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Exploring the effects of microaeration on the conversion of hexadecane to methane

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RESUMO

A grande variedade de resíduos e fontes de águas residuais contaminadas com hidrocarbonetos, juntamente com o alto rendimento energético dos hidrocarbonetos, torna-os potenciais candidatos à valorização por digestão anaeróbia em escala industrial para produção de biogás. Uma das principais barreiras à aplicação industrial de digestão anaeróbia de hidrocarbonetos é o longo tempo da biodegradação dos hidrocarbonetos, que pode requerer anos até degradação total. O *bottleneck* na conversão anaeróbia de hidrocarbonetos é a fase de ativação, na qual os hidrocarbonetos são convertidos a compostos mais facilmente biodegradáveis. Neste trabalho, teve como objetivo reduzir o período de ativação de hidrocarbonetos pela adição de quantidades vestigiais de oxigénio, aumentando assim a disponibilidade de compostos intermediários para microrganismos anaeróbios e, consequentemente, a produção de biogás e metano. Para atingir este objetivo, várias experiências foram planeadas de modo a estudar os efeitos da exposição do hexadecano e oxigénio a diferentes grupos de microrganismos que participam na biodegradação de hidrocarbonetos ao metano.

A toxicidade do hexadecano em culturas puras de microrganismos metanogénicos hidrogenotróficos foi avaliada em testes descontínuos. Culturas metanogénicas de *Methanobacterium formicicum* foram consideravelmente mais sensíveis ao hexadecano comparado com culturas de *Methanospirillum hungatei*, obtendo-se um IC_{50} de hexadecano entre 5 mM e 15 mM para *M. formicicum*, enquanto que em *M. hungatei* verificou-se uma redução de 27 ± 3% na taxa de produção de metano a 30 mM de hexadecano.

Ensaios de toxicidade com oxigénio em lamas anaeróbias demonstraram que a adição de etanol estimulou o consumo de O_2 por parte culturas e forneceu maior proteção contra a exposição ao oxigénio a populações metanogénicas, como se pode observar pela taxa de produção de metano significativamente maior na concentração de O_2 mais elevada (5% O_2 *headspace*) nas culturas com etanol, ao contrário da total inibição de produção de metano observada nos ensaios com H_2/CO_2 e acetato, *i.e.* 0,24 ± 0,05 mM/h (etanol) vs. 0,02 ± 0,01 mM/h (H_2/CO_2) e 0,01 ± 0,00 mM/h (acetato). Nas incubações com hexadecano, as diferentes percentagens de oxigénio testadas (2,5% e 5% e 21%) não tiveram efeito significativo na produção de metano e, até ao momento, não se podendo confirmar biodegradação anaeróbia do hexadecano adicionado.

Foram operados três biorreatores sob diferentes condições de arejamento: anaeróbio (AnR), anaeróbio com pulsos periódicos de ar (O2P) e adição contínua de quantidades vestigiais de O_2 dissolvido (O2C). Não foi possível confirmar a biodegradação de hexadecano devido à instabilidade da operação, tendo-se diagnosticado vários problemas experimentais, sendo o sistema modificado e adaptado de acordo. Melhoramentos no modo de operação de fluxo descendente e a implementação de um sistema de recuperação e reciclo de lamas eficiente são propostos. Os valores de ORP medidos foram semelhantes nos três biorreatores apesar das diferenças nas condições de arejamento aplicadas, contudo o reator O2C mostrou maior capacidade de captação de O_2 do meio.

ABSTRACT

The variety of waste residues and wastewater sources combined with the high energetic yield of hydrocarbons, makes hydrocarbon-contaminated wastes a potential candidate for valorization in industrial-scale anaerobic digestion for biogas production. One of the major barriers preventing industrial scale-up of hydrocarbon anaerobic digestion is the extensive degradation time, reaching periods of years before complete biodegradation. The bottleneck in anaerobic hydrocarbon bioconversion to methane is the activation phase, where the hydrocarbons are converted to more readily degradable compounds. In this work, an attempt was made to decrease the hydrocarbons activation period in the presence of limited amounts of oxygen, thus increasing the availability of the intermediary compounds for anaerobic microorganisms and ultimately increasing biogas and methane production. To achieve this goal, several experiments were design in order to study various effects of hexadecane and oxygen exposure to different microbial groups that participate in hydrocarbon biodegradation to methane.

Hexadecane toxicity towards pure cultures of hydrogenotrophic methanogens was evaluated in batch tests. Methanogenic cultures of *Methanobacterium formicicum* were substantially more sensitive to hexadecane than *Methanospirillum hungatei,* with a hexadecane IC_{50} between 5 mM and 15 mM for *M. formicicum*, while for *M. hungatei* a 27 ± 3 % decrease in methane production rate was observed at 30 mM hexadecane.

Oxygen toxicity assays on an anaerobic sludge showed that ethanol addition stimulated oxygen consumption by the cultures and provided increased shielding against oxygen exposure to methanogenic populations in all headspace O_2 percentages tested, with significant methane production rate observed even at the highest O_2 concentration tested (5% O_2 headspace), *i.e.* 0.22 ± 0.05 mM/h, contrary to the complete inhibition in the toxicity assays with H_2/CO_2 or acetate as substrate, 0.02 ± 0.01 mM/h and 0.01 ± 0.00 mM/h, respectively. In hexadecane amended cultures, addition of oxygen (2.5%, and 5% and 21% O_2 headspace) did not have a significant effect on methane production and anaerobic biodegradation of the added hexadecane is not evident thus far.

Three bioreactors were operated under different aeration conditions: anaerobic (AnR), anaerobic with periodic air pulses (O2P) and continuous supply of trace amounts of dissolved oxygen (O2C). Hexadecane biodegradation could not be confirmed due to instability of the operation, but several experimental problems could be identified, and the system was changed accordingly. Improvements for a downflow operation mode and implementation of an efficient sludge recovery and recycle system are proposed. ORP values measured were similar between conditions, despite the differences in aeration conditions, although the O2C reactor showed higher capacity for O_2 scavenging.

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ABBREVIATIONS

AAB	Acetic acid bacteria
AD	Anaerobic digestion
BTEX	Benzene, toluene, ethylbenzene and xylene
COD	Chemical oxygen demand
DO	Dissolved oxygen
FDH	Formate dehydrogenase
GC	Gas chromatography
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
LCFA	Long chain fatty acid
MPR	Methane production rate (mM/h)
$\bullet O_2^-$	Superoxide
OFMSW	Organic fraction of municipal solid waste
ORP	Oxidation-reduction potential
P	Maximum methane production
PAH	Polycyclic aromatic hydrocarbons
Rm	Methane production rate (per day)
ROS	Reactive oxygen species
$_r$RNA	Ribosomal ribonucleic acid
SMA	Specific methanogenic activity
S_r	Slope ratio
SRB	Sulphate-reducing bacteria
STP	Standard temperature and pressure
USBR	Upflow sludge bed reactor
VFA	Volatile fatty acid
VS	Volatile solids
$\Delta G^{o'}$	Free Gibbs energy
λ	Lag-phase

1. INTRODUCTION

1.1 Context and Motivation

Nowadays, there is an increasing concern regarding the environmental issues of oil exploration and processing. Petroleum is a valuable and essential resource in society (Varjani & Upasani, 2017), being one of the most important energy sources and a key raw material for a wide range of products. These can be categorized according to the associated industry: oil refinery products such as fuels (diesel, gasoline, kerosene, aviation fuel, etc.), non-fuel products such as solvents and lubricants, and raw materials for the petrochemical industry such as naphtha, ethane, propane, butane, ethylene, propylene, and others, essentially olefins and aromatic compounds; petrochemical industry products include synthetic polymers, purified chemicals, fuel and lubricant additives (Jafarinejad, 2017). If we include the upstream (exploration and production), the midstream (storing, transportation and wholesale of crude oil) and downstream activities (refining, byproduct distribution), the oil industry retains one of the largest shares of the global economy. As of 2011, the worldwide distribution of oil demand is overwhelmingly dominated by transportation (59 %), followed by industry (25 %), residential/commercial/agriculture (6 %) and energy sectors (4 %) and this trend is projected to remain ("Distribution of global oil demand in 2011 and 2040, by sector," 2017). Gasoline and gasoil/diesel alone represent over 50 % of the total worldwide demand of oil products ("Global demand outlook for selected oil products worldwide from 2015 to 2040 (in million barrels per day)," 2017).

