CNT/Ct ratio represents the number of times the production of methane increased relatively to the control assay.

[b]The percentage of methane produced was calculated relatively to the maximum methane produced during the experiment, which correspond to the theoretical methane production expected assuming total conversion of the acetate provided at the beginning of the incubation.

Table 3.5 – Acetate consumption and methane produced by *M. mazei* with and without CNT

CNT (g/L)	*[acetate]i* (mM)	*[acetate]f* (mM)	*[CH4]f* (mM)
0	21.90 ± 1.84	1.65 ± 0.36	17.20 ± 0.29
1	22.48 ± 0.70	1.49 ± 0.43	17.80 ± 0.22
5	21.45 ± 1.06	1.41 ± 0.41	17.24 ± 0.27

The pH did not vary much during the experiment (Table 3.6). pH measured at the beginning of the incubation was similar in all the assays (7.08 for assays without CNT and with 1 g/L CNT, and 7.06 in the assays with 5 g/L CNT) and slightly increased at the end of the experiment (7.36 for 0 and 1 g/L and 7.35 for 5 g/L). On the other hand, as it was also observed in the *M. concilii*'s assays, CNT presence seemed to cause a decrease in ORP values.

Throughout the assays with acetoclastic methanogens, pH did not suffer big alterations in either case. There was a very small increase in both cases that evolved very similarly for each of the concentrations of CNT used for both organisms. No major variation of these values was expected in these pure cultures since no products formed in the process which methane is produced through the use of acetate have a direct influence in the pH of the media (Equation 3.2).

SEM electrophotography showed that *M. mazei* cells appeared as the typical aggregates in the absence of CNT and also in their presence (Figure 3.4). However, in the assays without CNT the cell aggregates seem bigger (formed by a higher number of sarcina cells) while in the assays with CNT smaller aggregates could be observed.

Table 3.6 – pH and ORP values measured throughout the *M. mazei* assay in the presence of CNT

CNT (g/L)	*Sampling time*	*pH*	*ORP* (mV)
0	Beggining of incubation	7.08 ± 0.01	-275 ± 3
	End of incubation	7.36 ± 0.02	-282 ± 10
1	Beggining of incubation	7.08 ± 0.02	-286 ± 3
	End of incubation	7.36 ± 0.02	-287 ± 8
5	Beggining of incubation	7.06 ± 0.02	-298 ± 1
	End of incubation	7.35 ± 0.03	-317 ± 2

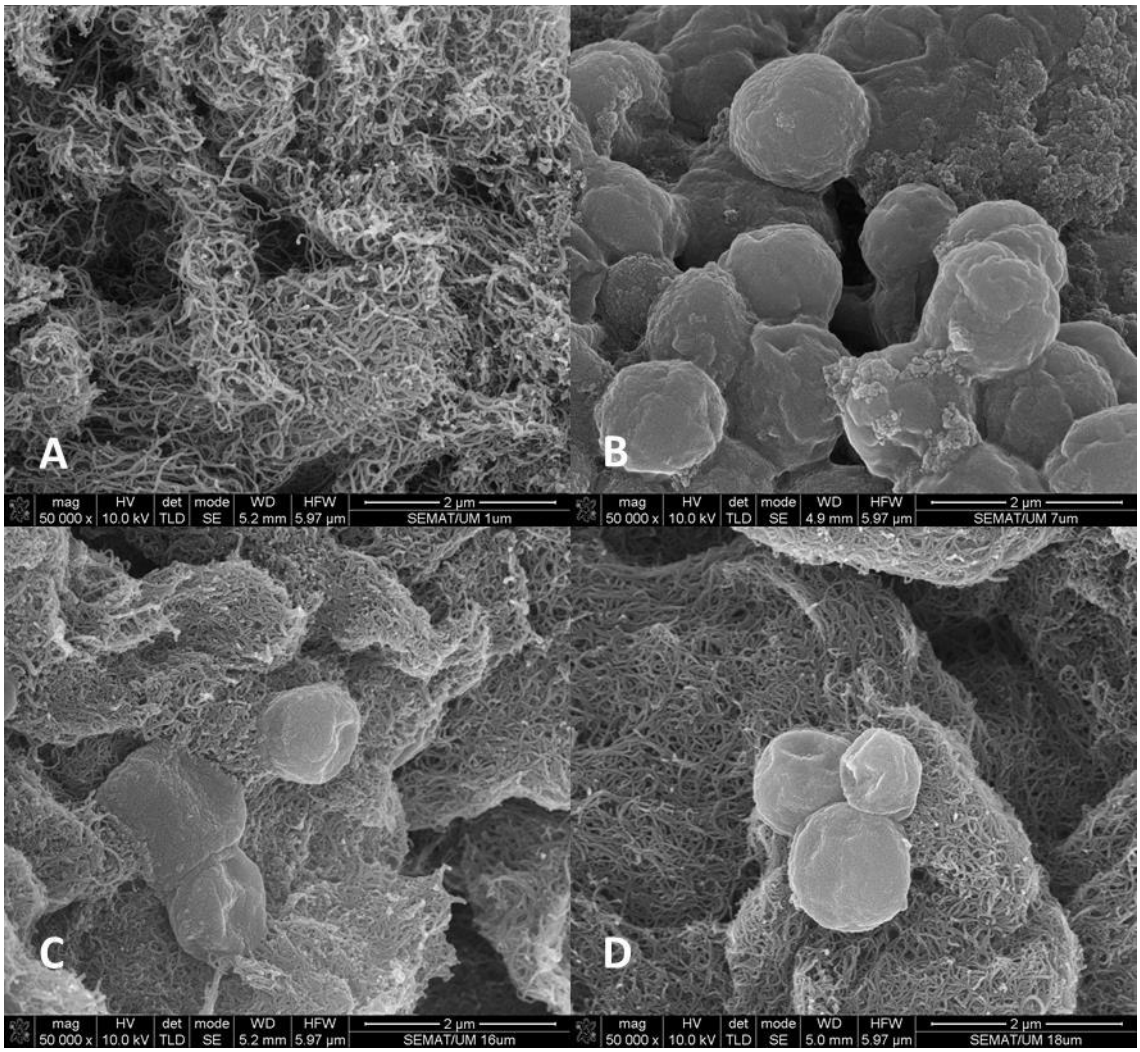


Figure 3.4 – SEM analysis of *M. mazei* with and without CNT. A) abiotic assay with only CNTs displayed with a concentration of 0.5 g/L; B) *M. mazei* cultures in the absence of CNT; C) *M. mazei* cultures with 1 g/L of CNT; D) *M. mazei* cultures with 5 g/L of CNT.

3.3.3 Methane production by pure cultures of *M. formicicum*

Pure cultures of *M. formicicum* were grown in the presence of CNTs. In Figure 3.5, the results of methane production along time can be observed.

As can be observed in Figure 3.5, the behavior of methane production by *M. formicicum* was different for increasing amounts of CNT. As with other methanogens, *M. concilii* and *M. mazei,* higher concentrations of CNT resulted in shorter (almost non-existent in the case of 5 g/L CNT) lag phases. The presence of CNT resulted in a faster growth rate, especially in cultures with 5 g/L CNT, with maximum methane yield being reached earlier than in the other assays, at around 9 days of incubation time. However, cultures containing 1 g/L of CNT only achieved the highest levels of methane after 14 days.

In the presence of 5 g/L CNT, approximately 31% of the H_2/CO_2 was already converted to methane during the first days of incubation, when compared to the assay without CNT (Table 3.7). This means that, after only 4 days of incubation, methane produced by *M. formicicum* cultures supplied with 5 g/L CNT increased more than sixteen times, when compared to the absence of CNT. After 8 days of incubation time, the difference between the methane produced in the control assay and in the assays with CNT was becoming progressively lower, but always higher than in the incubation without CNT. While the cultures incubated with 5 g/L CNT had already reached 91% of their maximum methane yield, control cultures had only reached approximately 63%.

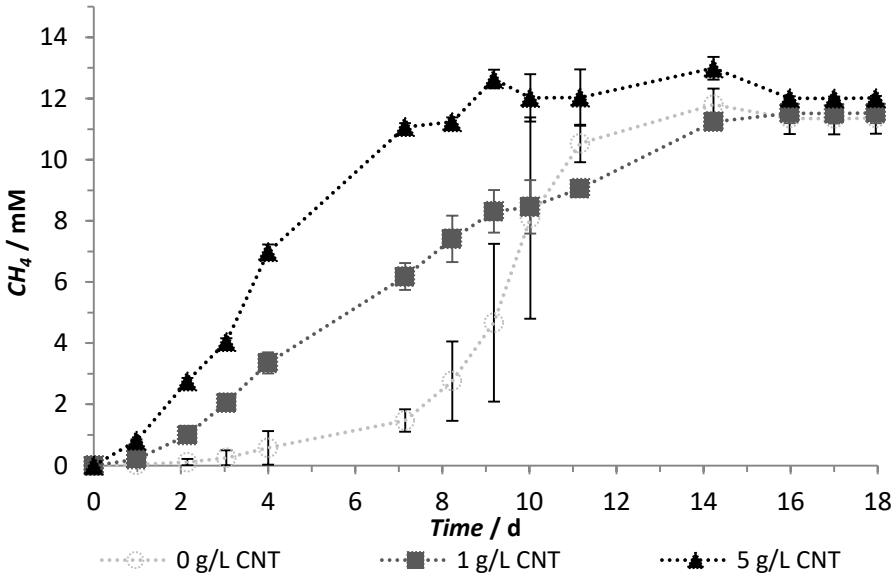


Figure 3.5 – Cumulative methane production by *M. formicicum* incubated with different amounts of CNT.

The hydrogen to methane conversion was calculated, with results being shown in Table 3.8. Unlike with acetate, in which the conversion to methane is from 1 mol to 1 mol, 4 mol of hydrogen are necessary to form 1 mol of methane, so the number of moles of hydrogen supplied to grow pure cultures of hydrogenotrophic methanogens was much higher when compared to the substrate supplied to the acetogens. All *M. formicicum* cultures completely consumed all H_2 supplied to the vials in the gas mixture (Table 3.8)

Table 3.7 – Cumulative methane production by pure cultures of *M. formicicum* incubated with and without CNT.

Incubation time (d)	*CNT* (g/L)	*Methane produced* (mM)	*CNT/Ct ratio* [a]	*Methane produced (%)* [b]
3	0	0.25 ± 0.25	—	2 ± 2
	1	2.04 ± 0.10	8.2 ± 4.0	16 ± 1
	5	4.04 ± 0.11	16.2 ± 0.5	31 ± 1
4	0	0.58 ± 0.55	—	4 ± 4
	1	3.25 ± 0.35	5.6 ± 0.6	25 ± 3
	5	6.99 ± 0.24	12.1 ± 0.4	53 ± 2
7	0	1.47 ± 0.37	—	11 ± 3
	1	6.49 ± 0.44	4.4 ± 0.3	49 ± 3
	5	11.07 ± 0.13	7.5 ± 0.1	84 ± 1
10	0	8.09 ± 3.29	—	63 ± 26
	1	9.07 ± 0.87	1.1 ± 0.1	69 ± 7
	5	12.02 ± 0.78	1.5 ± 0.1	91 ± 6

[a]CNT/Ct ratio represents the number of times the production of methane increased relatively to the control assay.

[b]The percentage of methane produced was calculated relatively to the maximum methane produced during the experiment, which correspond to the theoretical methane production expected assuming total conversion of the H_2/CO_2 provided at the beginning of the incubation.

Regarding the analysis of pH, an increase was detected after the experiment reached its end. All cultures reached values very close to pH 8 by the end of the assay, having started at a pH around 7.3. ORP values were much lower in the presence of CNT and decreased even further over the course of the assay, reaching a minimum of -365 ± 13 mV in the assays conducted with 5 g/L CNT (Table 3.9).

Regarding SEM analyses (Figure 3.6), it could be observed that in the absence of CNT, *M. formicicum* produces extracellular polysaccharides (EPS) over the cells, forming end-to-end connections (Figure 3.6B). In the presence of CNT (1 g/L and 5 g/L) the cells no longer seem to connect end-to-end and changed their aspect, adopting a more elongated and thinner morphology.

Table 3.8 – Initial and final hydrogen concentrations detected in *M. formicicum* cultures incubated with and without CNT and final methane concentrations obtained

CNT (g/L)	$[H_2]i$ (mM)	$[H_2]f$ (mM)	$[CH_4]f$ (mM)
0	51.46 ± 0.87	not detected	11.47 ± 0.22
1	52.66 ± 0.89	not detected	11.45 ± 0.14
5	52.99 ± 0.30	not detected	12.26 ± 0.49

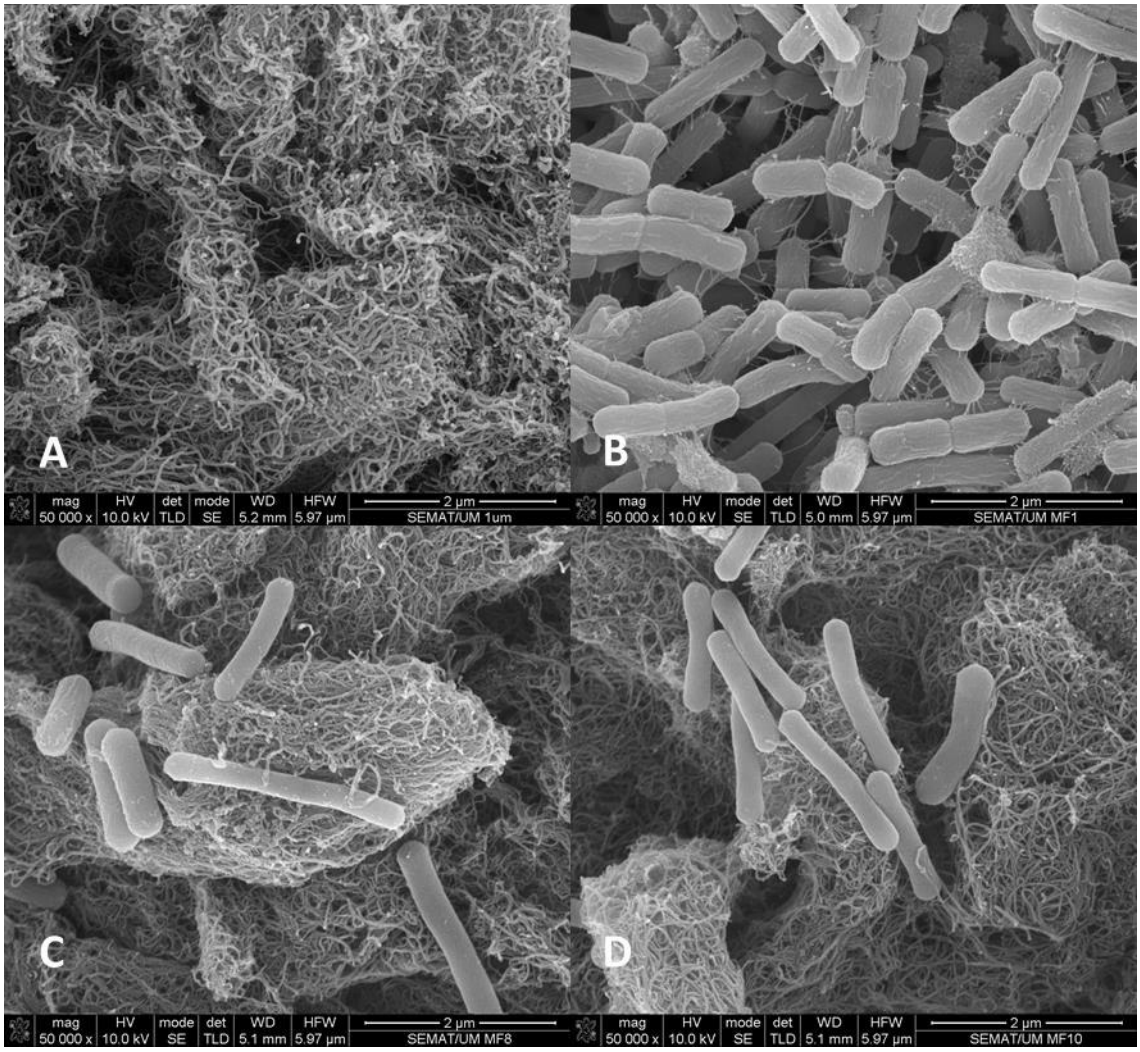


Figure 3.6 – SEM analysis of *M. formicicum* with and without CNT. A) abiotic assay with only CNTs displayed with a concentration of 0.5 g/L; B) *M. formicicum* cultures in the absence of CNT; C) *M. formicicum* cultures with 1 g/L of CNT; D) *M. formicicum* cultures with 5 g/L.