Given that the industry is prevalent worldwide, and the rate of oil consumption is predicted to increase, humanity will continue to face challenges to minimize the environmental hazardous consequences of oil dependency. It is certain that until equality versatile and cheap raw materials are found, crude oil will remain a high demand valuable resource. Hydrocarbons affect not only human health, but the entire ecosystem (marine or land based) which they come in contact with, which includes all life forms, from plants, to animals and microorganisms, particularly, groundwater contamination is severe consequence from drainage of contaminated soils.

In this work, a strategy was devised to enhance hydrocarbon biodegradation to methane based on recent results obtained by Duarte and collaborators (Duarte et al., 2018) in the research group BRIDGE. The researchers showed that the conversion of hydrophobic substrates (long-chain fatty acids) to methane was facilitated by continuous supply of trace oxygen amounts into a bioreactor, by keeping the feeding tank open to the air. Given that during anaerobic degradation hydrocarbons are activated and eventually converted to fatty acids, it would be interesting to replicate the results in the bioremediation of hydrocarbon/petroleum wastewaters.

1.1.1 Environmental sustainability and economic feasibility

Hydrocarbons are toxic molecules and some, particularly benzene, toluene, ethylbenzene and xylene (BTEX) and polycyclic aromatic hydrocarbons (PAHs), are particularly problematic due to their recalcitrant nature and highly hemotoxic, carcinogenic and teratogenic effects (Varjani et al., 2017). As such, various methods for remediation have been proposed and employed, for example, the more traditional physico-chemical methods (Chaudhry et al., 2005; Varjani et al., 2017) however, due to high costs and low efficiency of these methods, bioremediation has received special attention, especially since its successful application during the 1989 Exxon Valdez oil spill (Bragg et al., 1994). Bioremediation is the use of microorganisms for detoxification or removal of pollutants and it is shown to be a non-invasive and cost-effective process (April et al., 2000; Leahy & Colwell, 1990). The first microorganisms capable of natural bioremediation were isolated from an oil reservoir as early as 1926 (Bastin et al., 1926) but the extent of this natural occurring microbial degradation was only recently understood. Nowadays, crude-oil *in situ* bioconversion to methane (biogas) in oil reservoirs has proven to be an important economic argument for increased research on this topic (Jones et al., 2008), not only for future improvement of oil extraction techniques but also *in situ* or *ex situ* bioconversion of contaminants in oil refining and petrochemical plants. This opens the possibility of combined bioremediation and production of added-value commodities from waste materials, giving a major incentive for investments in understanding the mechanisms of these specific microbial communities, the development and improvement.

The oil industry produces a multitude of waste most of which hazardous to the environment and to human health. Over the various stages of oil processing, the number of sources for wastewater and sludge at a risk of hydrocarbon leakage and contamination is staggering (Jafarinejad, 2017). In the upstream stage, during drilling, extraction and production, maintenance, decommissioning and reclamation (plugging and well abandonment mostly), the primary source of wastewaters in these activities are the productions waters in during oil production (Bashat, 2003), while sludge is originate primarily in tank/pipping sludges. For example, British Petroleum treated wastewater discharges from upstream and downstream stages totaled 260 m^3 per minute. Other sources of wastewater include processed water from machinery cooling and washing, ballast water, contaminated rain/drainage water, fracking fluids, drilling fluids, water and oil-based muds, while sludge source also include soil contamination, spill clean-up, and waste management (sandblast, greases and filters).

Both production and refining are associated with the midstream stage of oil processing, which include storage, manipulation, distribution and transportation. Production sites, transportation terminals and refineries maintain a considerable amount of stored crude oil, while refineries also store finished

products (Cholakov, 2009). Liquid tank bottoms (water and oil emulsions) are the main wastewater source from refinery storage tanks. Depending on the quality of underground storage tank, groundwater leakage may occur. Sludges are also generated mainly from storage and transportation tanks. In petroleum refineries, water is used on a continuous basis for a number. Sources of oil contamination include distillation units, hydrotreating, vis-breaking, catalytic cracking, hydrocracking, lube oil for machinery, spent caustic, ballast water and also rain water contamination from utility areas (European Commission & Joint Research Center, 2013). The variety of waste residues and wastewaters sources combined with the high energetic yield of hydrocarbons (Jiménez et al., 2016) makes hydrocarbon-contaminated wastes a potentially feasible candidate for industrial-scale valorization.

With continuous increase in annual production growth of rate of biogas, the installation of biogas production units in Europe as boomed due to financial incentives (Eurostat, 2015; Kampman et al., 2016), however, further expansion is limited by the lack of profitable valorization strategies. Nowadays, biogas is often used in situ for heat or power at a low overall energetic efficiency, disregarding its potential as a C1 feedstock (Pöschl et al., 2010). The valorization of renewable CH_4 through reforming can be a promising first step towards biomethane based production of biochemicals or biofuels. Such a strategy allows a more widespread use as feedstock and facilitating its valorization by reaching out to new markets such as the chemical industry (Gallezot, 2012), enabling the biomethane end-users to profile themselves as more sustainable and reducing its reliance on financial support (Verbeeck et al., 2018), making it an economically viable solution. Additionally, biogas can be upgraded in delocalized small scale producers to green gas or biomethane, which has the same quality as natural gas, and subsequently injected into the main natural gas grid, allowing for wider and long-distance distribution to industrial scale sites. (Hengeveld et al., 2014; Jury et al., 2010). Thus, to establish a competitive and economically viable process, it is important to optimize the biodegradation of the target waste materials, namely hydrocarbon-contaminated wastewaters and sludges. To achieve this goal, several aspects regarding the microbial degradation should be considered such as identifying the key microorganisms involved in the biodegradation of hydrocarbon, understanding their metabolism and associated metabolic pathways, and defining the optimum conditions (oxic or anoxic environment, nutrients availability, temperature and others) which boost biodegradation.

1.2 Objectives

The bottleneck in anaerobic hydrocarbons biodegradation is the first step of hydrocarbon biodegradation, the compound activation. In this work, we propose to perform hydrocarbons activation in the presence of limited amounts of oxygen, and then promote the degradation of the intermediary compounds to methane by anaerobic microorganisms. This approach combines the activity of facultative and anaerobic microorganisms generally present in anaerobic mixed communities. The specific objectives proposed are as follow:

- **Evaluate the effects of hexadecane on the methanogenic hydrogenotrophic activity**

  Monitoring the methane production in batch assay of pure cultures of *Methanobacterium formicicum* and *Methanospirillum hungatei* in the presence of increasing concentrations of hexadecane.

- **Evaluate the effects of microaerobic conditions on the conversion of hexadecane to methane in batch assays**

  Perform batch essays in bottles under strict anaerobic conditions and at increasing O_2 concentrations in the headspace, using direct methanogenic substrates, acetate and H_2/CO_2, and syntrophic methanogenic substrates, ethanol and hexadecane; monitor methane production rates, substrate consumption, intermediary and product formation; measure headspace O_2 uptake.

- **Optimize the conversion of hexadecane to methane in bioreactors**

  Operate bioreactors in different conditions: strict anaerobic, anaerobic with periodic air pulses and continuous supply of dissolved O_2; monitor the biogas production, methane yield, monitor chemical oxygen demand (COD) removal percentage and VFA accumulation. Achieve a stable continuous operation.

2. LITERATURE REVIEW

2.1 Overview of microbial anaerobic metabolism

Anaerobic degradation (or digestion) is a process by which a complex mixture of symbiotic microorganisms transforms organic materials under oxygen-free conditions into biogas, nutrients and additional cell matter, leaving salts and refractory organic matter (Merlin et al., 2014). Anaerobic digestion stabilizes the organic matter in wastewater solids, reduces pathogens and odors, and the total solids by converting part of the volatile solids fraction into biogas. It is both a waste treatment technology, which enhances environmental quality, and a sustainable energy producing technology.

In anaerobic digestion, there are four key biological and chemical steps of anaerobic digestion: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The first step is the depolymerization and hydrolysis of complex organic matter. During hydrolysis, insoluble polymers such as polysaccharides, proteins and nucleic acids are broken down into smaller units such as monosaccharides, fatty acids, glycerol and amino acids, by action of different hydrolytic enzymes secreted by a variety of hydrolytic microorganisms (Cirne et al., 2012). The products of hydrolysis are further converted to smaller molecules by fermentative microorganisms in a process referred to as acidogenesis (Merlin et al., 2014) and, depending on the anaerobic microbial species and incubation conditions, can lead to the production of several C1-C4 compounds such as volatile fatty acids (VFAs), e.g. acetate, propionate, butyrate, other organic acids such as formate and lactate, as well as alcohols, ketones and aldehydes. Carbon dioxide (CO_2) and hydrogen (H_2) gases are also products in this step. Acidogenesis is usually the fastest reaction in the anaerobic conversion of complex organic matter in liquid phase digestion (Mosey & Fernandes, 1989). The acidogenesis products, mainly VFA and alcohols, are then utilized by a group of strict anaerobes known as acetogens. Acetogens are slow growing microorganisms, sensitive to oxygen (O_2) exposure and organic content and surrounding environmental conditions. Furthermore, acetogens form metabolic partnerships with methanogenic hydrogenotrophs for the degradation of acidogenesis products to acetate, CO_2 and H_2. This is because the presence of methanogenic Archaea that consume hydrogen is essential for the oxidation of acetogenic substrates otherwise the process is energetically endergonic, thus thermodynamically unfavorable. If hydrogen partial pressure is are kept at low levels, then fermentation becomes exergonic. This partnership between microorganisms is referred to as syntrophy (Gieg et al., 2014; Meslé et al., 2013; Morris et al., 2013).

The last step in anaerobic digestion is methanogenesis, biogenic methane formation carried out by strictly anaerobic microorganisms. Methanogenesis, is generally understood as the final step on the respiratory chains in anoxic, organic-rich, electron acceptor-poor environments (Bernhard Schink, 1997) and it is dependent on the conjugated activity of several microorganisms of distinct phylogeny and

metabolisms. It is the least exergonic process when compared to aerobic degradation or the alternative anaerobic respiration (Mbadinga et al., 2011; Bernhard Schink, 1997). The small amount of energy available in methanogenic conversion is therefore one of the driving forces for the development of efficient syntrophic cooperation.

2.1.1 Insight into syntrophic partnerships

Syntrophy is a tightly coupled mutualistic interaction where intermediates exchanged between the partners must be kept at low concentrations for efficient cooperation among the partners to occur (Sieber et al., 2012). The first identified syntrophic interaction was the ethanol oxidizing *Methanobacillus omelianskii* and *Methanobacterium bryantii* strain MoH co-culture (Bryant et al., 1967), previously thought to be a pure culture of a direct ethanol oxidizing methanogen, *Methanobacterium omelianskii* (Barker, 1940). *M. omelianskii* fermented ethanol to acetate and H_2 and the latter was used by *M. bryantii* MoH, creating thermodynamically favorable conditions for growth of the fermenter. The prevalence and range of syntrophic metabolism in different anoxic environments emphasizes that metabolic cooperation in microbial communities is often the rule rather than the exception and that the microbial consortium is the catalytic unit of anaerobic metabolism. Syntrophic interactions can be divided into two groups (Stams & Plugge, 2009), the facultative syntrophic interactions, where the substrate is fermented solely by a fermentative bacteria but the growth and fermentation products are affected by the presence of hydrogenotrophs, and the obligately syntrophic interactions, in which the presence of both the bacterium and archaeon species is essential for the degradation of specific organic compounds.

In obligately syntrophic interactions, the distances between syntrophic partners can affect the biodegradation and specific growth rates leading to the formation of aggregates, each comprising a functional unit (Shen et al., 2016; Stams & Plugge, 2009), having evolved biochemical mechanisms that allow sharing of chemical energy. Even under optimal syntrophic growth conditions, free energy changes are small, and all available energy is shared by the different microorganisms, maintaining a community that operates in thermodynamic equilibrium (Sieber et al., 2012). Syntrophic metabolism is clustered in the *δ-proteobacteria* class, as shown by 16S rRNA sequencing, including genera *Syntrophus*, *Syntrophobacter*, *Desulfoglaeba*, *Geobacter*, *Desulfovibrio* and *Pelobacter*. Other groups of species are comprised within the class *Syntrophomonadaceae*, particularly the *Syntrophomonas* genus (Mcinerney et al., 2008; Sieber et al., 2012).

In these methanogenic syntrophic communities, reducing equivalents are transferred between the bacteria and archaeon by shuttle components, through a process known as interspecies electron transfer.

The most commonly described of such mechanisms is the indirect H_2/formate transfer. Hydrogen forming bacteria and hydrogenotrophic methanogens sense redox conditions, influencing each other's metabolism. At high H_2 concentrations, the metabolism of the fermentative partner (acetogen) is inhibited and that of the H_2 scavenger (hydrogenotrophic methanogen) is stimulated. H_2 transfers between microorganisms occur by diffusion, and its ability to act as a shuttle for syntrophic interactions requires both syntrophic partners to contain hydrogenases, enzymes that catalyze the reversible conversion of hydrogen into protons and electrons, as shown by studies where the H_2 metabolic pathway was deleted (Hillesland & Stahl, 2010; A. Rotaru et al., 2012). Physical disruption of anaerobic aggregates can lead to a severe loss of methanogenic activity with substrates that require interspecies hydrogen transfer (Grotenhuis et al., 1991; Schmidt & Ahring, 1993).

Interspecies formate transfer can happen alongside H_2 transfer (Sieber et al., 2014) and is even essential for specific syntrophic partners (Mcinerney et al., 2008). Two pathways have been proposed (Shen et al., 2016), an indirect path with formate being split into H_2 and HCO_3^- (or CO_2) by a formate dehydrogenase and subsequently converted to methane or formate can be directly oxidized to methane by a formate hydrogenlyase system, consisting of a formate hydrogenase coupled with a formate dehydrogenase (FDH), and has been reported in both bacterium (Sawers, 2005) and archaeon (Wu et al., 1993). Studies have shown that syntrophic propionate (Dong et al., 1994; Dong & Stams, 1995) and butyrate degradation (Dong et al., 1994) were only possible if the syntrophic partner was capable of utilizing both H_2 and formate. Indeed, the levels of formate hydrogenase were elevated in both syntrophic partners, consistent with interspecies formate transfer (Bok et al., 2002).

2.2 Hydrocarbon biodegradation

2.2.1 Crude oil and hydrocarbons

Petroleum or crude oil is a naturally occurring, toxic, flammable dark brown liquid consisting of a complex mixture of organic compounds, mainly hydrocarbons, oxygen, nitrogen and sulfur-containing organic compounds, as well as inorganic components such as metals (Varjani & Upasani, 2017). The main components of crude oil can be broadly divided into 4 main categories: aliphatic (alkanes and naphthenic compounds), aromatics, such as the monoaromatics BTEX and PAHs, resins and asphaltenes (Meslé et al. 2013; Varjani, 2017). Saturated alkanes are hydrocarbons with simple bonds and range from linear chain alkanes, branched chain alkanes and cycloalkanes and represent the highest

percentage of crude oil components. Aromatic hydrocarbons have one or more aromatic rings usually substituted with other alkyl groups. Resins and asphaltenes contain very complex and mostly unknown carbon structures that contain non-carbon polar groups with nitrogen, sulfur and oxygen atoms, though these two components are not considered hydrocarbons. The susceptibility of hydrocarbons to microbial degradation depends primarily, on the type of hydrocarbon, which follows a decreasing susceptibility: linear alkanes > branched alkanes > small aromatics > cyclic alkanes > polycyclic aromatics.

2.2.2 Aerobic pathway

The aerobic biodegradation of hydrocarbons occurs in the presence of oxygen, which is used as the reactant for the activation of the hydrocarbon molecules and as the final electron acceptor in the respiration process (Wentzel, Ellingsen, Kotlar, Zotchev, & Throne-Holst, 2007). The initial intracellular attack of hydrocarbon is an oxidative process, activation and incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases (Abbasian & Lockington, 2015; Peixoto, Vermelho, & Rosado, 2011). The use of oxygen in the initial activation allows the microorganisms to overcome the low chemical reactivity of hydrocarbons by generating reactive oxygen species, resulting in faster degradation and assimilation into the metabolic pathways.