Table 3.9 – pH and ORP values measured throughout the experiment with *M. formicicum* pure cultures

CNT (g/L)	*Incubation time* (d)	*pH*	*ORP* (mV)
0	0	7.25 ± 0.07	-296 ± 14
	19	8.07 ± 0.01	-297 ± 4
1	0	7.28 ± 0.01	-329 ± 17
	19	7.92 ± 0.02	-332 ± 4
5	0	7.27 ± 0.03	-345 ± 35
	19	7.96 ± 0.02	-365 ± 13

3.3.4 Methane production by pure cultures of *M. formicicum* without the addition of a reducing agent (sodium sulfide)

To better understand the role of redox potential on methane production, another test was performed with *M. formicicum,* but this one differed from the previous, because it focused on the effect of the CNT in the presence and in the absence of a reducing agent in the growth medium. The reducing agent, sodium sulfide (Na_2S), was only added to one set of control cultures with no CNT, while all other vials were inoculated without the addition of reducing agent. The results obtained for cumulative methane production over time are shown in Figure 3.7.

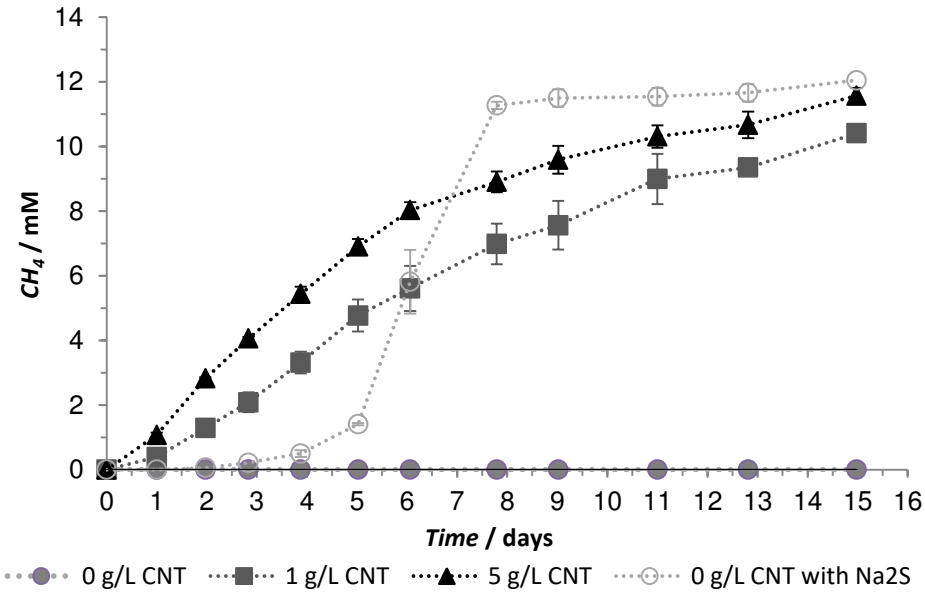


Figure 3.7 – Cumulative methane production in pure cultures of *M. formicicum*, without the addition of Na_2S.

Control cultures inoculated without the reducing agent and no CNT showed absolutely no methane production until the end of the experiment (Figure 3.7), and after 27 days of incubation still no methane could be detected in the vials headspace (data not shown), while the cultures growing in the absence of CNT but with the addition of Na_2S were active and showed a similar cumulative methane production curve to that of the previously presented assay conducted under the same conditions (Figure 3.5). Interestingly, the cultures containing CNT, but without sodium sulfide, grew in a somewhat similar way than what they did before, showing curves that resemble those displayed in Figure 3.5, even though cultures with 5 g/L CNT produced methane at much faster rates in the previous assay. Maximum methane yield was, however achieved earlier in this control culture (after 8 days).

M. formicicum displayed consistent results in growth with and without CNT in the sense that, while CNT presence greatly accelerated methane production in an initial phase, their absence resulted in a highly accentuated exponential phase that quickly went from a minimum amount of methane produced to the maximum methane yield obtained. Even so, for the first three days of incubation, the cultures supplied with 1 g/L of CNT produced over ten times the amount of methane that accumulated in control cultures with sodium sulfide, while for cultures with 5 g/L CNT, the increase was of almost twentyfold.

Table 3.10 - Methane production by *M. formicicum* pure cultures without the addition of a reducing agent.

Incubation time (d)	*CNT* (g/L)	*Methane produced* (mM)	*CNT/Ct ratio* [a]	*Methane produced (%)* [b]
2.8	0	0.21 ± 0.04	—	2 ± 0
	1	2.25 ± 0.31	10.5 ± 1.4	17 ± 2
	5	4.06 ± 0.05	19.0 ± 0.2	31 ± 0
5	0	1.41 ± 0.03	—	10 ± 0.2
	1	5.05 ± 0.49	3.6 ± 0.4	37 ± 4
	5	6.90 ± 0.23	4.9 ± 0.2	53 ± 2
6	0	5.81 ± 0.98	—	42 ± 7
	1	5.99 ± 0.70	1.0 ± 0.1	44 ± 5
	5	8.03 ± 0.24	1.4 ± 0.0	62 ± 2
7.8	0	11.27 ± 0.11	—	81 ± 1
	1	7.34 ± 0.63	0.7 ± 0.1	54 ± 5
	5	8.90 ± 0.32	0.8 ± 0.0	69 ± 3
11	0	11.54 ± 0.29	—	83 ± 2
	1	9.43 ± 0.78	0.8 ± 0.1	70 ± 6
	5	10.31 ± 0.35	0.9 ± 0.0	80 ± 3

[a]CNT/Ct ratio represents the number of times the production of methane increased relatively to the control assay.

[b]The percentage of methane produced was calculated relatively to the maximum methane produced during the experiment, which correspond to the theoretical methane production expected assuming total conversion of the H_2/CO_2 provided at the beginning of the incubation.

The overall conversion percentage from hydrogen to methane was better in this assay performed without the addition of Na_2S, when compared to the previous done with Na_2S in pure cultures of *M. formicicum*. Higher methane yields were achieved for all cultures except the ones without CNT and without reducing agent, which did not consume any of the hydrogen available. The cultures of *M. formicicum* growing with 5 g/L CNT had a good performance, showing a 92% H_2 to CH_4 conversion and,

despite showing a lower conversion percentage to the previous assay without Na_2S, cultures with 1 g/L still managed to convert 82% of hydrogen to methane (Tables 3.8 and 3.11).

Table 3.11 – Hydrogen to methane conversion by *M. formicicum* pure cultures without the addition of the reducing agent Na_2S.

CNT (g/L)	*[H₂]i* (mM)	*[H₂]f* (mM)	*[CH₄]f* (mM)
0 (without Na_2S)	55.77 ± 0.33	not detected	12.18 ± 0.27
1	54.14 ± 1.22	4.36 ± 0.73	11.14 ± 0.17
5	51.81 ± 0.33	2.34 ± 0.20	11.91 ± 0.36

Without the addition of any reducing agent, pH values behaved in a similar way than what they did otherwise, despite being slightly lower, as can be seen in table 3.12. The major difference resided in redox potential, as ORP values measured in cultures with CNT but without sodium sulfide were less negative. ORP values at the beginning of the assays were less negative (approximately -205 mV for cultures with 1 g/L CNT and -187 mV for cultures with 5 g/L) when compared to the assays with sodium sulfide (approximately -268 mV) and, by the time the experiment was finished, cultures with 1 g/L CNT reached -78 mV and cultures with 5 g/L showed an ORP of -26 mV which comes relatively close to 0. Nevertheless the activity of *M. formicicum* was not compromised.

Table 3.12 – ORP and pH values measured in *M. formicicum* pure cultures without the addition of Na_2S

CNT (g/L)	*Incubation time* (d)	*pH*	*ORP* (mV)
0 + Na_2S	0	7.29 ± 0.05	-268 ± 3
	27	8.05 ± 0.08	-341 ± 6
1	0	7.19 ± 0.04	-205 ± 15
	27	7.89 ± 0.03	-78 ± 24
5	0	7.17 ± 0.03	-187 ± 32
	27	7.92 ± 0.04	-26 ± 5

3.3.5 Methane production by pure cultures of *M. hungatei*

Figure 3.5.1 shows the methane production by pure cultures of *M. hungatei* over the time, with hydrogen as the substrate.

As it can be observed, some differences were registered between the concentrations of CNT used for growing *M. hungatei*, with lag phases being shortened by the presence of nanotubes. The lag phase in vials containing 1 g/L CNT was less than 1 day and in vials with 5 g/L CNT the lag phase was even lower (exact number of hours cannot be determined), while it lasted almost 2 days in the absence of CNT. Despite that, all cultures reached the plateau phase at around the same time (3.8 days).

In the first two days of cumulative methane production by *M. hungatei* (Table 3.13), the methane produced was over ten times higher in cultures with 1 g/L CNT and with 5 g/L, the methane produced increased approximately 18 times.

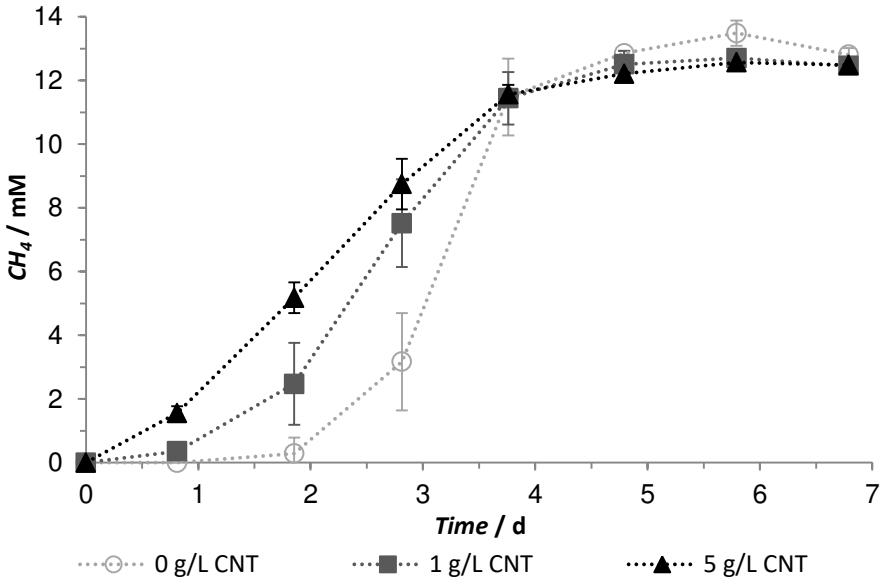


Figure 3.8 – Cumulative methane production by the hydrogenotrophic methanogen *M. hungatei* in the presence and absence of CNT.

In the presence and in the absence of CNT, hydrogen was fully consumed by *M. hungatei* cultures and final methane concentrations obtained were lower than what was expected in case of complete conversion of hydrogen to methane which would be of 1 mol of methane for each 4 mol of H_2 (approximately 16.78 mM, 15.33 mM and 15.20 mM for cultures with 0 g/L, 1 g/L and 5 g/L of CNT, respectively).

Table 3.13 – Cumulative methane production by pure cultures of *M. hungatei* with and without CNT in different points of the course of the assay.

Incubation time (d)	*CNT* (g/L)	*Methane produced* (mM)	*CNT/Ct ratio* [a]	*Methane produced (%)* [b]
1.9	0	0.29 ± 0.495	—	2 ± 3
	1	3.07 ± 1.283	10.7 ± 4.5	20 ± 8
	5	5.18 ± 0.483	18.1 ± 1.7	34 ± 3
2.8	0	3.17 ± 1.528	—	19 ± 9
	1	8.21 ± 1.378	2.6 ± 0.4	54 ± 9
	5	8.75 ± 0.790	2.8 ± 0.2	58 ± 9
3.8	0	11.48 ± 1.207	—	68 ± 7
	1	11.70 ± 0.825	1.0 ± 0.1	76 ± 5
	5	11.56 ± 0.300	1.0 ± 0.0	76 ± 2

[a]CNT/Ct ratio represents the number of times the production of methane increased relatively to the control assay.

[b]The percentage of methane produced was calculated relatively to the maximum methane produced during the experiment, which correspond to the theoretical methane production expected assuming total conversion of the H_2/CO_2 provided at the beginning of the incubation.

ORP values obtained in these pure cultures reached very negative values, as lowest as -409±5 mV in vials containing 5 g/L CNT after a week of incubation. Also, these values decreased for cultures with 0 g/L and 1 g/L of CNT, since the ORP registered in the end of the experiment (approximately -347 and -404 mV, respectively) was of about 50 mV lower than it was when it started (approximately -297 and -350 mV). An increase was registered in the pH as hydrogen was consumed, which is consistent with what was observed in the *M. formicicum* assays.

Table 3.14 – Concentrations of hydrogen and methane at the beginning and at the end of the incubations of *M. hungatei* with different CNT concentrations

CNT (g/L)	*[H₂]i* (mM)	*[H₂]f* (mM)	*[CH₄]f* (mM)
0	67.13 ± 0.49	not detected	13.05 ± 0.38
1	61.33 ± 0.57	not detected	12.56 ± 0.13
5	60.79 ± 0.19	not detected	12.42 ± 0.19

Regarding pH values measured throughout these experiments with both hydrogenotrophic methanogens, results were similar for both methanogens. pH increased from around 7.2-7.3 to 8 in all cultures grown and no major influence of the presence of CNT was verified in this parameter. This

increase in pH can be related to the reactions occurred in the midst of the process involving methane production, since hydrogenotrophic methanogens also consume CO_2 beside H_2, which reduces the media's buffer capacity, causing the oscillation in the pH values (Equation 3.1).

The SEM analysis performed in the pure cultures of *M. hungatei* resulted in the obtaining of the images displayed in Figure 3.9. Apparently there are no big differences in the cells' morphology and arrangement in the cultures incubated with and without CNT.