Various enzymes have been identified in different pathways during aerobic degradation of hydrocarbons and are extensively described in several reviews (Abbasian & Lockington, 2015; Das, Chandran, Das, & Chandran, 2011; Varjani, 2017; Wentzel et al., 2007), but the most commonly described pathways involve the alkane monooxygenase/hydroxylase enzyme group (Abbasian & Lockington, 2015). The aforementioned reviews also explore the different genera of microorganisms capable of aerobic degradation, and particular interest is given to *Rhodococcus* (Sorkhoh, Ghannoum, Lbrahim, Stretton, & Radwan, 1990; Whyte et al., 1998), *Acinetobacter* (Chung & Kubo, 2001; Yuste, Puyet, & Y, 2000) and *Pseudomonas* (Beilen et al., 2002; Naik & Sakthivel, 2006) genera, capable of degrading hydrocarbons ranging from C6 to C36. Some of the bacteria are facultative anaerobes, meaning that they can function under very low oxygen levels or even anoxic conditions, which is an important characteristic when considering complex microbial consortia for biogenic methane production. *Pseudomonas*, for example, has been detected in anoxic oil reservoirs (Li et al., 2012; Zhang et al., 2012) and has also been associated with oil degradation under anoxic conditions in laboratory (Ranchou-peyruse, 2008; W. Wang, Zhang, Wang, Shen, & Pan, 2014), substantiating its role in anaerobic and possibly methanogenic environments.

2.2.3 Anaerobic pathway

Methanogenic hydrocarbon degradation occurs in a series of steps requires close syntrophic relations between fermentative bacteria and methanogenic *Archaea* (Zengler et al., 1999). First the hydrocarbons need to be activated. Mechanisms for hydrocarbon activation in anaerobic biodegradation pathways are more diverse than aerobic ones and five different mechanisms have been proposed (Abbasian & Lockington, 2015): fumarate addition to methylene or methyl groups of hydrocarbons, oxygen-independent hydroxylation on the second or third terminal atoms (to make secondary or tertiary alcohols), carboxylation of unsubstituted carbon atoms of aromatics, hydration of the double and triple bond of alkenes and alkynes and reverse methanogenesis, although addition of fumarate is the most accepted. Further degradation involves reactions that are endergonic and become thermodynamically feasible only if the end products (formate, hydrogen or acetate) are kept at low concentrations (Jiménez et al., 2016). The methanogenic transformation of alkanes is possible at hydrogen partial pressures lower that 4×10^{-5} atm (Dolfing et al., 2008). Figure 2.1 illustrates the various stages of microbial hydrocarbon conversion to methane.

Biodegradation of n-alkanes under methanogenic conditions was first reported in highly enriched cultures obtained from ditch mud, with the ability to metabolize hexadecane into methane (Zengler et al., 1999). Ribosomal 16S analysis showed that all the bacteria were affiliated to δ-proteobacteria, three of closely related to the genus *Syntrophus.* All *Archaea* were related to methanogenic Archaea, three particular genera were detected, *Methanosaeta*, which comprise acetoclastic methanogens, and *Methanospirillum* and *Methanoculleus*, associated with hydrogenotrophic methanogens. The low concentration of acetate and hydrogen (H_2) towards the end of the experiments and free-energy calculations were in agreement with the authors initially proposed syntrophic partnership: a syntrophic acetogen oxidizes the hexadecane into acetate and H_2, a group of *Archaea* cleaves acetate into methane and CO_2 and another group of *Archaea* that converts CO_2 and H_2 to methane. The authors suggested that over two thirds of the methane was produced from acetate.

In a recent review, Mésle and co-workers summarized the genus frequently found in oil reservoirs (Meslé et al., 2013). Recovered methanogens include the hydrogenotrophic genera *Methanocalculus*, *Methanoculleus*, *Methanobacterium*, *Methanothermobacter*, the obligatory acetoclastic *Methanosaeta* and the versatile *Methanosarcina*. Syntrophic bacterial genera include the δ-proteobacteria *Syntrophus* and *Smithella,* the Firmicutes *Clostridium* and *Syntrophomonas,* and several fermentative bacteria including *Clostridium*, the *Acinetobacteria*, *Rhodococcus* and *Propionicella* and β-proteobacteria *Azoarcus* and *Acidovorax*, among others. *Smithella* and *Syntrophus* have been identified in multiple environments

and are prevalent in both n-alkane and crude-oil degrading cultures, making them key genera in syntrophic hydrocarbon metabolism (Gieg et al., 2014).

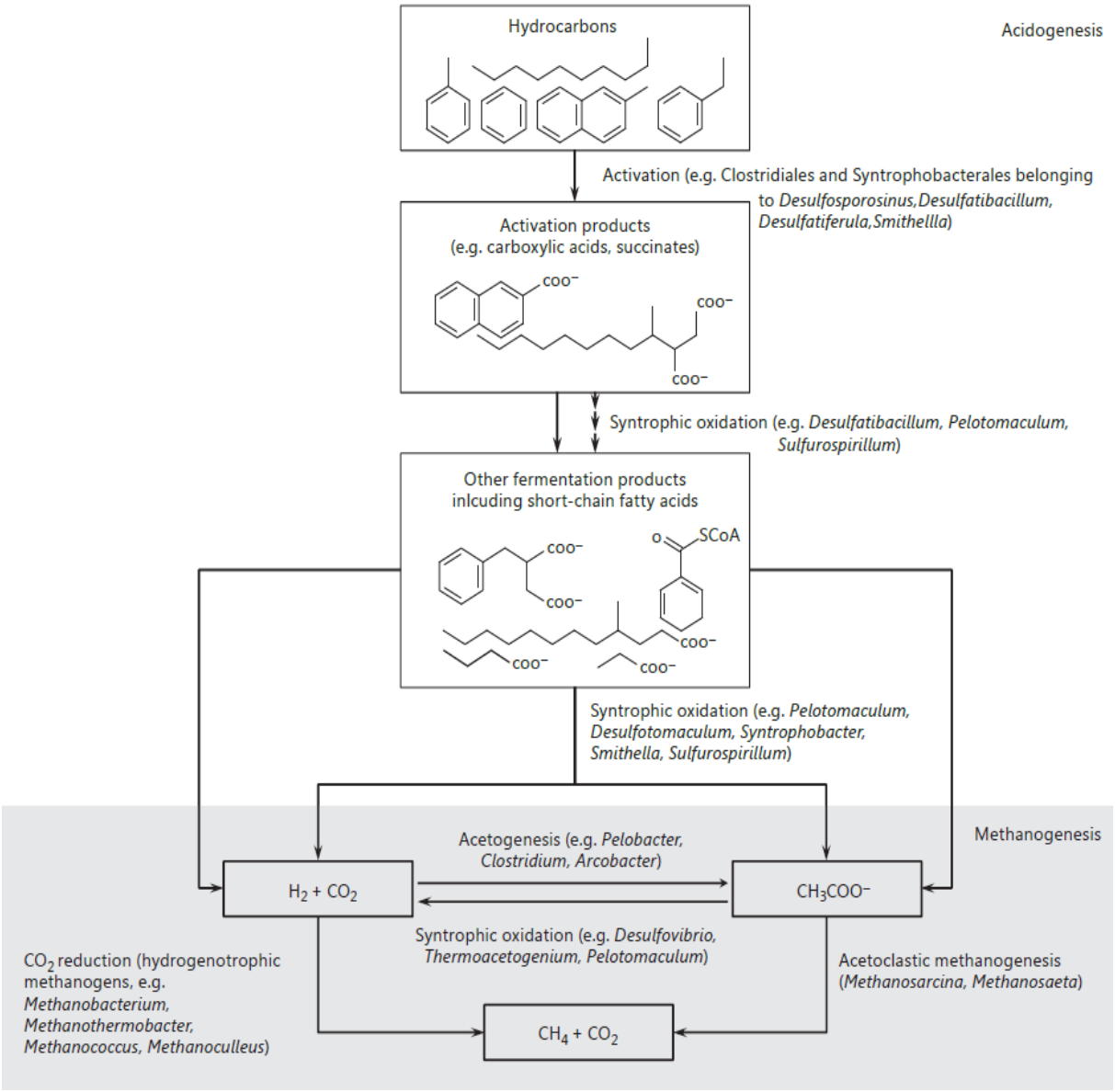

Figure 2.1 – Schematic diagram of the various stages and associated microbial communities involved in microbial hydrocarbon conversion to methane (adapted from Jiménez et al., 2016).