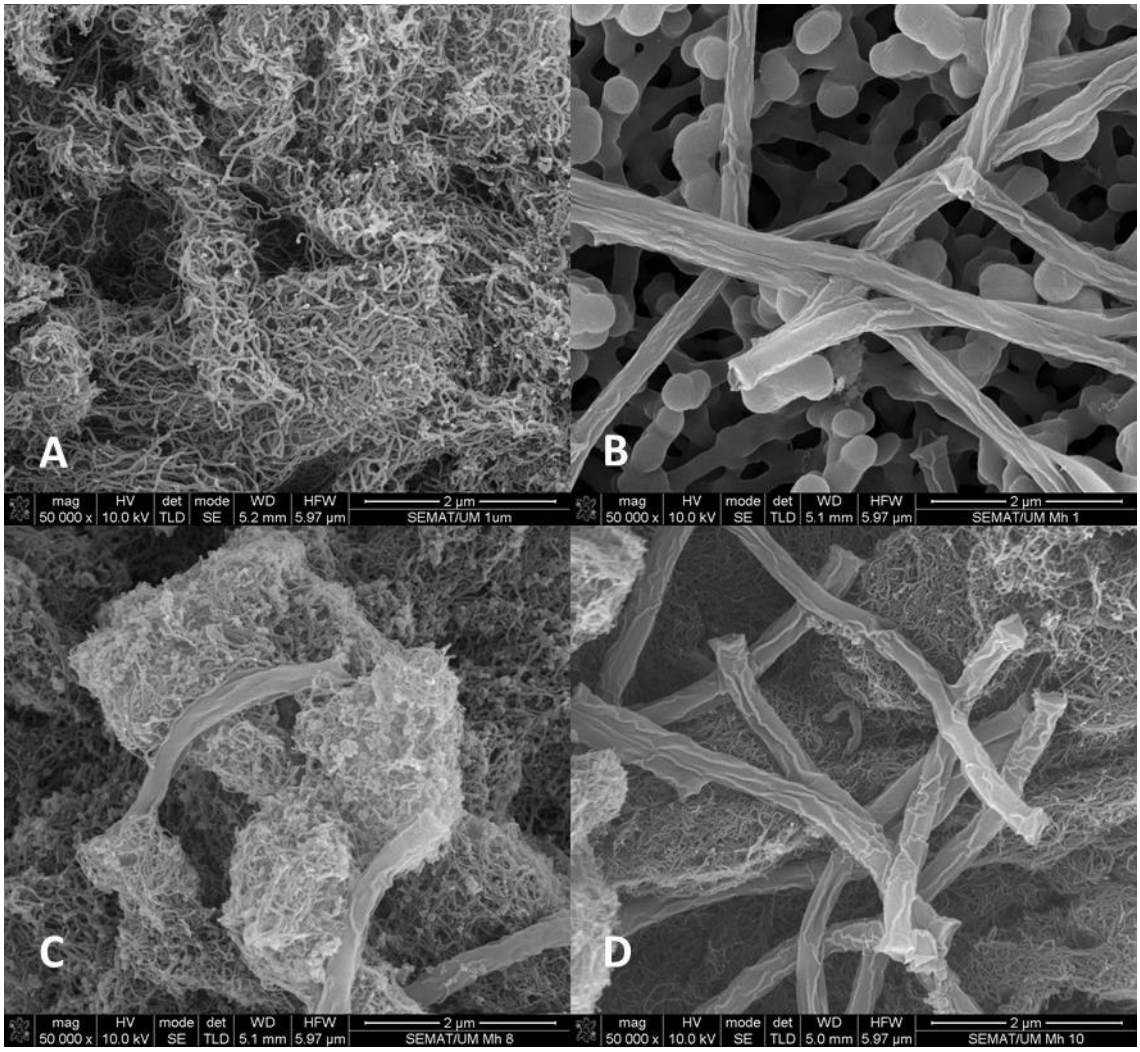


Figure 3.9 – SEM analysis of *M. hungatei* with and without CNT. A) abiotic assay with only CNTs displayed with a concentration of 0.5 g/L; B) *M. hungatei* cultures in the absence of CNT; C) *M. hungatei* cultures with 1 g/L of CNT; D) *M. hungatei* cultures with 5 g/L.

Table 3.15 – pH and ORP values in the beginning and in the end of the experiment with *M. hungatei* pure cultures

CNT (g/L)	*Incubation time* (d)	*pH*	*ORP* (mV)
0	0	7.31 ± 0.01	-297 ± 6
	7	8.21 ± 0.05	-347 ± 9
1	0	7.27 ± 0.05	-350 ± 4
	7	7.95 ± 0.13	-404 ± 9
5	0	7.26 ± 0.00	-391 ± 5
	7	8.01 ± 0.05	-409 ± 5

3.4 Discussion

These studies revealed that CNT stimulate the methanogenesis performed by pure cultures of methanogenic archaea. CNT presence induced faster methane production rates, although this effect was more evident with the hydrogenotrophic than with the acetoclastic cultures. It was also verified that CNT in the lack of a reducing agent, allowed *M. formicicum* to produce methane faster when compared with the assay where no CNT were added but in the presence of a reducing agent.

For both pure cultures of acetoclastic methanogens, the results showed that 1 g/L CNT accelerates the conversion of acetate to methane, while 5 g/L CNT seemed to inhibit their growth, especially for *M. concilii*. Li et al. (2015) performed an experiment with anaerobic sludge exposed to 1 g/L single-walled carbon nanotubes (SWCNT) and identified *M. concilii* as the most abundant methanogen. Although the CNT used in this work were multi-walled, the *M. concilii* pure cultures also had a positive development in the presence of 1 g/L CNT. However, the same cannot be said for exposure to higher concentrations, giving the idea that CNTs may be toxic for this methanogen when present in high amounts. Higher concentrations of CNT prompt an increase in surface area and a consequent increase in direct contact with the methanogens' cell walls, which is said to be one of the major causes for the cytotoxicity of CNT towards bacteria (Kang et al. 2007; Pasquini et al. 2012). A cytotoxicity effect may explain why *M. concilii* took 35 d to reach maximum methane yield in the presence of 5 g/L of CNT, while it took less than 20 days to reach the same plateau when exposed to 1 g/L of CNT or when it is growing without CNT. The same effect could be observed with *M. mazei* cultures although the toxicity effect was not as intense. These results show that both acetoclastic microorganisms were affected by CNT, that despite having a positive effect at low concentrations, such as 1 g/L, are deterrent to methane production by acetoclastic methanogens, when provided in high amounts, such as 5 g/L.

Concerning ORP values, *M. concilii* and *M. mazei* also reacted similarly to the presence of CNT. These values were much lower when the cultures were supplied with CNT and decreased further with higher amounts. An average was calculated for ORP values measured throughout each assay and it was observed that *M. concilii* registered a decrease of 6% and 14% in ORP values for the concentrations of 1 g/L and 5 g/L of CNT respectively, when compared to the control cultures and in *M. mazei*, this decrease was 7% and 18% for the same amounts of CNT. A relationship between the concentration of CNT and the ORP values, in anaerobic medium for growing acetoclastic methanogens, could be established (Figure 3.10).

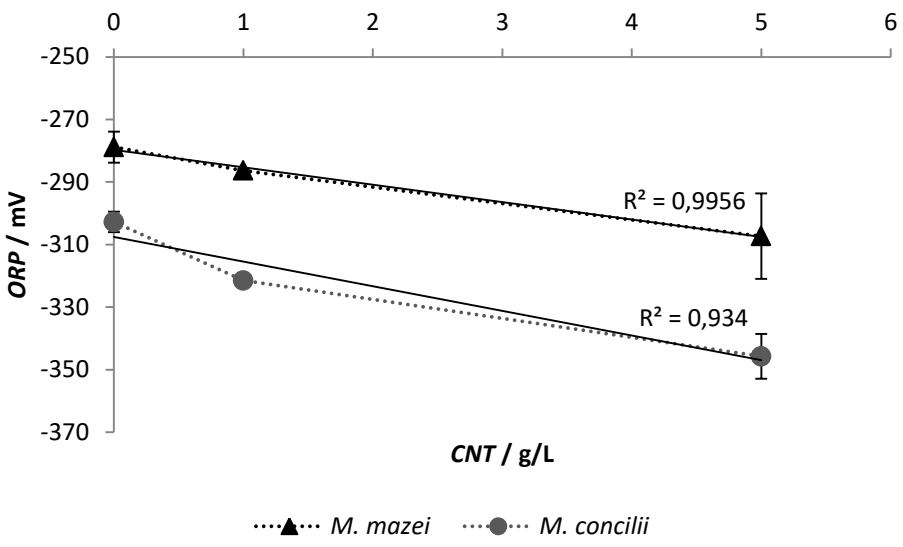


Figure 3.10 – ORP averages determined in the experiments with *M. concilii* and *M. mazei* when incubated with and without CNT.

ORP values seem to decrease linearly according to the CNT concentrations of the vials. This decrease in ORP is related to the unique properties of the CNT and their strong electroconductivity that causes these values to become much more negative in the presence of higher amounts of CNT (Pereira et al. 2014). It is established that in order for obligate anaerobes to grow, it is necessary to maintain the media at ORP values between -200 mV and -350 mV (Morris 1975). Observing these results and taking into account the cumulative methane productions obtained in both cases, it can be said that lower ORP values do not necessarily mean better development for methanogenic pure cultures, despite the fact that methanogens prefer to inhabit in environments with ORP values of about -400 mV (Archer & Harris 1986).

Unlike what happened in the pure cultures of *M. concilii* and *M. mazei*, CNT did not slow down methane production by any of the hydrogenotrophic methanogens studied. Instead, pure cultures of both *M. formicicum* and *M. hungatei* grew faster when supplied with 5 g/L of CNT, than what they did with 1 g/L of CNT. In the first few days of incubation time, cultures with 5 g/L of CNT produced more than 15 times the amount of methane produced by control cultures (Tables 3.5 and 3.8). From this observation derives the hypothesis that high CNT concentrations promote these methanogens' activity, as opposed to what was verified for the acetoclastic methanogens. It is difficult to relate these results to those of other works since there are no studies with pure methanogenic cultures and carbon materials. Nevertheless, a number of studies claim that the addition of carbons materials and other conductive materials to co-cultures and mixed cultures promotes the occurrence of DIET (Liu et al. 2012; Liu et al.

2014; Chen et al. 2014a; Chen et al. 2014b). However, in this work, it was verified that CNT improved methane production by pure cultures of methanogens where DIET is not expected to occur.

It was also interesting to observe the effects of sodium sulfide (or the lack of it) in pure cultures of *M. formicicum*. Without a reducing agent and in the absence of CNT, there was absolutely no substrate consumption and hence, methane production. Nevertheless, in the presence of CNT, the cultures in this assay performed very similarly to what they did for each CNT concentration with the addition of the reducing agent. This shows that although the ORP seem too high to allow methanogenesis to occur, CNT could provide the necessary conditions for growing active *M. formicicum* cultures, even in the absence of a reducing agent. Note that when a reducing agent is added, the ORP was much more negative (Table 3.12).

As was observed for *M. mazei* and *M. concilii*, the ORP values decreased as the amount of CNT supplied to the cultures increased. The connection between these two parameters can be verified by the results presented in Figure 3.11, although it is not as linear in this case as it was for the acetoclastic methanogens. On the other hand, the absence of a reducing agent had a completely different effect. As CNT increased in these cultures, so did ORP values, meaning that they actually reduced the media, reaching relatively high negative values for the concentration of 5 g/L of CNT.

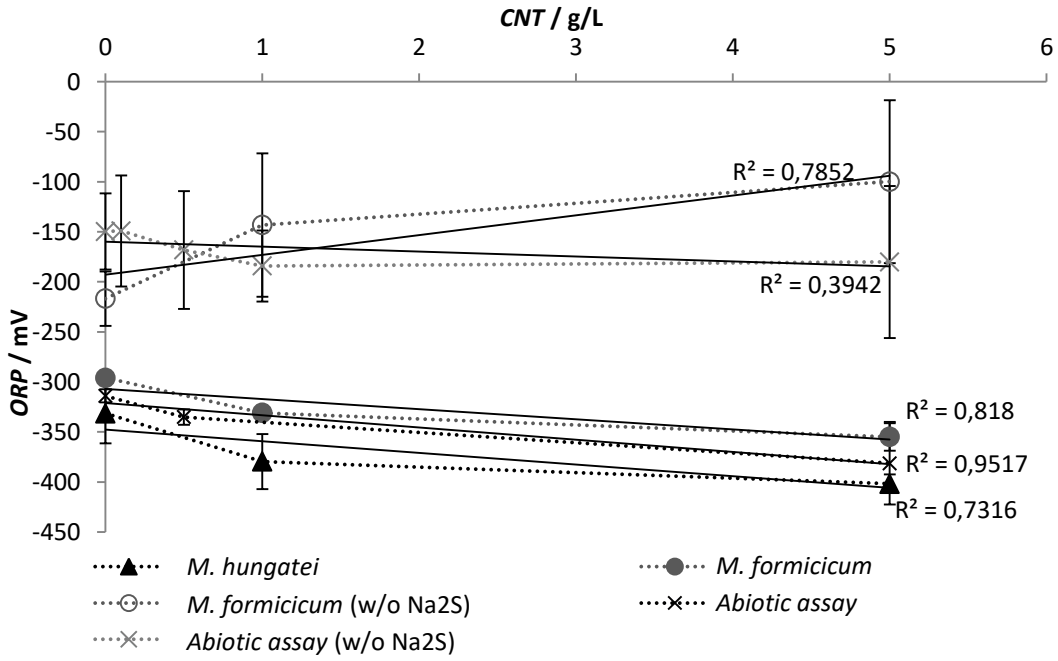


Figure 3.11 – ORP averages determined in the experiments with *M. formicicum* and *M. hungatei,* incubated with and without CNT and in the abiotic assay performed with different CNT concentrations.

In the absence of the reducing agent, ORP values became less negative in the presence of CNT, and these results are difficult to explain. Nevertheless, during the ORP measurement, the sample exposition

to the air might explain the part of the ORP in the assays without the addition of the reducing agent, as well as the high standard deviation.

Finally, in this chapter, the effect of CNT on the activity of pure cultures of methanogenic archaea was investigated. Incubations with increasing CNT concentrations resulted in an increase of methane production rates as well as a decrease on the lag phases preceding methane production by the hydrogenotrophic methanogens *M. formicicum* and *M. hungatei*. ORP turns more negative as CNT concentration increases. The activity of acetoclastic methanogens, *M. concilii* and *M mazei*, was also improved, although inhibited by high CNT concentrations. The mechanisms by which CNT influences methanogenic activity may be related to the decrease of the ORP, but other factors should also contribute and deserve further investigation.

4. EFFECT OF CNT IN CO-CULTURES OF *S. WOLFEI* AND *M. HUNGATEI*

4.1 Introduction

Syntrophic communities have the ability of transforming fatty acids such as propionate, acetate and butyrate, into methane, by means of electron exchange between species (Schink 1997). Mediation by hydrogen/formate and direct interspecies electron transfer (DIET) are some of the mechanisms that have been reported for this process (Stams & Plugge 2009; Summers et al. 2010). *Syntrophomonas wolfei* is a well-known syntrophic bacteria, able to use a limited substrate range of fatty acids with four- to eight-carbon atoms (Sieber et al. 2010). In co-cultures, it can oxidize butyrate through the ß-oxidation pathway by action of acetoacetyl coenzyme (CoA), forming two acetate molecules and one ATP molecule by substrate-level phosphorylation. In the midst of this process, hydrogen is formed by reduction of protons (or formate is formed by CO_2 reduction) and electrons are released (Equation 4.1) (Schink 1997).

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2 \quad (\Delta G^{o\prime} = 48.6\ kJ) \qquad \text{(Eq. 4.1)}$$

This reaction is thermodynamically unfavorable, with a positive Gibbs free energy. However, parallel to this reaction the presence of a methanogenic partner helps to maintain a low hydrogen partial pressure or formate concentration, which makes this conversion (Equation 4.1) thermodynamically favorable, with a Gibbs free energy of -39.2 kJ (Sieber et al. 2012; Stams et al. 2012). Sieber et al. (2014) noted that hydrogen transfer is essential for syntrophic metabolism of butyrate by *S. wolfei*, by performing inhibition studies with pure cultures of this bacteria and co-cultures of *S. wolfei* and *Methanospirillum hungatei.*

M. hungatei is a hydrogen and formate-utilizing methanogen that is unable to utilize acetate as a substrate for methanogenesis and therefore, in normal conditions, acetate formed by butyrate oxidation is not consumed in these co-cultures for energy generation proposes. Instead, *M. hungatei* utilizes the resulting hydrogen from this reaction as an electron donor, while most of the acetate accumulates in the cultures (Equation 4.2).

$$2CH_3CH_2CH_2COO^- \rightarrow 4CH_3COO^- + CH_4 \qquad (\Delta G^{o\prime} = 48\ kJ) \qquad \text{(Eq. 4.2)}$$

Recently, conductive nanoparticles of magnetite and carbon nanotubes have been studied for their influence in the butyrate oxidation in lake sediments and paddy soil enrichments (H. Li et al. 2015; Zhang & Lu 2016). These materials have been reported to accelerate methane production in

methanogenic communities, with the *Syntrophomonas* genus being one of the bacteria detected as responsible for syntrophic butyrate oxidation. Also, studies have been performed with carbon materials in syntrophic consortia in order to study their effect on methane production and determine their influence in the interspecies electron transfer mechanisms. It has been found that materials such as biochar and GAC can promote DIET, thus enhancing methane production (Liu et al. 2012; Chen et al. 2014b; Zhang & Lu 2016).

Therefore, in this chapter, co-cultures with *S. wolfei* and *M. hungatei* were grown in the presence of CNT in order to assess the influence of these carbon materials in methane production.

4.2 Materials and methods

4.2.1 Preparation and incubation of the co-cultures

Co-cultures of *Syntrophomonas wolfei* and *Methanospirillum hungatei* (DSM 2245B) were grown in anaerobic medium with and without CNT, to assess the effect of CNT on syntrophic conversion of butyrate to methane. For each co-culture, different concentrations of CNT were tested; 0 g/L, 0.5 g/L, 1 g/L, 1.5 g/L and 5 g/L, and all assays were carried out in triplicate. The CNT used in this assay were the same as described in chapter 3.

The experimental assays were conducted in 120 mL serum bottles containing 45 mL of basal medium and 5 mL of inoculum (10 % v/v). Inoculation was performed with 4 mL of a pre-grown co-culture of *S. wolfei* and *M. hungatei* and 1 mL of a *M. hungatei* pure culture. Basal medium was prepared as described in chapter 3, and butyrate, cysteine-HCl and biotin solutions were added to the vials for final concentrations of 20 mM, 0.28 g/L and 0.05 mg/L, respectively. Vials were closed with black butyl rubber stoppers and aluminium caps and then flushed with a gas mixture of 80%/20% of N_2/CO_2, as it was done for the pure cultures in chapter 3. The medium was sterilized at 120 ^{o}C, 1 Bar for 20 min. Parallel to this, and as a control of methanogenic activity, pure cultures of *M. hungatei* were also grown in the same conditions as described in chapter 3, with 0 g/L, 0.1 g/L, 0.5 g/L, 1 g/L and 5 g/L CNT concentrations tested.

4.2.2 Analytical methods

Methane accumulated in bottles as well as acetate and butyrate were monitored throughout the experiment following the procedure described in Chapter 3.

4.3 Results

Co-cultures of *S. wolfei* and *M. hungatei* behaved differently according to the amounts of CNT supplied to them. Figure 4.1 depicts the results obtained by the growing these co-cultures in the presence and in the absence of CNT.

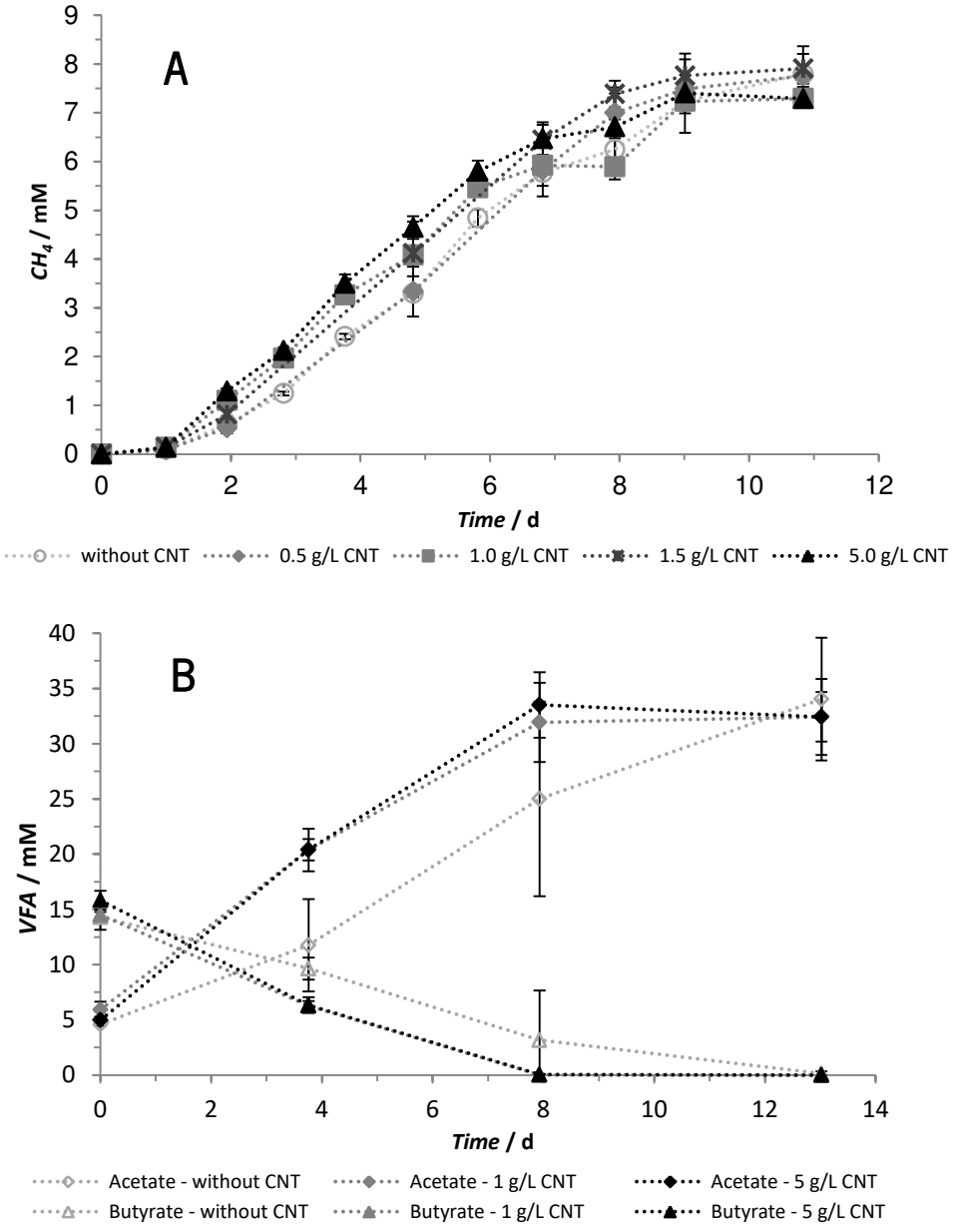


Figure 4.1 – Cumulative methane production (A) and conversion of butyrate to acetate (B) by the co-culture of S. wolfei and M. hungatei in the presence and in the absence of CNT.

CNT presence induced a faster methane production, especially during the first days of incubation. Nevertheless, the assay with 0.5 g/L of CNT behaves similarly to the control assay without CNT. Lag phases of the co-cultures shortened in the presence of CNT and methane was produced in much larger quantities in the first few days of incubation. For example, after two days of incubation the double of the

methane produced could be quantified in incubations with CNT (0.6 mM in control assay without CNT and 1.1 and 1.3 mM in assays with 1 g/L and 5 g/L CNT, respectively). However, these differences vanished along time.

In stoichiometric proportions, it is expected the formation of 4 mol of acetate and 1 mol of methane from each 2 mol of butyrate consumed. Regarding methane yields, the results shown in Table 4.1 state that the reactions occurred as expected, since for all assays, methane concentrations in the end of the assay were about half of the initial butyrate concentration. For acetate accumulation in the vials, the amount generated by the *S. wolfei* was twice the amount of butyrate consumed.

Table 4.1 – Total conversion of butyrate to acetate

Concentration of CNT (g/L)	*Butyrate* (mM)	*Acetate* (mM)	CH_4 (mM)
0	14.30 ± 1.14	29.46 ± 5.56	7.52 ± 0.34
0.5	15.50 ± 1.06	28.79 ± 1.46	7.62 ± 0.26
1	14.50 ± 0.26	26.52 ± 2.23	7.26 ± 0.13
1.5	15.87 ± 0.91	26.69 ± 4.68	7.83 ± 0.32
5	15.82 ± 0.85	27.45 ± 3.43	7.35 ± 0.45

Pure cultures of *M. hungatei* were also grown in the same conditions, serving as a control assay for this experiment. The cumulative methane production by these cultures is displayed in Figure 4.2. Hydrogen conversion to methane by *M. hungatei* was enhanced by CNT concentration ranging from 0.5 g/L to 5 g/L CNT (Figure 4.2). Notably, after approximately 3 days of incubation, cultures incubated with 5 g/L CNT produced around threefold more methane when compared with control cultures, and almost no lag phase can be observed. Hydrogen concentrations in the co-cultures were measured along with methane in GC analysis and the results show that there was a small accumulation of hydrogen in the vials that decreased as the experiment advanced (data not shown). The hydrogen concentrations detected at 2 and 3 days of incubation time were approximately 0.04 mM in the absence of CNT, while in the presence of 1 g/L and 5 g/L of CNT the hydrogen detected was approximately 0.01 mM for both concentrations.

Using the data from the first 4 days in the case of the co-culture and 3 days in *M. hungatei*, initial methane production rates were also determined and compared for each concentration of CNT supplied (Table 4.2).

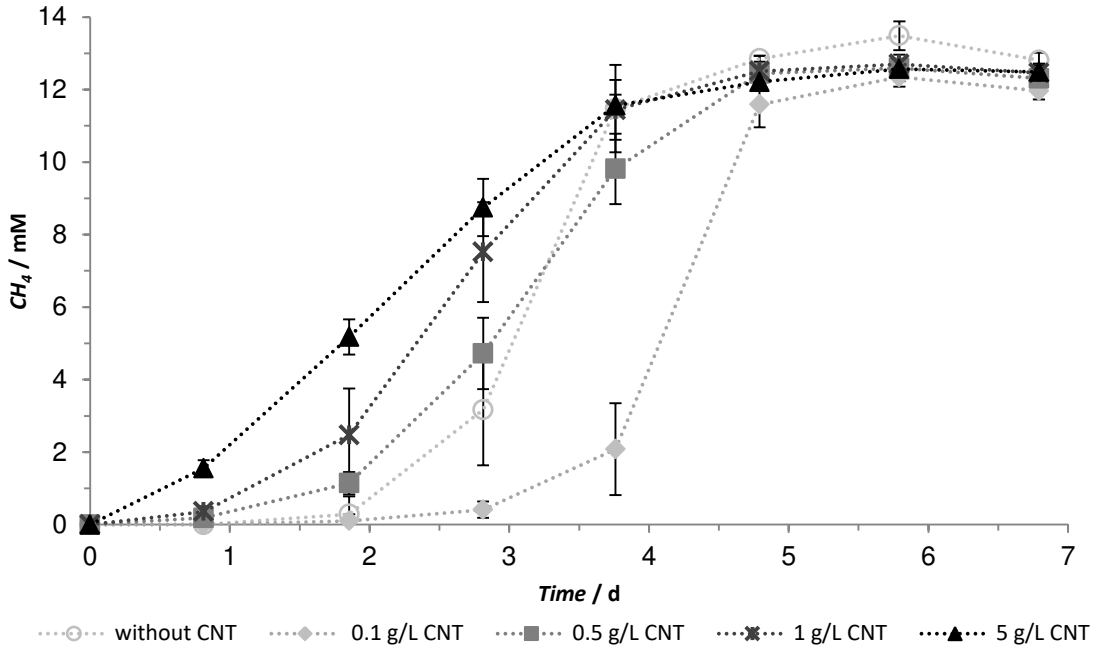


Figure 4.2 – Cumulative methane production by *M. hungatei* pure cultures in the presence and absence of CNT.

For all assays of pure and co-cultures, the methane production rates increased in the presence of CNT (Table 4.2). The exception was the assay with the pure cultures of *M. hungatei* and 0.1 g/L CNT. In co-cultures, the increase of methane production rate was not as intense as it was verified for pure cultures of *M. hungatei*, with the values being only 1.4 times higher than the control, comparing with the 5.5 times higher for the 5 g/L CNT in *M. hungatei* pure culture. Regarding ORP values, they were less negative in the case of the co-culture, ranging from -291 ± 17 mV to -327 ± 14 mV.

Table 4.2 – Methane production rate, lag phase, time of maximum methane and ORP values in co-cultures and pure cultures

Microorganism	*CNT* (g/L)	*Methane production rate* (mM/d)	R^2	*Lag phase time* (d)	*Time of maximum methane* (d)	*ORP* (mV)
M. hungatei + *S. wolfei*	0	0.72 ± 0.01	0.93 ± 0.01	1	9	-296 ± 16
	0.5	0.74 ± 0.11	0.93 ± 0.00	1	9	-296 ± 12
	1	0.92 ± 0.12	0.96 ± 0.01	<1	9	-306 ± 18
	1.5	0.91 ± 0.10	0.95 ± 0.00	<1	9	-291 ± 17
	5	1.03 ± 0.03	0.96 ± 0.00	<1	9	-327 ± 14
M. hungatei	0	0.57 ± 0.13	0.71 ± 0.14	2 to 3	5	-331 ± 30
	0.1	0.18 ± 0.01	0.71 ± 0.14	3 to 4	6	-327 ± 25
	0.5	1.61 ± 0.37	0.81 ± 0.03	<2	5	-349 ± 27
	1	2.91 ± 0.56	0.88 ± 0.06	<1	5	-380 ± 28
	5	3.17 ± 0.29	0.99 ± 0.01	<1	5	-402 ± 9

4.4 Discussion

Within this work, it was possible to verify that, in line to that was observed with methanogenic pure cultures (chapter 3) syntrophic conversion of butyrate to methane by *S. wolfei* and *M. hungatei* were enhanced in the presence of CNT, with butyrate oxidation coupled to methanogenesis being performed at a faster rate, when compared to the control without CNT. It is known that CNT have high conductivity and are good redox mediators (Pereira et al. 2014; Tessonnier et al. 2009), however, it is unclear if DIET actually took place in the present experiments, since both hydrogen and formate were detected during the process of butyrate oxidation acting as an electron shuttles (Sieber et al. 2014; Sieber et al. 2010). In addition, it is known that *S. wolfei* contains no genes for outer membrane c-type cytochromes, which are said to play an major role in DIET (Sieber et al. 2014).