Another study with a methanogenic consortium from gas-condensate-contaminated subsurface sediments was enriched and then exposed to crushed residual-oil-bearing core material collect from a mature oilfield undergoing secondary recovery, showed similar results (Gieg et al., 2008). Ribosomal 16S analysis showed that acetoclastic methanogens of the *Methanosaeta* genus were the primary *Archaea* genera in the consortium, although hydrogenotrophic methanogens from the *Methanobacterium* and

Methanoculleus genera were also detected after selective cultivation. Bacteria phyla comprised mainly of δ-proteobacteria. The *Smithella* genus was detected as well as sulfate-reducing bacteria (SRB) genera such as *Desulfobulbus*, *Desulfosporosinus*, *Desulfovibrio* and the syntrophic *Desulfotomaculum.* Firmicutes also had a significant presence in the consortia. Interestingly, the presence of sulfate-reducing bacteria did not affect the methane production, which indicates that these genera may directly oxidize hydrocarbon or utilize other metabolites such as fatty acids from other microorganisms, and don't directly compete with methanogens for acetate. It has also been suggest that methanogens of the *Methanosaeta* genus are capable of competing with acetate-utilizing sulfate-reducing bacteria in sulfate-reducing conditions (Struchtemeyer et al., 2005), which explains its recurring dominance on *Archaea* from consortia collected from different environments.

A recent study used an inoculum from gas condensate-contaminated aquifer sediments that were found to degrade whole crude oil under methanogenic conditions (Fowler et al. 2016). The initial inoculum was treated with crushed sandstone reservoir material (residual oil), two n-alkanes, n-hexadecane and n-octadecane, and their correspondent hypothesized fatty acids, palmitate and stearate, respectively. All five cultures were dominated by *Firmicutes*, predominantly by the *Clostridium* genus. Residual oil and palmitate were the only enrichment cultures where *Smithella* was prevalent and coincidently both these cultures presented the highest percentage of δ-proteobacteria. Both n-alkane cultures exhibited lower taxa diversity and had the highest percentage of methanogens, however, n-hexadcane cultures had higher abundance of acetoclastic methanotrophs of the *Methanosaeta* genus while n-octadecane ones presented higher abundance of hydrogenotrophic methanotrophs, namely *Methanoculleus* and *Methanolinea*. The results indicated that substrate variations for the same initial enrichment culture may lead an adaptation and establishment of different syntrophic partnerships.

The role of acetoclastic methanotrophs on the methanogenic degradation of crude oil/n-alkanes, particularly n-hexadecane, cannot be understated, though other reports show both acetoclastic and hydrogenotrophic in similar abundance, or even absent, in the consortia *Archaea*. Siddique and coworkers (Siddique et al., 2011) observed prevalence of the *Archaea* genus *Methanosaeta* with mature oil tailing samples as substrate though an equivalent percentage of Methanomicrobiales and Methanosarcinales was detected when n-tetradecane, n-hexadecane, and n-octadecane were used as substrate, the dominant genera being *Methanoculleus* for the former and *Methanosaeta* for the later. The bacteria taxa were predominantly comprised of *Syntrophus* genus in the 3-alkane culture while *Chloroflexi*, *Firmicutes* and *β-proteobacteria* dominated the mature oil tailing cultures.

Another study (Berdugo-Clavijo & Gieg, 2014a) compared the microbial consortia between two setups, a planktonic and a sessile (sand-stone column). A methanogenic enrichment culture was initially obtained from a mixture of production waters of a low temperature reservoir and later amended with crude oil, which was also used as substrate. A considerable difference of the dominant microbial populations was observed between both setups. For the planktonic community, at the genus level, *Smithella* was the dominant bacteria, along with other minor occurrences of δ-proteobacteria such as *Sedimentibacter*, *Desulfotomaculum* and *Desulfobacterium*. Similar abundance of *Methanosaeta* and *Methanoculleus* was observed, with a smaller percentage of *Methanocalculus* and *Methanolinea* detected. In contrast, in the sand-stone column culture, the dominant bacteria belonged to the *Pseudomonas* genus, although *Methanobacterium* was overall the most abundant genus with over 50% of total reads. *Smithella* and *Methanosaeta* are barely above detection levels and in fact at the end of the essays, significant amounts of acetate had accumulated in the column. This study clearly shows the effects of the surrounding environment on the development of hydrocarbon degrading microbial communities.

In contrast, Jones and coworkers utilized a methanogenic oil alkane-degrading enrichment from estuarine sediments with North Sea crude oil as substrate and found that the predominant methanogenic process involved CO_2-reductio (Jones et al., 2008). The consortia showed abundance of *Syntrophus sp*. and hydrogenotrophic methanogens. They propose that the principal route whereby n-alkanes are converted to methane involved syntrophic acetate oxidation to CO_2 and H_2 couple with hydrogenotrophic methanogens. This assessment is supported by highly enriched CO_2 isotopic signatures in heavily degrade oils of mesothermic Peace River Oil Sands (Western Canada), which suggests that hydrogenotrophic methanogens predominate in subsurface oil reservoirs. Other studies regarding production waters in oil reservoirs also support this assessment (Mayumi et al., 2011; Nazina et al., 2006; Nercessian et al., 2005; Orphan et al., 2000).

Overall, the methanogenic route followed by a given consortia is shown to be dictated by the sampling source, the microbial community, the substrate utilized and even the surrounding environment. Anaerobic degrading consortia are very complex and further studies are required to understand the syntrophic interactions and in what circumstances and which microorganisms are responsible for said interactions.

2.3 Effects of oxygen exposure to anaerobic microorganisms

The microorganisms belonging to the *Archaea* domain generally live under extreme conditions, frequently under strict anaerobic conditions. Unlike aerobic or facultative microorganisms, exposure to oxygen can be detrimental to archaeon species due to the formation of reactive oxygen species (ROS) such as superoxide ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2). Previously, it was believed that these microorganisms lacked the mechanisms to cope with oxidative stress, but several studies have shown that methanogens can survive exposure to O_2 for hours or days (Fetzer & Conrad, 1993; Jasso-Chávez et al., 2015; Kiener & Leisinger, 1983; Patel et al., 1984), and that active methanogenic communities were found in typical oxidative (aerobic) environments (Angel et al., 2012; Angel et al., 2011; Ueki et al., 1997).

For a deeper understanding on the evolution of oxidation resistance of methanogens, a recent study using hierarchical clustering analyses, based on functional gene content, analyzed the genomes of six well-established methanogen orders, and were able to distinguish two separate groups (Lyu & Lu, 2017): Class I, comprised of *Methanobacteriales*, *Methanopyrales* and *Methanococcales*, and Class II comprised of *Methanomicrobiales*, *Methanosarcinales* and *Methanocellales*. By means of comparative genomics, the authors discussed the overall potential adaptability of both groups to oxidative stress and recovery and sampling from microaerophilic or oxic environments. Three distinct mechanisms were used to assess overall antioxidant capability: limiting ROS production, reducing accumulation of ROS in the cell and self-repairing for ROS damage. Previous studies have revealed that all three strategies are essential for the survival of both aerobes and facultatives under oxygen stress (Hillmann et al., 2008; Imlay, 2008). The authors found that Class II microorganisms, which include the species *Methanosarcina barkeri*, *Methanosarcina mazei*, *Methanosarcina acetivorans* and *Methanospirillum hungatei*, had been more frequently recovered from oxic/microaerophilic environments and had higher antioxidant properties than those of Class I. Indeed, KEGG data base shows that the genera *Methanosarcina*, *Methanosaeta*, *Methanospirillum* and *Methanobacterium* are among those with the highest and most diverse gene coding for oxidative stress protection proteins (Jasso-Chávez et al., 2015). Other notable genera include *Methanocella* and *Methanobrevibacter*. These findings show that, although perceived as strict anaerobic microorganisms, methanogens have in fact the potential to endure and adapt to oxidative environments.