Recent studies indicated that hydrogen transfer is essential in syntrophic butyrate conversion to methane by *S. wolfei* coupled with *M. hungatei*, as opposed to formate transfer, which is more important in methane production by crotonate (Sieber et al. 2014). *S. wolfei* cells have genes for three hydrogenases and for five formate dehydrogenases (Sieber et al. 2010). This bacteria seems to adapt differently according to the growth conditions that it is subjected to and changes can be verified in its metabolic features (Schmidt et al. 2013). When grown syntrophically with butyrate, the overall expression of genes of hydrogenases is much higher to that of the expression of formate dehydrogenases (Sieber et al. 2014). A small accumulation of hydrogen was detected in the first two days of incubation in co-cultures in the absence of CNT and the hydrogen accumulated in co-cultures grown in the presence of CNT were four times lower. The results obtained in chapter 3 show that hydrogen consumption by pure cultures *M. hungatei* (section 3.3.5), and in this chapter (Figure 4.2) was accelerated by the presence of CNT, giving support to this data and confirming that CNT presence does increase the methane production by the hydrogenotrophic methanogen *M. hungatei*. Therefore, hydrogen most likely had a major role in electron transfer in these co-cultures and this process was enhanced in the presence of CNT. Formate was also detected at the start of incubation, but the concentrations were very low in all assays (approximately 0.1 mM) and decreased with incubation time, probably meaning that it also had a role in interspecies electron transfer between *S. wolfei* and *M. hungatei*.

DIET in the syntrophic butyrate conversion to methane is only a possibility and further studies are required to better understand the effect of conductive materials in methane production from butyrate by *S. wolfei* and *M. hungatei*. To date, what is clear is that CNT presence does influence the methane production by methanogenic co-cultures in a positive way and somehow facilitates butyrate oxidation by

S. wolfei (note that butyrate conversion to acetate was faster in the assays with CNT (Figure 4.1). The activity of pure cultures or co-cultures is much higher in the presence of CNT, which might be related to the effect of CNT on the redox potential of the growth medium (see Table 4.2), since higher methane production rates were obtained when ORP values became more negative and vice-versa.

In conclusion, the addition of CNT to syntrophic consortia proved to be an interesting approach, since it seems to produce positive effects in methane production.

5. ADAPTATION OF ANAEROBIC SLUDGE TO ETHANOL AND VFA MIXTURE IN REACTOR OPERATION

5.1 Introduction

The inevitable depletion of fossil fuels forces humanity to come up with new ways of obtaining energy in order to maintain its living standards. AD technology has been evolving in the last decades as a way for producing renewable energy and also as an effective and sustainable strategy for waste management (Chen et al. 2008). Effluents with high ethanol and VFA concentrations, such as winery, domestic or industrial wastewaters, can and should be treated by AD, since they can become an environmental hazard, and many studies have been conducted in order to optimize this process (Yu et al. 2006; Li et al. 2008; Wang et al. 2009; Rodriguez-Caballero et al. 2012; Franke-Whittle et al. 2014; Welz et al. 2016).

Abreu et al. (2007) attempted the recovery of a granular sludge whose specific acetoclastic activity (SAA) had been severely damaged. The sludge was inoculated in an expanded granular sludge bed reactor (EGSB) with ethanol as the sole substrate and results showed that the SAA increased significantly with this method. *Methanosaeta* were the most abundant methanogens observed in the granules analyzed at the end of the experiment. In another study, Wang et al. (2009) performed anaerobic digestion tests in order to determine the effect of VFA concentrations in methane yield and methanogenic archaea, where various reactors were fed with mixtures of acetic acid, propionic acid, butyric acid and ethanol at different concentrations. Propionic acid posed as the most harmful of these VFA for methane production, with a significant inhibitory effect being observed at concentrations of 900 mg/L, resulting in the accumulation of ethanol and VFA and the reduction in methane yield. In contrast, the other substrates used (ethanol, propionic acid and acetic acid) did not result in any significant inhibition of methanogenic activity. Franke-Whittle et al. (2014) studied the methanogenic communities in two different reactors, under mesophilic and thermophilic conditions, in order to determine the effect of different concentrations of VFA in anaerobic digester plants. The results showed that changes in VFA concentrations did not alter the methanogenic communities' composition significantly, with *Methanosaeta*, *Methanoculleus*, *Methanobacterium* and *Methanosarcina* as the dominant species. The *Methanosaeta*'s presence in most of studies in anaerobic digesters may be related to their high affinity for acetate, which is commonly accumulated in anaerobic reactor operation, enabling them to prevail at low residual VFA concentrations among other acetoclastic methanogens, belonging to *Methanosarcina* genus (De Vrieze et al. 2014).

Carbon materials have been studied as a potential stimulator of methane production by anaerobic sludge in anaerobic digesters (Zhao et al. 2015). Zhao et al (2015) studied the effect of carbon cloth,

graphite and biochar in UASB reactors and found that these carbon materials accelerate syntrophic metabolism, with carbon cloth standing out among them in the conversion of ethanol to methane.

In this Chapter, anaerobic bioreactors were operated for adapting the methanogenic communities to degrade ethanol or VFA. This is the first step of a study that seeks the better comprehension of the interspecies electron transfer mechanisms that take place in syntrophic communities that degrade ethanol and VFA. In the follow up of this study, methanogenic communities will be characterized in order to identify the microorganisms present over the course of the adaptation period. The biomass obtained by adaptation in continuous bioreactor will be used later as an inoculum for the study of the effect of CNT in the interspecies electron transfer performed by these communities, which is the ultimate objective of this study. The results presented in this Chapter are only referring to the first stage of biomass adaptation in an UASB reactor.

5.2 Materials and Methods

5.2.1 Reactor setup

Four UASB reactors, made of acrylic glass with 200 mL of work volume (L = 50 cm; d = 2 cm) were set up. Reactors 1 (R1) and 2 (R2) were set to be fed with ethanol as an energy and carbon source and reactors 3 (R3) and 4 (R4) with a mixture of VFA. The reactors setup can be seen in Figure 5.1.

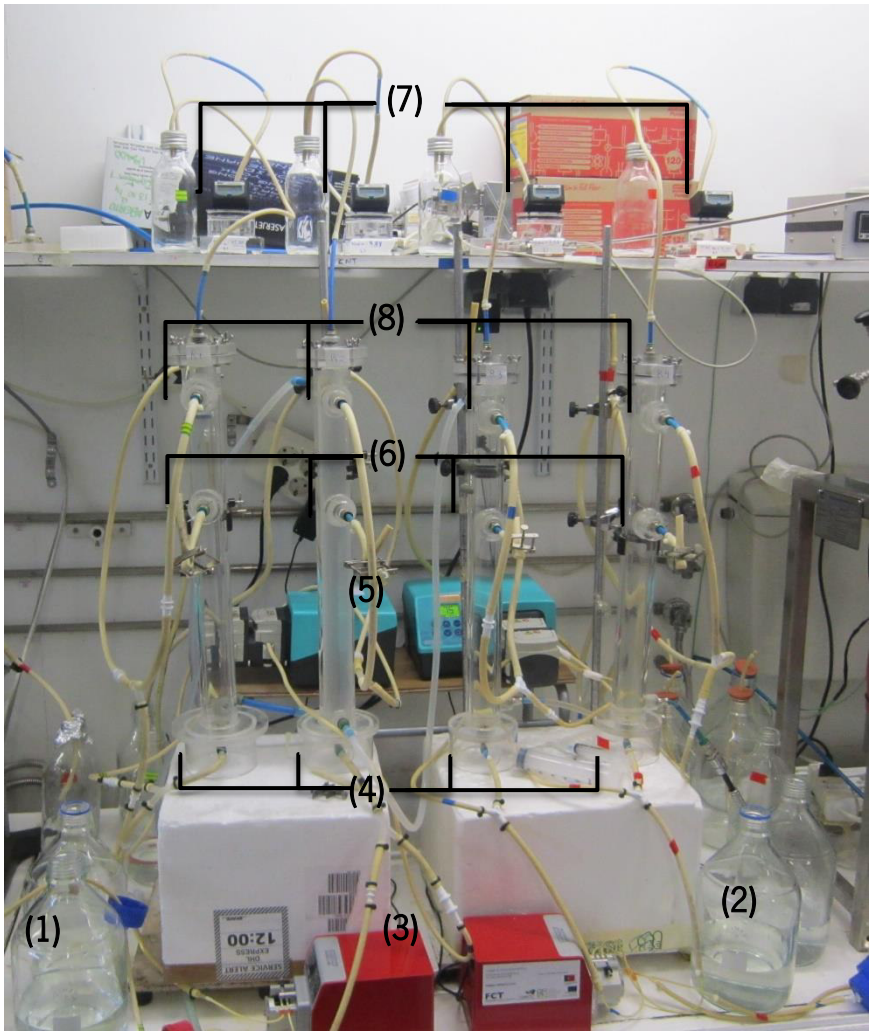


Figure 5.1 – Reactor setup; (1) – 2L glass bottles with feeding solutions for R1 and R2 (ethanol); (2) – 2L glass bottles with feeding solutions for R3 and R4 (VFAs); (3) – Peristaltic pumps for reactor feeding; (4) – Reactor entrance; (5) – Peristaltic pumps for recirculation; (6) – Exit for recirculation; (7) – Biogas counter; (8) – Reactor exits.

A gas trap was set at the top of each reactor in order to count the volume of biogas produced. Four peristaltic pumps were used to set up the operation, one for feeding R1 and R2, another for mixing the content of R1 and R2 by recirculation, and the other 2 pumps were used for the same propose for R3

and R4. All reactors were fed at a flow rate of 200 mL/d, corresponding to a hydraulic retention time (HRT) of approximately 24 h. In addition, reactors were operated, at 37 ± 1 °C.

5.2.2 Anaerobic sludge preparation

As an inoculum to the reactors, a sample of granular sludge was collected from a full-scale UASB reactor treating brewery wastes (Central de Cervejas, Portugal). Prior inoculation, the granular sludge was washed with a mineral solution and the granules were smashed with a mill. Finally, the granular sludge was distributed by the reactors in order to obtain a final concentration of approximately 10 gVSS/L in each reactor.

5.2.3 Preparation of feeding solutions

One liter of feeding solution was prepared with ethanol (3 gCOD/L) for R1 and R2 and a mixture of VFAs with a 24:32:24 COD ratio of acetate, propionate and butyrate (3 gCOD), respectively for R3 and R4. Feeding solution for R1 and R2 were prepared with demineralized water supplemented with micronutrients and macronutrients solution, which were prepared according to Zehnder et al. (1980). Feeding solution was then boiled and cooled down with ice under a N_2 atmosphere. After adding 1.44 g of absolute ethanol, the solution was distributed by two 2 L glass bottles and pressurized with N_2, before being sterilized at 120 °C for 20 min. Finally, both solutions were supplemented with bicarbonate and Na_2S for a final concentration of 5 g/L and 0.12 g/L, respectively. The procedure for preparing feeding solutions with VFAs was the same, with the exception of the substrate added. Instead of ethanol, three VFA were added: sodium acetate (1.53 g/L), sodium propionate (0.87 g/L) and sodium butyrate (0.87 g/L). The detailed composition of all feed solutions are described in Appendix II.

5.2.4 Routine analysis

Three times a week, samples of each feeding solution and from the exit of the reactors were collected for routine analysis of VFAs and ethanol, pH, redox potential (ORP) and chemical oxygen demand (COD).

For VFA analysis, samples were collected from each feeding solution and each reactor, and centrifuged for 5 mins at 15000 rpm. Afterwards, the supernatant was transferred to another tube and stored at -20ºC until being subjected to HPLC analysis. After defrosting, 800 µL of filtered samples were added to

the vials, along with 200 μL of crotonic acid, used as internal standard. Samples were filtered through a syringe with a 0.20 μm filter. The equipment used for this purpose was the same as described in Chapter 3 for VFAs quantification (HPLC (Jasco, Tokyo, Japan) with a Chrompack column (67H) coupled to a UV detector at 210 nm and a Aminex HPX-87H column coupled to a RI detector at 160 nm. The procedure for HPLC analysis of ethanol was the almost the same, but internal standard was not added to the vials before going to HPLC analysis. All HPLC analyses were performed in duplicate.

Methane content of the biogas in the trap bottles headspace was quantified as described in Chapter 3, by using a GC BRUKER SCION 456 connected to a TCD detector (MOLSIEVE/PLOT). These analyses were performed three times a week in order to determine the percentage of methane contained in the biogas produced by each reactor.

COD was also determined in feeding solutions as well as in the reactors. The sample collection was done as described for VFA and ethanol determination, where they were also centrifuged in the same conditions. Instead of being stored, samples were processed immediately using COD determination kits (Hach Lange LCK 515; LCK 114 and LCK 014) according to manufactures' instructions.

For pH and redox potential, measurements were made with a mV meter (Consort 533 VWR: 662-1409) and a pH/mV meter (WTW pH7110 VWR: 662-1767), respectively.

For determination of volatile suspended solids (VSS), a random volume of samples was collected for each reactor, which was then filtered, using a 47 mm glass microfiber filter. Prior to filtering the samples, these filters were dried at 550 ^{o}C for 2 h and then weighed. After the samples were filtered, the filters were then dried at 105 ^{o}C for 24 h and weighed a second time for determination of total suspended solids (TSS). Filters were then dried again at 550 ^{o}C for 2 h, in order to determine VSS. These measurements were made in triplicate.

To determine the amount of TSS and VSS, calculations were made by using the following Equations 1 and 2, where *a*, *b* and *c* are the weights of the filters after each drying process in order and *V*, the volume of filtered samples for each filter:

$$TSS = \frac{(b-a)*1000}{V} \quad (Equation\ 5.1) \quad ; \quad VSS = \frac{(b-c)*1000}{V} \quad (Equation\ 5.2)$$

In addition, samples for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) extraction were also taken at three points: at the time of inoculation, 24h after the initiation of the continuous operation and in the end of the operation. For DNA extraction samples (10 mL of bulk volume) were collected from each reactor, which were then centrifuged at 15000 rpm for 10 mins, with the supernatant being

discarded afterwards. The pellet was then resuspended in previously sterilized PBS buffer and stored at -20 °C. Samples for RNA extraction, around 30 mL were collected each time and also centrifuged at 15000 rpm for 10 minutes. After discarding the supernatant, the pellet was resuspended in RNA later solution + sterile PBS buffer (prepared with DEPC treated water) and stored at -80 °C, until RNA extraction was performed. DNA and RNA extraction were done using the Fast DNA spin kit for soil and the Fast RNA Pro Soil kit (MP Biomedicals), respectively, following the manufacturer instructions.

5.3 Results

An overview of methane production rates in all reactors is presented in Figure 5.2.