Previous works studies on the effects of oxygen exposure to methanogens had already shown potential for oxidative tolerance, and several recent ones denote the striking differences oxygen exposure resistance, supporting the genomic data and comparative analysis previously mentioned (Table 2.1 - Studies on effects of oxygen exposure to pure and co-cultured methanogenic species.).

Table 2.1 - Studies on effects of oxygen exposure to pure and co-cultured methanogenic species.

Microorganism(s)	Substrate	Operation mode	O_2	O_2 supply	Results	Reference
Methanosarcina barkeri	Methanol	Batch	0,005%/0,05%/0,5 %/0,1%/1%	Single injection	(Concentrated cells) $[O_2] > 0.5\%$ immediately inhibit CH_4 production; $[O_2] \leq 0.005\%$ shows tolerance; rapid increase in redox potential (above 100mV) and cell damage likely causes of inhibition.	(Fetzer & Conrad, 1993)
Methanobacterium thermoautotropbic, *Methanobrevibacter arboriphilicus, Methanosarcina barkeri, Methanococcus voltae* and *Methanococcus vannielii*	Formate/acetate	Batch	-	Direct air exposure	*M. thermoautotrophicum*, *M. arboriphilicus* and *M. Barkeri* show remarkable resistance to air exposure for several hours before considerable viability was lost (10-30h)*; M. vannielii and M. Voltae* rapid viability loss (1h-2h).	(Kiener & Leisinger, 1983)
Methanospirillum hungatei, *Methanobacterium thermoautotrophicum*, *Methanobacterium bryantii*	Acetic acid/H_2/CO_2	Batch	0.001% (Dissolved O_2)	Single injection	*M. Hungatei* was the most sensitive to oxygen exposure, with 90% death rate in less than 4 hours of exposure; *M. bryantii* was the most resistant, with 90% death rate at 7.5 hours of exposure.	(Patel et al., 1984)
Psalteriomonas vulgaris (strict aerobe) and *Methanobacterium formicicum*	Peptone/Formate	Batch	Up to 5%	Single injection	Methanogenesis inhibited at only 5% O_2	(Broers et al., 1993)
Methanobrevibacter arboriphilicus, Methanobacterium formicicum and Methanosarcina mazei		Suspended culture/dried biomass	-	Direct air exposure	Significant viability after up to up to 30 days of aeration in the presence of paddy soil; in liquid culture, M. mazei showed the highest resistance (14 days of exposure); lower recovery period for M. formicicum	(Liu et al., 2008)
Methanosarcina barkeri and *C. testosteroni*;	Methanol (*M. barkeri*)/Formate (*M. formicicum*)	Chemostat (0,26 h^{-1})	0,7% and 2% (v/v)	Continuous supply	Growth of *M. formicicum* and *M. barkeri* affected at $[O_2]$ lower than the equipment's detection limit (0,2 µM); *M. barkeri* and *C. testosteroni* at 2% (v/v) O_2 show an increase in *C. testosteroni* population while the methanogen flora maintained stability (though at 25% less methane production capability); results show that distinct metabolisms can co-exist and thrive in similar conditions.	(Gerritse & Gottschal, 1993)
Methanobacterium formicicum and *C. testosteroni*		Chemostat (0,26 h^{-1})	0,18% (v/v)			
Methanobacterium formicicum and *Methylocystis* sp.		Chemostat (0,28 h^{-1})	0,18% (v/v)			

Most notably, a recent study showed that after successive periods of oxygen exposure and culture transfer, pure cultures of the methanogen *Methanosarcina acetivorans* grown in acetate and methanol were able to achieve methane production similar to anaerobic controls (16 μM O_2 and 40 μM O_2 in the headspace, respectively) (Jasso-Chávez et al., 2015). Moreover, pure cultures subjected for the first time to O_2 exposure reached 60% methane production of the anaerobic controls. Significant increase in biofilm formation and antioxidant enzyme content in air-adapted cells showed that not only was *M. acetivorans* resistance to oxygen exposure but also able to adapt and increase that resistance.

2.3.1 Effects of oxygen during anaerobic digestion

Aerobic treatment has been shown to improve the overall anaerobic digestion (AD) process, as long as the process is not too prolonged It can be applied at various stages of the AD process: pre-treatment, during the AD process or to the digestate. Recent review showed compiled several studies reporting enhancement of AD performance through aeration by improving hydrolysis, aiding in reactor start-up, reducing the accumulation of VFAs thus participating in pH control and increased removal of inhibitor compounds such as polyphenols or sulfides (Girotto et al., 2018). The response to aeration is dependent on several aspects such as hydrolysis rate coefficient, biomass concentration, hydraulic retention time and organic loading rate (for continuous reactors) and sludge age (Botheju & Bakke, 2010).

In the case of aerobic pre-treatment, some studies in reactors using different types of waste reported increase in methane yield, the majority of which identified improvement of hydrolysis as the main effect of pre-aeration. Various methods were used to supply the air to the AD vessel such as air pumps, air injection, compressed air. In the case of batch bottle studies, a simple method of open-air bottle proved to be effective. A summary of pre-treatment analysis is shown in Table 2.2.

During AD, aerobic stimulation can be performed by micro-aeration or limited oxygen injection throughout the process. Microaeration can be defined as the introduction of small amounts of oxygen into an anaerobic biochemical process to enable both aerobic and anaerobic activities to occur in a single vessel (Botheju & Bakke, 2011). The main goal is to stabilize the anaerobic process thus enhancing the methane production yield. By keeping the concentration of oxygen at low levels but high enough so that facultative communities are also stimulated and improve the efficiency of the overall process, as increased diversity of the microbial community is a crucial step in complete substrate decomposition (Girotto et al., 2018). Moreover, microaeration as the potential to shorten lag-phases, increase specific growth rates (Ghaly & El-Taweel, 1994) and improve tolerance against substrate inhibition (Duarte et al., 2018). Table 2.3 details studies where microaeration was applied.

Table 2.2 – Studies on the effects of aerobic pre-treatment on anaerobic digestion processes (adapted from Girotto et al., 2018).

Substrate	Aeration method and amount	AD process set-up	Optimum results	Effects of pre-aeration	References
Corn straw	Oxygen injection under thermophilic conditions (0, 5, 10, 20, 30, 40 mlO_2/g VS)	Batch mesophilic BMP test	16 % methane yield improvement (5 mlO_2/g VS)	Micro-aerobic condition enhanced hydrolysis	(Fu et al. 2015a)
Digestate from primary fermentation of corn stover	Oxygen injection under thermophilic conditions (5, 10, 20 mlO_2/g VS)	Batch mesophilic BMP tests	28% methane yield improvement (10 mlO_2/g VS)	-	(Fu et al., 2015b)
Food waste and brown water	Oxygen injection (37.5 mlO_2/L_R/d; 4 days)	Batch mesophilic BMP tests	10% acetate increase in the hydrolysate and 23.0% methane yield improved (37.5 mlO2/LR/d, 4 days)	The enhanced solubilization of organics and control of VFA accumulation improved methane yield	(Wei & Wang, 2013)
Organic fraction of municipal solid waste (OFMSW)	Air injection (0.0067 L_{air}/kg/min; 2 h run/4 h stop)	Mesophilic CSTR	Biogas production doubled in AD reactors with micro-aeration	Better hydrolysis and acidification were observed	(Nguyen et al., 2007)
OFMSW	Partial composting as pre-aeration (20 °C 0.21 L_{air}/kg-OFMSW/min)	Batch mesophilic BMP tests	20% of VS was degraded during partial composting and 40% methane yield was lost	Start-up of the dry anaerobic batch digestion of OFMSW was accelerated	(Brummeler & Koster, 1990)
Olive mill wastewater	Air injection for 5 days (5 L_{air}/L_R/min)	Mesophilic CSTR	Methane production doubled from 0.16 m^3/kgCOD without pre-treatment to 0.39 m^3/kgCOD	Polyphenol concentration was reduced	(González-González & Cuadros, 2014)
Primary sludge and waste activated sludge	Natural aeration (bottles were left open to maintain semi-aerobic conditions) at 20 °C (12, 24, 48, 72, 96 h)	Batch mesophilic BMP test	VFA recovery (43 gCOD VFA/kg VS) and 14% methane potential improved (20 °C, 72 h)	Pre-aeration positive effect was linked to growth of fungi which have a strong biodegradation activity	(Peces et al., 2016)
Sewage sludge	Air injection under thermophilic conditions (air rate 2.5 L_{air}/L_R/min; 1 day)	Batch mesophilic BMP tests	42% higher methane production rate and 15% higher TCOD removal (air rate 2.5 L/L_R/min, 1 days)	A greater diversity of bacteria and archaea populations was observed during mesophilic anaerobic digestion	(Min et al., 2013)

Table 2.3 – Studies on the effects of microaeration during anaerobic digestion (adapted from Girotto et al., 2018).