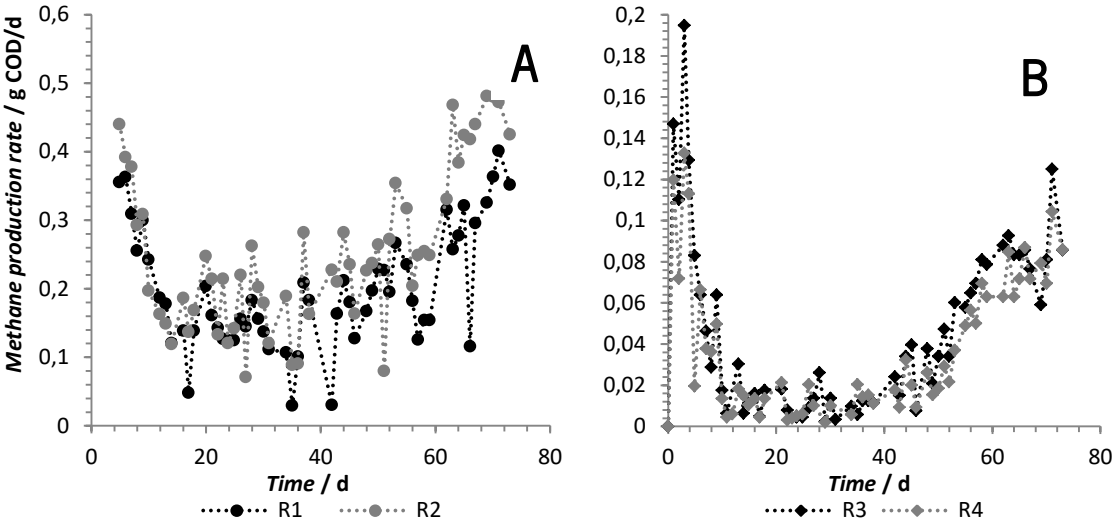


Figure 5.2 – Methane production rates in reactors; A) Reactors fed with ethanol feeding solution, B) Reactors fed with VFA feeding solution.

As can be observed in Figure 5.2, reactors operated with ethanol produced more methane than reactors operated with VFAs over the course of the experiment and, of the four, R2 performed the best overall. All reactors produced large amounts of methane at inoculation time (approximately 24 h of batch operation) and, afterwards production rates stabilized at lower levels. After 40 days of operation, the methane production rates started to slowly increase, which was more evident in R3 and R4 (Figure 5.2 B). In the final phase – from day 60 to the end – the methane production registered was more than the double (except for R1) of the rates verified in the first half.

Figure 5.3 shows the cumulative methane production rates along with COD removal efficiencies obtained by operation the four reactors. While R1 and R2 reached an average of 18.1 ± 3.73 gCOD/d, R3 and R4 only reached an amount of 3.8 ± 0.51 gCOD/d by the end of the experiment.

COD removal was also much more efficient in reactors R1 and R2 with percentages above 80 for the most part of the operation (Figure 5.3). On the other hand, R3 and R4 only started to show COD removal efficiencies above 50% in the final phase of the reactors operation.

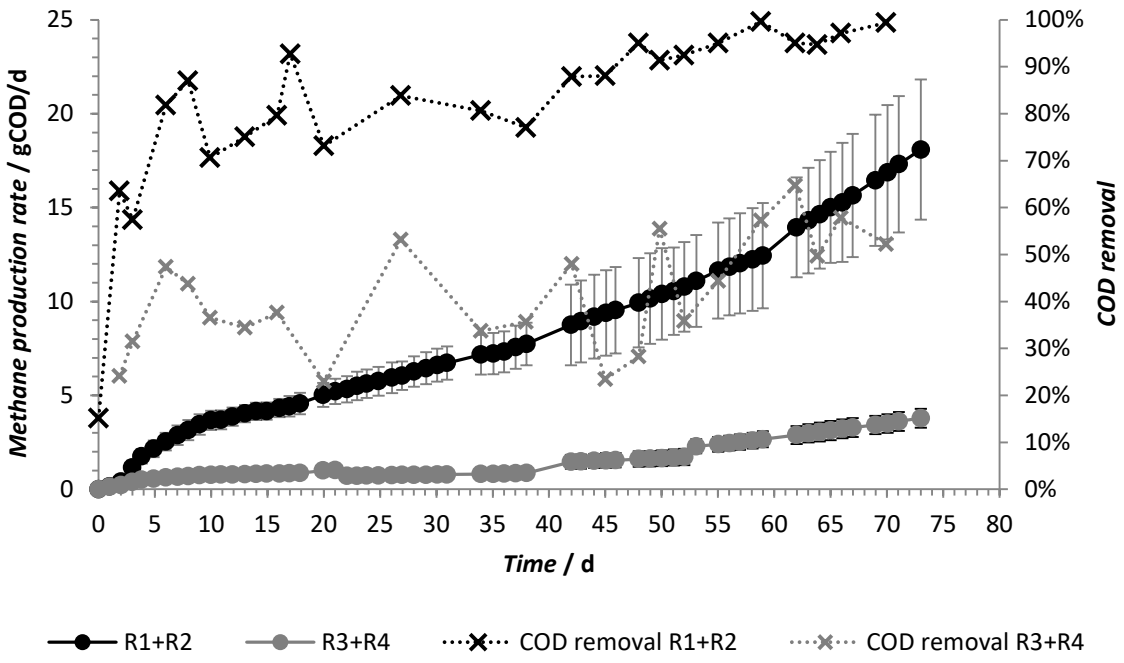


Figure 5.3 – Cumulative methane production rates and COD removal efficiencies in reactors fed with ethanol and reactors fed with VFAs. Results are presented in averages of the duplicates.

As observed in Figure 5.4, the ethanol that was fed to both reactors R1 and R2 was completely consumed throughout the whole experiment and no trace of this substance was found in all samples. In the other side, higher amounts of acetate were detected during the first 40 days. After this time, it started to decrease, disappearing from the reactors after 55 days. This decrease coincides with the increase in methane production rates and the higher percentages of COD removal. In addition, the COD of R1 and R2 was also higher in the beginning and started to decrease to very low values, around the same time that acetate did. Since there was no ethanol at the exit of the reactors at any point of the operation, it might indicate that the soluble COD content was comprised fully by acetate.

Regarding the reactors with VFAs, the COD of feeding solutions was maintained at around 3 g/L (Figure 5.5A). In the exit of the reactors the values dropped to values around 2 g/L of COD during the first 40 days. Afterwards, COD values started to decrease gradually until the end of the operation (Figure 5.5B). Despite being the most abundant VFA in feeding solutions, butyrate was mostly consumed through the whole experiment, whereas some considerable amounts (around half of what was fed) of propionate were still detected at the exit of both reactors (Figure 5.5B).

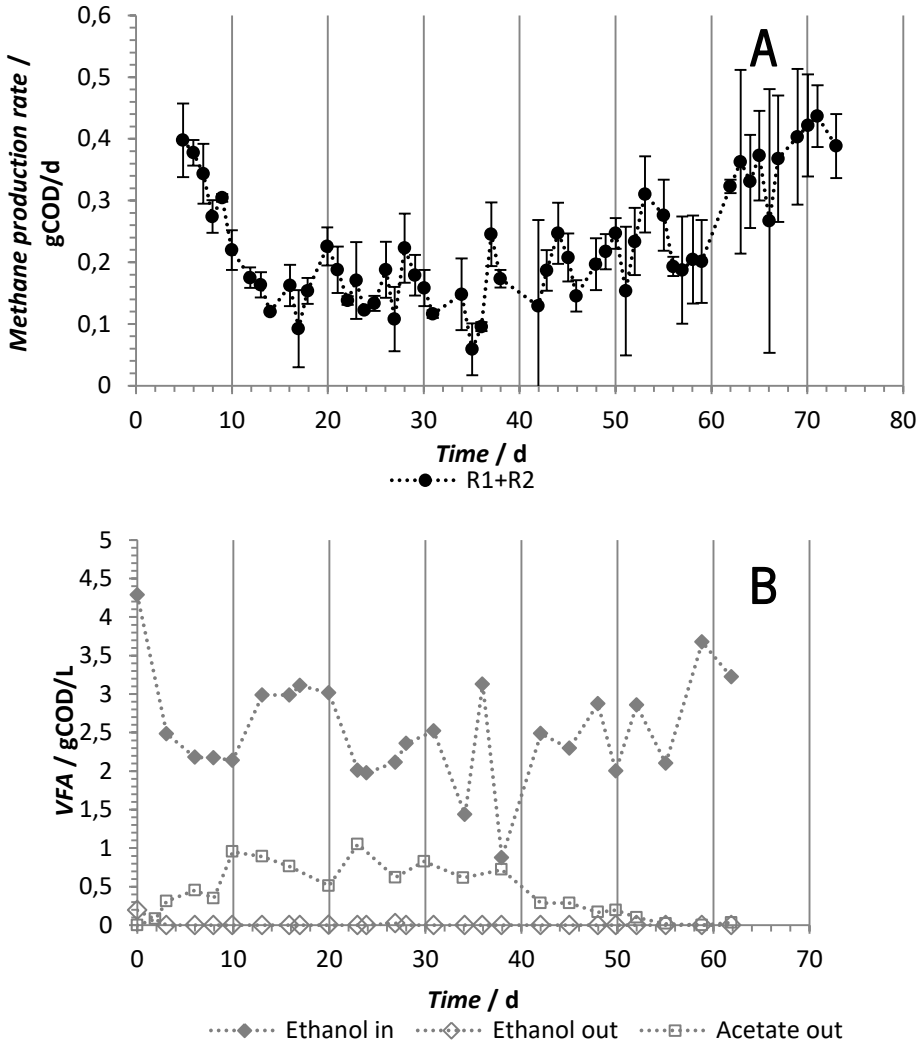


Figure 5.4 – Methane production, ethanol and acetate averages present in R1 and R2; A) Methane production rates; B) ethanol and acetate in entrance and exit of the reactors

Regarding the methane production rates (Figure 5.5B), they started to increase as the COD decreased. However, the amount of acetate and propionate at the exit only started to decrease after methane production rates had already reached the higher levels. Butyrate was kept at low concentrations throughout the whole assay. As was verified in R1 and R2, COD in R3 and R4 was composed mostly by acetate, despite the presence of other VFA.

VSS measured at the exit of the reactors reflected the biomass wash-out that was verified during operation (Table 5.1). The results showed that reactors R1 and R2 had high washout in the first day after continuous operation, decreasing afterwards, while reactors R3 and R4 the washout was higher at day 2.

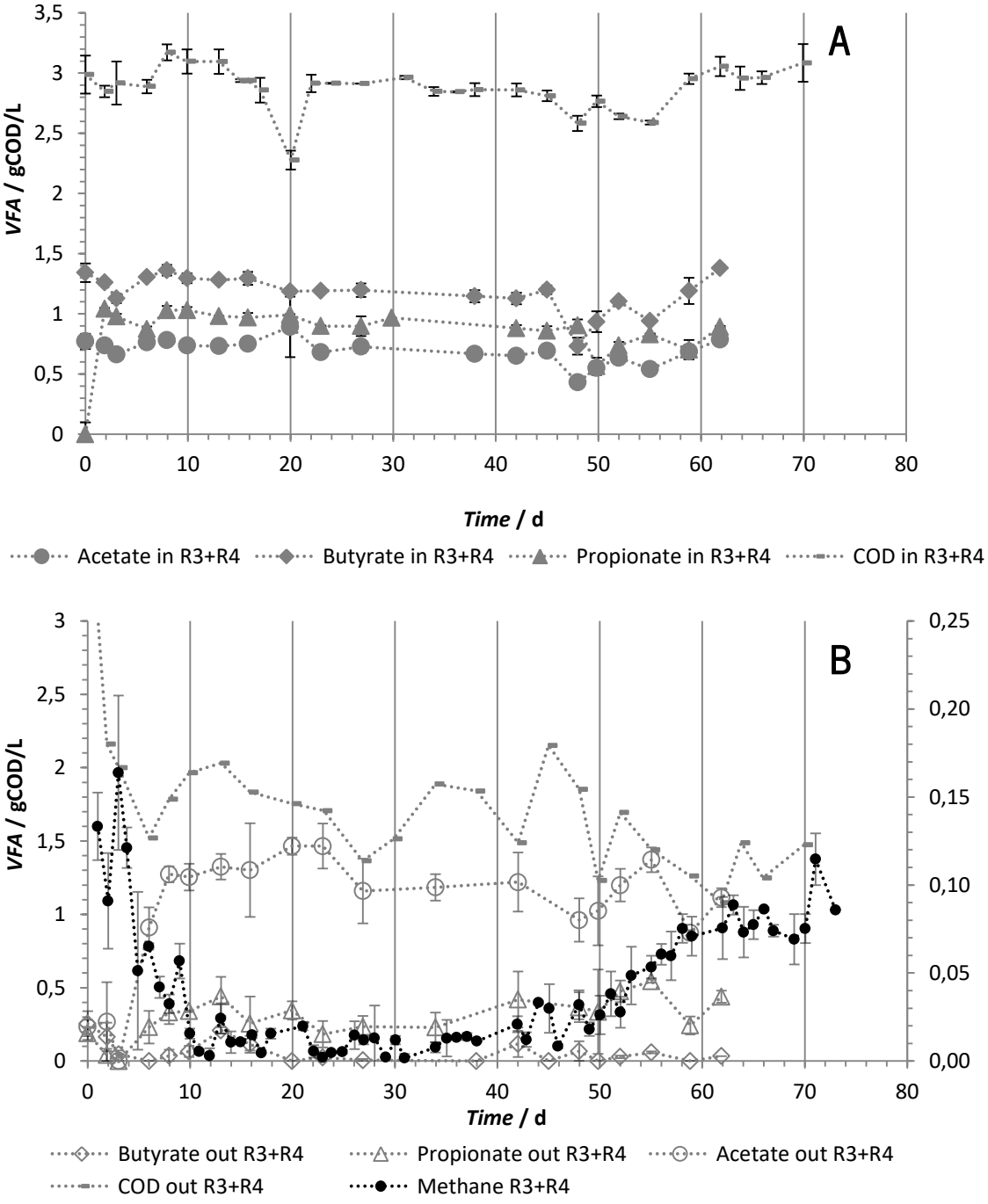


Figure 5.5 – COD and VFA along time for R3 and R4: A – feeding solution; B - exit of R3 and R4 .

pH was also monitored during reactor operation and the values are presented in Table 5.2. During the first 50 days, the pH of the feeding solution was not controlled and the values were around 9 for all reactors. After that, pH was adjusted to values around 7 by adding HCl (5 M) after autoclaving the feeding solution. This fact did not result in a significant change on the pH inside the reactors, with the values varying around 7.7 ± 0.27 to 8.3 ± 0.25. ORP values were also determined at the entrance and exit of all four reactors, as shown in Table 5.3.

Table 5.1 – VSS determined in each of the four reactors throughout the assay

t	VSS (mg/L)			
	R1	R2	R3	R4
1	4898 ± 1846	10117 ± 4326	667 ± 29	1394 ± 319
2	783 ± 153	7667 ± 954	1083 ± 558	1572 ± 256
5	150 ± 71	787 ± 186	207 ± 45	500 ± 413
7	120 ± 80	953 ± 255	600 ± 608	227 ± 117
15	160 ± 72	410 ± 36	147 ± 110	400 ± 433
22	29 ± 14	42 ± 6	96 ± 130	150 ± 10
44	45 ± 18	100 ± 13	71 ± 19	90 ± 24
65	250 ± 29	40 ± 10	213 ± 0	258 ± 7

Table 5.2 – pH averages of reactors 1 to 4

t	R1		R2		R3		R4	
	in	out	in	out	in	out	in	out
0-15	8.7 ± 0.6	7.8 ± 0.9	8.6 ± 0.6	7.6 ± 0.4	8.7 ± 0.4	8.1 ± 0.4	8.8 ± 0.3	7.9 ± 0.2
15-30	9.1 ± 0.3	8.4 ± 0.6	9.1 ± 0.3	7.8 ± 0.6	9.0 ± 0.3	8.2 ± 0.3	9.0 ± 0.3	8.1 ± 0.3
30-48	9.1 ± 0.5	8.4 ± 0.6	9.1 ± 0.5	7.8 ± 0.3	9.1 ± 0.4	8.1 ± 0.4	9.0 ± 0.6	8.0 ± 0.3
48-73	7.4 ± 0.3	8.3 ± 0.3	7.4 ± 0.2	7.7 ± 0.3	7.5 ± 0.2	7.8 ± 0.2	7.6 ± 0.4	7.8 ± 0.2

Table 5.3 – ORP values averages at the entrance and exit of R1, R2, R3 and R4

t	R1		R2		R3		R4	
	in	out	in	out	in	out	in	out
0-15	-287 ± 63	-256 ± 115	-273 ± 24	-238 ± 119	-303 ± 12	-315 ± 31	-311 ± 16	-329 ± 23
15-30	-331 ± 23	-235 ± 91	-263 ± 87	-279 ± 59	-314 ± 19	-301 ± 99	-321 ± 11	-321 ± 62
30-48	-326 ± 53	-256 ± 87	-343 ± 72	-269 ± 17	-310 ± 16	-332 ± 21	-302 ±24	-320 ± 17
48-73	-290 ± 32	-126 ± 88	-279 ± 25	-208 ± 57	-317 ± .0	-324 ± 29	-300 ± 28	-326 ± 22

5.4 Discussion

The adaptation of anaerobic biomass to specific substrates is a crucial point for the success of AD processes. Here we showed that the same inoculum could adapt to different substrates (ethanol in reactors R1 and R2 and a mixture of VFA in reactors R3 and R4. However, as verified, the adaptation to the different substrates takes different times and led to different rates of methane production (Figures 5.2 and 5.3).

The performance of all reactors was probably affected by the low acetoclastic activity, because in all reactors, and during the first 40 d, acetate remained in the reactors at a high concentration. Only after adjusting the pH of the feeding, acetate concentrations started to decrease. Indeed, in anaerobic reactors, about 70% of methane is produced by acetoclastic methanogens, while the other 30% is produced by the action of hydrogenotrophs (Abreu et al. 2007; Ali Shah et al. 2014). Acetate, aside from being a sub-product in the AD process, can also be directly consumed for methane production (Table 5.4) and its accumulation at high levels may indicate that the activity of acetoclastic methanogens present in the community is being inhibited (Abreu et al. 2007). Therefore, when acetate levels in the reactors started to decrease, the highest rates of methane production in this work were achieved, meaning that the acetoclastic methanogens were becoming active (Figure 5.4). In addition, in the first 40 days after the start of the operation, the hydrogenotrophic methanogens were probably responsible for most, if not all, methane produced. The anaerobic sludge used in this work displayed low acetoclastic activity (27.1 ± 1.6 mL CH_4@STP/gVSS d), determined in activity tests performed before inoculation (data not shown), which can explain the delay in the acetoclastic methanogens' adaptation, in contrast to the seemingly high activity displayed by hydrogenotrophs (326.1 ± 19.5 mL CH_4@STP/gVSS d).

Table 5.4 – Reactions occurred in the AD process with ethanol and acetate as the substrates for methane production

Substrate	Reaction	Total $\Delta G^{\circ\prime}$ (kJ/mol CH_4)
Ethanol	$2EtOH + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 4H_2$ $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ $2EtOH + HCO_3^- \rightarrow 2CH_3COO^- + H^+ + CH_4 + H_2O$	-116.3
Acetate	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-36

Another explanation for the long period of biomass adaptation and to the lower methane production rates obtained, could be the higher the biomass washout verified in all four reactors, in particular R3 and R4 (Li et al. 2008). Due to operational conditions, the washout of the granular sludge verified in the first days of operation probably caused a high washout of methanogenic microorganisms, specially the acetoclastic. The washout of the granular sludge may limit the biogas production and analysis of the methanogenic communities (Li et al. 2008).

The organic loading rate (OLR) used in the operation of these reactors was of 0.6 gCOD/L and calculations (Appendix II) were made in order to determine what was the expected amount of methane to be produced for the OLR fed to the reactors. Assuming an average of 60% of methane contained in the biogas produced, it was expected to be obtained a rate of 225.27 mL/d of methane. Since 350 mL of methane correspond to 1 gCOD, the expected amount of methane, assuming total conversion of the COD was of 0.64 gCOD/d. Therefore, the methane production rates obtained in all reactors, particularly in R3 and R4, were low in comparison to what was expected by total conversion of COD (the maximum conversion rates obtained were of 0.44 gCOD/d and 0.11 gCOD/d for averages of R1+R2 and R3+R4, respectively).

Ethanol was the sole organic substrate fed to R1 and R2 and was entirely consumed from beginning to end. As shown in Table 5.4, methane production from ethanol oxidation is a much more energetically favorable process than acetoclastic methanogenesis, with little over 3 times the amount of energy generated. Also, it is known that ethanol induces high specific acetoclastic activity in anaerobic sludge (Abreu et al. 2007) and, as it was already mentioned, a major part of the methane produced in AD derives from the activity of acetoclastic methanogens. These facts, coupled to the much higher COD removal efficiency observed in R1 and R2, might offer an explanation as to why the methane produced by the reactors fed with ethanol (R1 and R2) was far greater than the methane produced in reactors fed with VFA (R3 and R4). The accumulation of propionate in R3 and R4 fed with VFA, might also have been a growth-limiting factor, since it is known that its presence in high concentrations (900 mg/L) inhibits methanogenic activity (Wang et al. 2009), although in this case it was only detected at relatively low concentrations (lower than 450 mg/L for most of the experiment).

Finally, in this Chapter we could successfully adapt an anaerobic sludge to the degradation of ethanol and VFA and identify the critical points of the bioreactors operation. The results obtained were essential for starting the studies on the effect of CNT on the methanogenic activity of mixed microbial cultures. The adaptation is a crucial period for the success in UASB reactors, and full control of all the parameters involved in the process is necessary in order to prevent instability problems, and to promote

the growth and establishment of an active and efficient microbial community. For future studies, the addition of conductive materials during the adaptation period might be an interesting approach to improve methane production in bioreactors and to select for a different and eventually more efficient microbiome in the degradation of ethanol- and VFA-based effluents.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The experiments designed in the framework of this dissertation had the main objective of developing new strategies for the conversion of waste organic matter to methane, by adding CNT to methanogenic cultures, in order to determine its effect in methane production. A better understanding of the whole process of methane production by the methanogenic archaea alone and the methane producing syntrophic communities was, and is still necessary in order to maximize the conversion of waste organic matter to methane.

In Chapters 3 and 4 it was possible to verify the effects of CNT in the methane production by pure cultures of hydrogenotrophic and acetoclastic methanogens and by butyrate-degrading syntrophic co-cultures. The results obtained made it clear that the butyrate oxidation by *Syntrophomonas wolfei*, but specially the hydrogen consumption for methane production by *Methanospirillum hungatei* and *Methanobacterium formicicum*, is greatly accelerated by the presence of CNT. Also, we found a relationship between the media's redox potential and the concentration of CNT supplied. The redox potential increases with increasing concentrations of CNT, which resulted in higher methane production rates by methanogenic cultures. While CNT presence also accelerates methane production in pure cultures of acetoclastic methanogens, when supplied at high concentrations methane production is inhibited in these cultures. It remains unclear with the experiments conducted, if CNT have a role in interspecies electron transfer and further studies are necessary in order to determine its role in this mechanism.

In Chapter 5, anaerobic sludge was adapted to the degradation of different wastewater containing ethanol or VFA as substrate, in anaerobic bioreactors. Methane production rates increased in all four reactors after about 48 days of inoculation, with reactors fed with ethanol showing much better production by the end of the operation (over 4 times the methane produced by the reactors fed with VFA mixture). However, the washout in all reactors was a major problem, since most of the sludge was cleared out of the reactors in the first days of operation. The sludge washout might have also limited methane production in all reactors and, if this did not occur, methane yields would probably be higher. The microbial interactions within each one of the bioreactors still need to be studied.

For future works, it would be important to analyze the long-term effect of CNT in methanogenic co-cultures and pure cultures, in order to see if the cultures still maintain high methanogenic activity after adapting to the media supplied with CNT. To evaluate this, it would be necessary to re-inoculate the adapted cultures with more substrate and measure methane production over time, comparing to the results obtained in the first stage. It might also be an interesting approach, from a genetic point-of-view, to manipulate the methanogens and/or the fermentative bacteria, eliminating their ability to use

hydrogen, thus obligating them to use another mechanism for interspecies electron transfer, which will make it easier to determine if CNT do indeed have a role in the electron transfer.

Concerning reactor operation, it might be interesting to use higher work volumes, diminishing the washout effect, which might improve methane production and make it possible to analyze the methanogenic communities more effectively and more frequently, in order to observe their evolution during degradation of organic matter. This approach might also facilitate the application of CNT in the reactor operation, which is set to be a second stage of the work which is reported in Chapter 5 of this dissertation.

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7. APPENDICES

7.1 Appendix I – Chapter 3

7.1.1 Composition of basal medium and bicarbonate and salts and vitamins solutions

Basal medium:

- 900 mL of demi water
- 15 mL of KH2PO4 solution
- 15 mL of Na2HPO4.H2O solution
- 1 mL of trace elements H+ solution
- 1 mL of trace elements OH- solution
- 1 mL of resazurine solution

Bicarbonate solution:

- 100 mL of NaHCO3 solution
- 2 mL of Na2S.9H2O solution (added after autoclaving)

Salts and vitamins solution:

- 75 mL of demi water
- 25 mL of salts solution
- 2 mL of vitamins solution (added with sterile filter after autoclaving)

7.1.3 Substrate consumption by methanogenic pure cultures

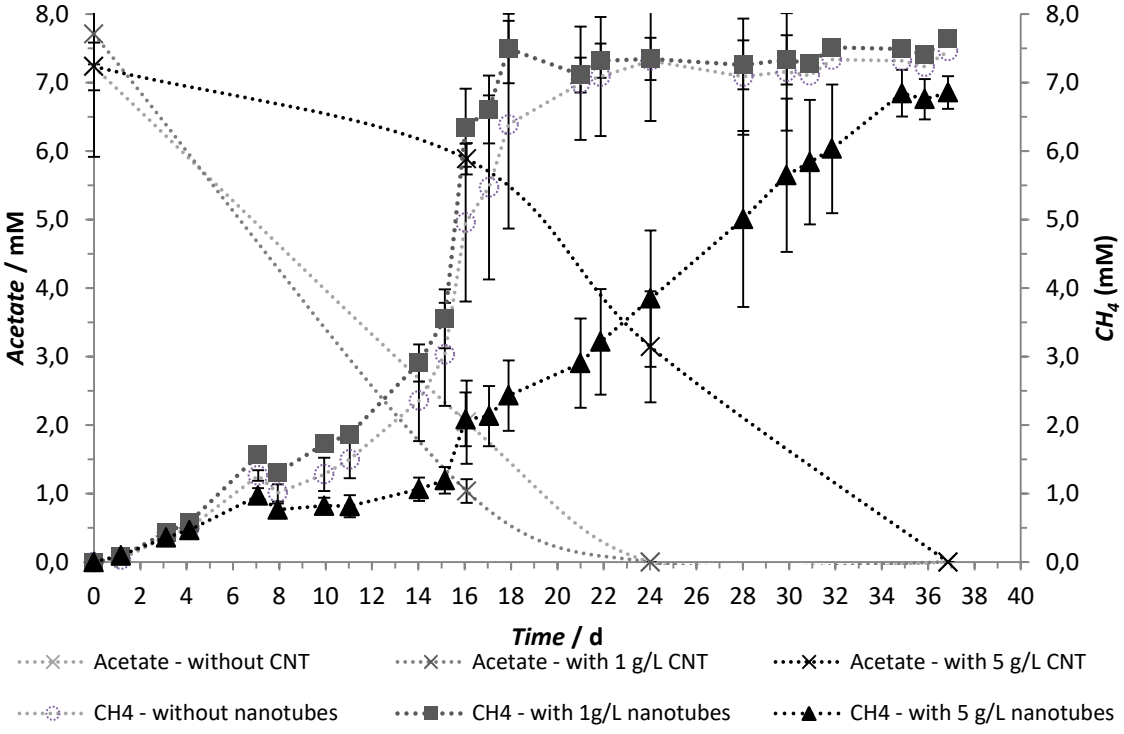

Figure 7.1 – Acetate consumption and methane production in *M. concilii* pure cultures

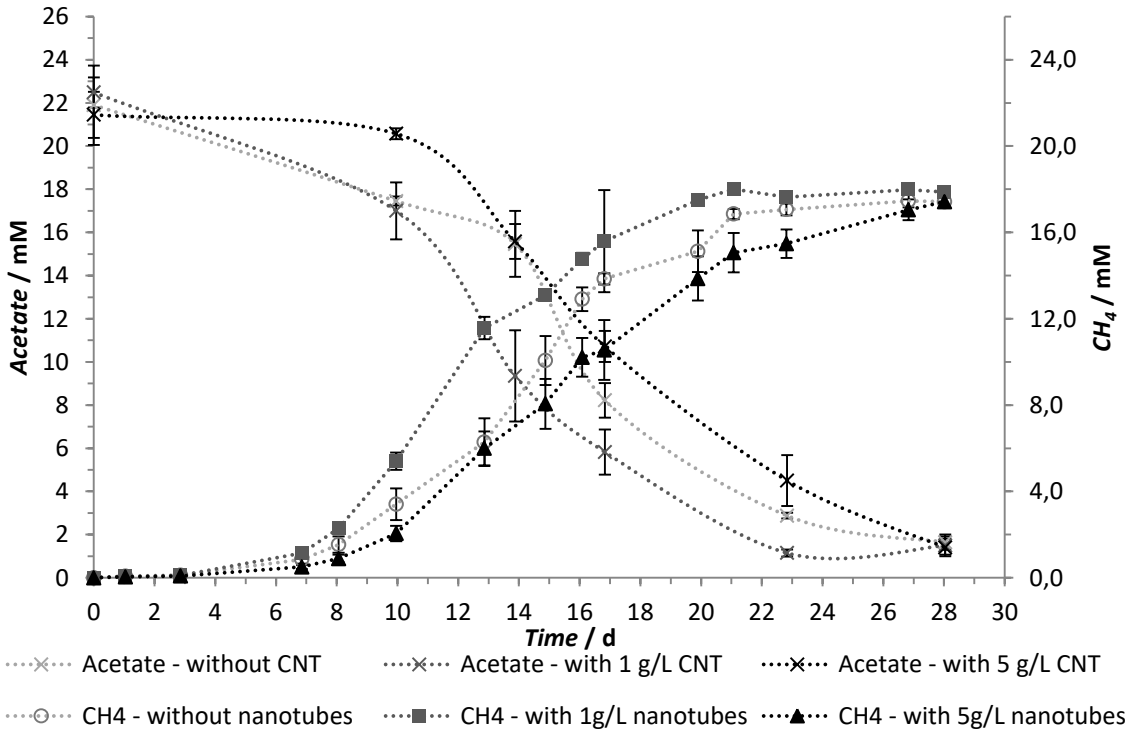

Figure 7.2 – Acetate consumption and methane production in *M. mazei* pure cultures