Substrate	AD process	Microaeration method	Results	Effects of microaeration	References
Starch	Batch	2%/4%/8%/16% (COD); single injection	15% increase in biogas production at 1.6%		
Synthetic wastewater	Batch UASB Reactor	3×10^{-4} mL O_2/min, from the electrolysis of water	-	26% increase in methane production	(Tartakovsky et al., 2011)
Brown water and food waste	Mesophilic two-phase CSTR	Oxygen injection to the liquid part of the reactor (5 $mlO_2/L_R/d$ and 7 $mlO_2/L_R/d$)	43% total VFA concentration increase with 5 $mlO_2/L_R/d$	Microaeration led to diversification of bacterial communities	(Wei et al., 2014)
Corn straw	Batch thermophilic BMP tests	Air injection (0, 12.5, 25, 50, and 100 $ml/L_R/d$)	16.5% methane yield improvement (12.5 $ml/L_R/d$)	Hydrolysis-associated microorganisms, *Firmicutes*, class *Clostridia* and order *Clostridiales* raised; Oxy–tolerant *Methanosarcina* doubled	(Fu et al., 2016)
OFMSW	Mesophilic CSTR	Air injection at the bottom of the anaerobic digestion reactor (1 L/min; 2 h run/4 h stop)	Reduction of DOC with an aeration time of 3 days	DOC reduced from 140 gC/kgTS (without aeration) to 127 gC/kgTS (with aeration)	(Nguyen et al., 2007)
Sewage sludge	Mesophilic CSTR	Pure oxygen supply into the sludge recirculation 0.0033 $LO_2/L_R/min$	Better capability to deal successfully with overloads	The growth of hydrogenotrophic methanogens was promoted.	(Ramos & Fdz-Polanco, 2013)
Sewage sludge	Mesophilic CSTR	Oxygen injection every 10 min into different O_2 dosing points with O_2 flow rate (0.005–0.034 $LO_2/L_R/d$)	Oxygen did not have a significant impact on methane production	O_2 transfer rate positively affected long-term microbial diversity	(Ramos et al., 2014)
Sewage sludge and food waste	Thermophilic CSTR	Compressed air injection (41.7 mlair/kgTS/min)	Aerobic treatment may amend occasional acidification problems	-	(Lagerkvist et al., 2015)
Synthetic food waste	Mesophilic two-phase UASB	Air pump at the bottom of the LBRs (0, 129, 258, and 387 L_{air}/kg TS/d)	5% COD faster leaching and 18% methane production enhancement with 258 $L_{air}/kgTS/d$	Microaeration enhanced acid fermentation and promoted production of VFAs	(Xu, Selvam, & Wong, 2014)
Oleate-based wastewater	Mesophilic Up-flow Continuous	Open air feeding tank	Avoided long term inhibition of methane production	Higher abundance of facultative anaerobes	(Duarte et al., 2018)

3. MATERIALS AND METHODS

3.1 Effects of hexadecane on pure methanogenic cultures

3.1.1 Cultures growth conditions and maintenance

Methanobacterium formicicum (DSM 1535^T) and *Methanospirillum hungatei* JF-1 (DSM 864^T) were obtained from Leibniz-Institute DSMZ, Braunschweig, Germany. These methanogens were grown in basal medium prepared according to Stams et al. (1993) (Table VI-1, Appendix VI). Once prepared, the media was heated to boiling point to promote degasification (removal of dissolved O_2) and immediately cooled in ice, with simultaneous flushing with N_2 to substitute as much residual O_2 as possible and avoid O_2 re-dissolution. Once cooled, 80 mL of medium was transferred to 200 mL serum bottles, sealed with butyl rubber stoppers and secured with aluminium crimp caps. The bottles were then subjected to several cycles of vacuum and pressure using a Manifold apparatus with a H_2/CO_2 (80/20 % v/v) gas mixture, adjusted so that at the end of the cycles the bottles had a final pressure of 1.7 atm. The bottles were then autoclaved at 120 °C for 20 min.

After cooling, and before inoculation, the bottles were supplemented with 5% sodium bicarbonate buffer solution with reducing agent (Na_2S), for a final pH of 7.0-7.2, and 5% salts and vitamins solution. The sodium bicarbonate solution was prepared with demineralized water and 80 g/L $NaHCO_3$ and 0.33 g/L acetate for internal cell maintenance, followed by headspace flush with N_2 and autoclaving at 120 °C for 20 min. After cooling, 2 mL of a 240.2 g/L $Na_2S \bullet 9H_2O$ solution (filter-sterilized, stored in the dark and under N_2 headspace overpressure) was added under sterile conditions to 100 mL of the sodium bicarbonate solution in 200 mL bottles (as detailed above). The salts and vitamin solution was prepared as follow: 25 mL salts solution composed of 24 g/L NH_4Cl, 24 g/L NaCl, 8 g/L $MgCl_2 \bullet 6H_2O$ and 8.8 g/L $CaCl_2 \bullet 2H_2O$ was added to 50 mL demineralized water. After headspace N_2 flush, the solution was autoclaved at 120 °C for 20 min. After cooling, 2 mL of a filter-sterilized vitamin solution composed of 20 mg/L biotin, 200 mg/L nicotinamid, 100 mg/L p-aminobenzoic acid, 200 mg/L thiamin (vitamin b1), 100 mg/L panthotenic acid, 500 mg/L pyridoxamine, 100mg/L cyanocobalamine (vitamin b12), 100 mg/L of riboflavin, 50 mg/L folate and 50 mg/L lipoate, was added under sterile conditions. The final culture volume never exceeded 50% of the total bottle volume. Once the whole media was prepared, 10 mL of inoculum (10% of final culture volume) was transferred, and the bottles were incubated at 37 °C and 110 rpm.

For culture maintenance and longevity, once the end of the exponential growth phase was reached (5-7 days), 10 mL were transferred to fresh media and incubated again. To verify the growth stage of the cultures, a visual assessment was performed and confirmed with a headspace pressure measurement

using a pressure transducer (Centerpoints Electronics; Galway, Ireland). It measures pressure increase or decrease and can measure variations of ± 2 atm (0 ± 202.6 KPa) through a range of -200 to +200 mV. In each measurement approximately 30 μL of biogas is lost, which represents a minimal fraction of the total biogas produced during the test, making unnecessary the use of a correction factor. Since the biogenic conversion of H_2 to CH_4 leads to a decrease in pressure (4 mol of H_2 lead to the production of 1 mol of CH_4), once the pressure reached -70/-80 mV, a new transfer was performed.

3.1.2 Toxicity assays with hexadecane

Hexadecane toxicity assays were prepared in triplicates at increasing concentrations of 1 mM, 5 mM, 15 mM, 30 mM, with a set of triplicates with no added hexadecane as the control. A mixture of H_2/CO_2 (80/20 % v/v) was used as substrate. The steps for media preparation followed the ones described in 3.1.1., though for this assay 120 mL bottles were used, with a final working volume of 55 mL. The hexadecane was added using a glass syringe before autoclaving the bottles. Incubations were performed at 37 °C and 110 rpm. The cultures growth was monitored by measuring the CH_4 production and H_2 uptake through gas chromatography (GC). At the end of the assays, oxidation-reduction potential (ORP) and pH (InoLab, WTW) were measured.