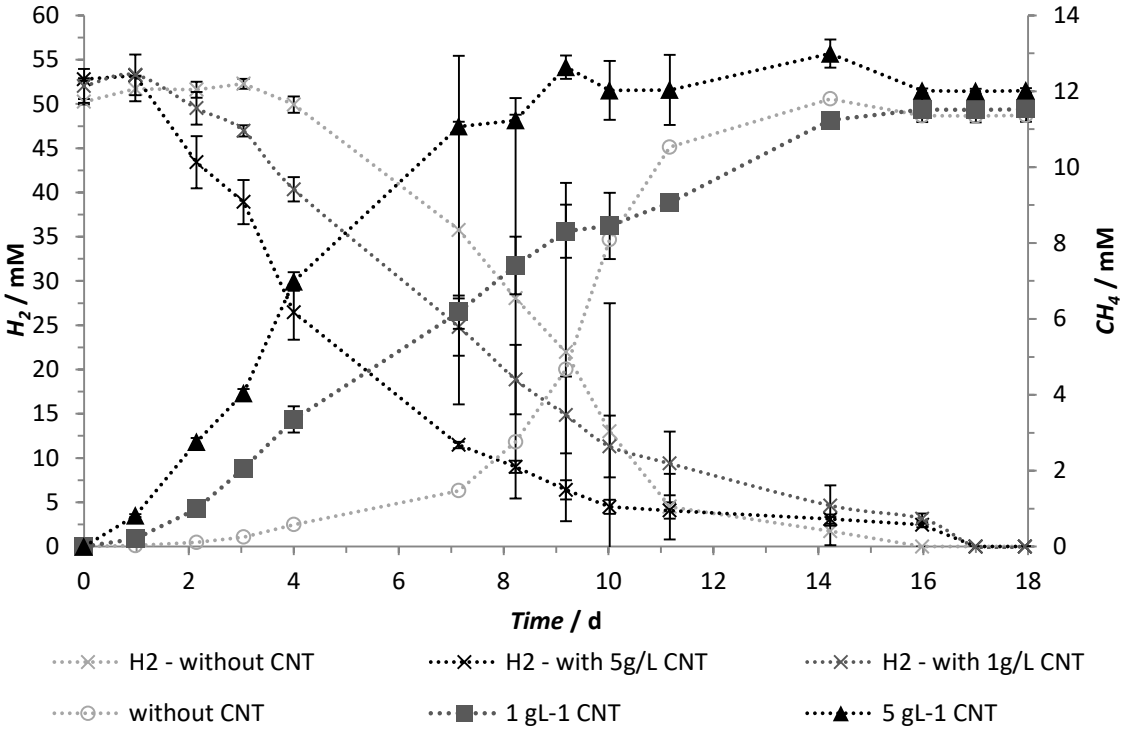

Figure 7.3 - Hydrogen consumption and methane production *by M. formicicum* pure cultures

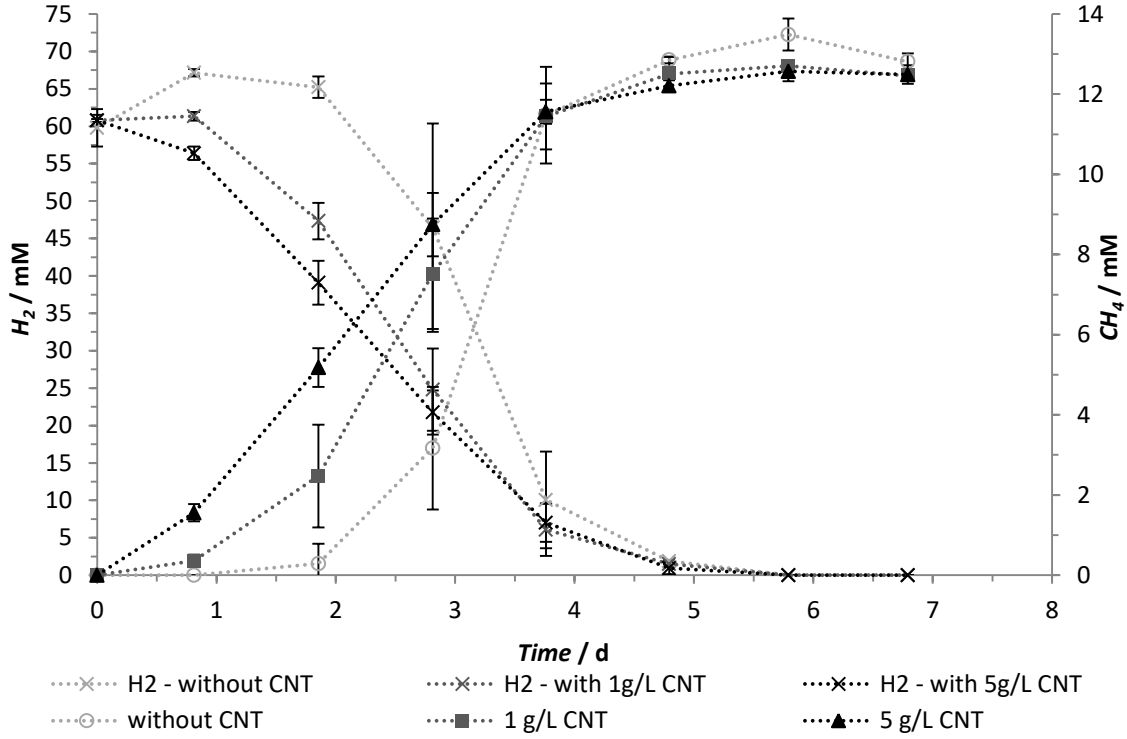

Figure 7.4 – Hydrogen consumption and methane production by *M. hungatei* pure cultures

7.2 Appendix II – Chapter 5

7.2.1 Calculations of expected methane production according to COD supplied to the reactors

$$0.25\ gCH4 \rightarrow 1\ gCOD \qquad ; \qquad 0.35\ L\ CH_4 \rightarrow 1\ gCOD$$

$$Expected\ CH_4\ gCOD = 0.22527\ \frac{L\ CH_4}{d} * \frac{1\ g\ COD}{0.35\ L\ CH_4} = \ 0.64\ \frac{gCOD}{d}$$

7.2.2 Routine analysis data

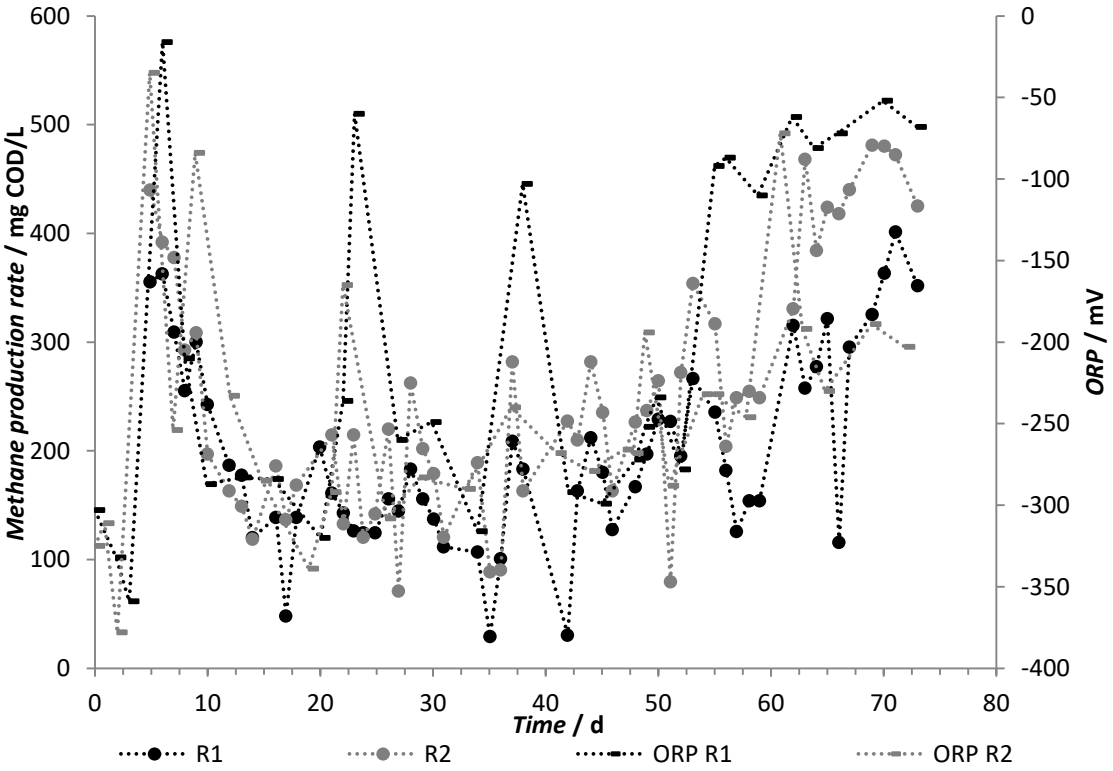

Figure 7.5 – Methane production rates and ORP values in R1 and R2

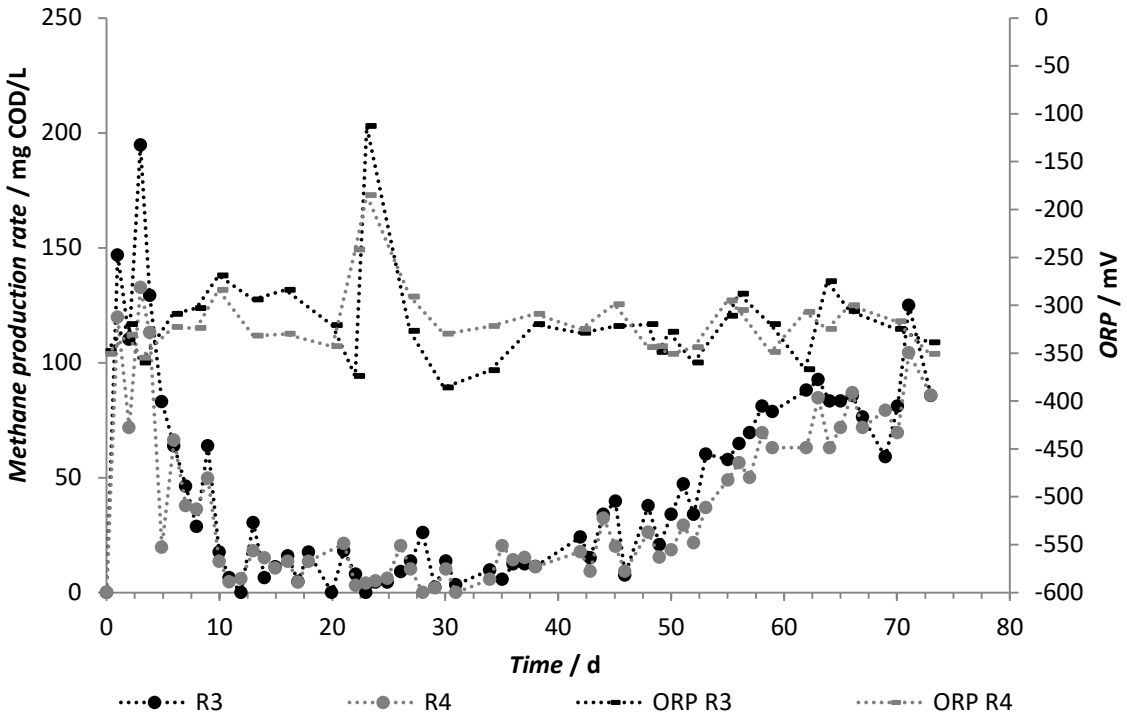

Figure 7.6 – Methane production rates and ORP values in R3 and R4

Table 7.1 – VSS and TSS concentrations in R1, R2, R3 and R4

t	R1		R2		R3		R4	
	TSS (mg/L)	VSS (mg/L)	TSS (mg/L)	VSS (mg/L)	TSS (mg/L)	VSS (mg/L)	TSS (mg/L)	VSS (mg/L)
0,0	6478 ± 1892	4898 ± 1846	11472 ± 4492	10117 ± 4326	3850 ± 229	667 ± 29	11622 ± 2812	1394 ± 319
0,9	8950 ± 2164	783 ± 153	8717 ± 855	7667 ± 954	9867 ± 475	1083 ± 558	9556 ± 214	1572 ± 256
4,0	1100 ± 113	150 ± 71	1020 ± 87	787 ± 186	1320 ± 92	207 ± 145	1253 ± 61	500 ± 413
5,9	473 ± 76	120 ± 80	1280 ± 85	953 ± 255	1040 ± 632	600 ± 608	800 ± 164	227 ± 117
13,8	737 ± 117	160 ± 72	547 ± 29	410 ± 36	343 ± 116	147 ± 110	790 ± 382	400 ± 433
20,9	123 ± 11	29 ± 14	67 ± 11	42 ± 6	198 ± 82	96 ± 130	405 ± 50	150 ± 10
28,0	239 ± 10	88 ± 4	300 ± 17	192 ± 8	358 ± 14	133 ± 5	458 ± 38	162 ± 22
34,9	279 ± 110	116 ± 101	151 ± 12	121 ± 09	225 ± 14	72 ± 10	264 ± 43	94 ± 17
42,9	145 ± 9	45 ± 18	133 ± 4	100 ± 13	236 ± 7	71 ± 19	233 ± 52	90 ± 24
50,0	194 ± 32	73 ± 16	324 ± 95	214 ± 66	459 ± 31	213 ± 15	570 ± 14	188 ± 19
60,0	265 ± 9	213 ± 9	182 ± 23	58 ± 10	497 ± 10	194 ± 13	545 ± 24	215 ± 14
63,9	294 ± 10	250 ± 29	153 ± 12	40 ± 10	388 ± 25	213 ± 0	458 ± 29	258 ± 7

7.2.3 Feeding solutions composition:

Feeding solution with ethanol (1 L solution)

- 950 mL of distilled water
- 1 mL of micronutrients solution
- 1.8 mL of macronutrients solution
- 1.44 g (= 1.825 mL) of absolute ethanol
- 5g of commercial bicarbonate
- 2 mL sodium sulfide (24.02 g/L)

Feeding solution with VFA (1L solution,)

- 950 mL of distilled water
- 1 mL of micronutrients solution
- 1.8 mL of macronutrients solution
- 1.5299 g of sodium acetate 3 hydrate
- 0.8742 g of sodium propionate
- 0.8689 g of sodium butyrate
- 5g of commercial bicarbonate
- 2 mL sodium sulfide (24.02 g/L)

Table 7.2 - Micronutrients and macronutrients solutions composition

	Element	Concentration (g/L)
Micronutrients	$FeCl_2.6H_2O$	2
	H_3BO_3	0.05
	$ZnCl_2$	0.05
	$CuCl_2.2H_2O$	0.038
	$MnCl_2.4H_2O$	0.5
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.05
	$AlCl_3.6H_2O$	0.09
	$CoCl_2.6H_2O$	2
	$NiCl_2.6H_2O$	0.092
	$Na_2SeO_3.5H_2O$	0.164
	EDTA	1
	Resazurin	0.2
	HCl 37%	1 (mL/L)
Macronutrients	$MgSO_4.7H_2O$	30.2
	KH_2PO_4	28.3
	NH_4Cl	170