3.1.3 Data and statistical analysis

Experimental CH_4 production data (expressed as mM) recorded during the toxicity assays performed with hexadecane was fitted by the modified Gompertz equation (Zwietering, Jongenburger, Rombouts, & Van't Riet, 1990):

$$\mathbf{M(t)} = \mathbf{P} \times \mathbf{exp}[-\mathbf{exp}\left[\frac{\boldsymbol{Rm} \times \boldsymbol{e}}{\boldsymbol{P}}(\boldsymbol{\lambda} - \boldsymbol{t}) + \mathbf{1}\right]] \qquad \textbf{(Equation 1)}$$

Where:

M(t) = cumulative CH_4 production (mM)

P = maximum CH_4 production (mM)

Rm = CH_4 production rate (mM/day)

e = 2.7182818

λ = lag phase duration (days).

To improve the data fitting, a restriction was applied to the P value, so that it should be less or equal to the maximum theoretical methane production calculated according to the stoichiometric balance of hydrogenotrophic CO_2 reduction (*i.e.*, 4 mole of H_2 consumed per mole of CH_4 produced), using the H_2 concentration measured at t = 0 h for each individual condition (mean of triplicates). R^2 values and the standard error for each variable obtained were calculated.

Statistical significance of the differences observed in the results achieved was evaluated using single factor analysis of variances (ANOVA). A F-test was applied between pairs of data (comparison between a control set and a treated set) to evaluate the equality of variances to determine the most appropriate statistical T-test. Statistical significance was established at the $p < 0.05$ level.

3.2 Effects of microaeration on the conversion of hexadecane to methane in batch assays

To assess the effects of microaeration on the bioconversion of hexadecane to methane, two assays were performed focusing on the activity of different trophic groups in complex microbial communities. The first assay targeted the activity of syntrophic bacteria, which are considered essential in the anaerobic biodegradation of n-alkanes to methane, as well as the activity of the methanogens in the mixed community. In the second assay, the activity of the different microbial groups directly involved in hexadecane bioconversion to methane was investigated.

3.2.1 Inocula source

In the first assay, the inoculum was an anaerobic granular sludge collected from a brewery wastewater treatment plant (Superbock, Porto). Sludge was kept at 4 °C and was degranulated immediately before use. For the second assay, sludge was collected from a treatment plant performing *ex-situ* bioremediation of petroleum-contaminated groundwater in France. This sludge was mixed with the anaerobic degranulated sludge at a ratio of 1:3 (v/v).

3.2.2 Inocula characterization

3.2.2.1 Volatile solids analysis

To determine the VS content, crucibles in triplicates were first put in a muffle furnace at 550 °C for 30 min to remove any trace of organic content. After cooling in a desiccator, the crucibles were weighted, then 5 mL of sludge was dispensed to each crucible and weighted again. The crucibles were then put in an oven at 105° C for 24 h. Once cooled in the desiccator, the crucibles were weighted (W_{105}) and then put in a muffle furnace at 550 °C for 2 hours. After cooling in the desiccator, the crucibles were weighted (W_{550}). The VS content was then calculated according to Equation 1:

$$\mathbf{VS\ (g/L)} = \frac{\mathbf{W_{105}(g) - W_{550}(g)}}{\mathbf{V_{sample}\ (L)}} \qquad \text{(Equation 2)}$$

3.2.2.2 Specific methanogenic activity

Specific methanogenic activity (SMA), in the presence of acetate, ethanol and H_2/CO_2, was determined for the anaerobic degranulated sludge. The method used is based on the pressure transducer technique (Coates et al., 1996; Colleran et al., 1992). It consists in the measurement of the pressure increase or decrease inside sealed vials, caused by the production of biogas as liquid substrates (ethanol or acetate) or gaseous substrates (H_2/CO_2) are degraded, respectively. To measure the pressure variations on the gaseous headspace, a pressure transducer was used (see section 3.1.1).

The basal medium used in the tests had the same composition as the one described in 3.1.1, with addition of sodium bicarbonate (3 g/L) to stabilize the pH at 7.0-7.2 (when needed, 1M HCl and 1M NaOH solutions were used to adjust). No salts or vitamins were added. SMA tests were performed in triplicate with 25 mL vials for the liquid substrates (acetate and ethanol) and 70 mL for the H_2/CO_2, with blank vials (without substrate) also being prepared in triplicate. The working volume was 12.5 mL for both types of substrate, with a VS concentration of approximately 4 g/L. The vials were sealed with butyl rubber stoppers and secured with aluminium crimp caps followed by headspace flush with a N_2/CO_2 (80/20 % v/v) gas mixture (see section 3.1.1). The vials were then depressurized to 1 atm and placed at 37 °C and 110 rpm overnight to deplete residual substrate.

In the following day, the biogas produced was removed from the vials by headspace flushing with N_2/CO_2 (80/20 % v/v) for the ethanol and acetate assays and H_2/CO_2 (80/20 % v/v) for the H_2/CO_2 assays. The former were then depressurized to 1 atm and the latter were adjusted at 1 atm overpressure (2 atm final pressure). A volume of 0.125 mL of acetate and ethanol was added to the corresponding vials from 3 M stock solutions, to obtain a final concentration of 30 mM of substrate. In the case of blank assays, no substrate was added for the liquid substrates and a N_2/CO_2 (80/20 % v/v) gas mixture was used for the H_2/CO_2 assays.

At the end of the assay, once the pressure variations were not significant and a plateau stage had been reached, the methane content of the biogas produced was determined in triplicate for each individual vial by GC. The headspace volume of all the vials was individually determined by recording the pressure increase when 10 mL of air was injected in the closed vial (mV/mL). This headspace correction factor (mV/mL) is used to convert the recorded pressure variation rate from the initial linear zone (mV/h) to mL of biogas produced per hour (mL/h). The VS content of each vial was determined according to the method described in 3.2.2.1.

To determine the SMA, Equations 2 and 3 were used for the liquid and gaseous substrates, respectively:

$$\mathbf{SMA} = \frac{\mathbf{mL/h}_{biogas} \times \mathbf{MP} \times \mathbf{CF} \times \mathbf{24}}{\mathbf{VS}} \quad \text{(Equation 3)}$$

$$\mathbf{SMA} = \frac{\mathbf{mL_{CH4}/h} \times \mathbf{CF} \times \mathbf{24}}{\mathbf{VS}} \quad \text{(Equation 4)}$$

CF represents the calibration factor for the conversion to Standard Temperature and Pressure conditions, or STP. MP is the methane percentage in the biogas produced in each individual vial during the test:

$$\mathbf{MP} = \frac{\mathbf{Vh} + \mathbf{Vc}}{\mathbf{Vc}} \times \%\mathbf{CH_4} \quad \text{(Equation 5)}$$

Where:

$$\mathbf{Vc} = \frac{\mathbf{Final\ mV\ reading\ (mV)}}{\mathbf{mV/mL}} \quad \text{(Equation 6)}$$

$$\mathbf{Vh} = \frac{\mathbf{mV\ reading\ at\ 1\ atm\ pressure}}{\mathbf{mV/mL}} \quad \text{(Equation 7)}$$

For the assays with H_2/CO_2, the periodic mV values were directly converted to methane by following the stoichiometry of 4:1 mole of H_2 consumed per mole of methane produced and using the previously determined headspace correction factor (mV/mL). The cumulative methane production during the test is calculated by addition of the CH_4 volumes produced between each measurement. The CH_4 production rate (mL_{CH4}/h) was determined through the initial slope of the CH_4 production curve:

$$\mathbf{mL_{CH4}/h} = \frac{\mathbf{P(n-1) - P(n)}}{\mathbf{mV/mL} \times \mathbf{4}} \quad \text{(Equation 8)}$$

Where:

P(n-1) = mV reading at time n-1

P(n) = mV reading at time n

Background methane production determined in the blank controls was subtracted and the specific methanogenic activity was obtained, expressed as $mL_{CH4@STP}/gVS/day$.

3.2.3 Effects of microaeration on syntrophic and methanogenic communities

The effects of microaeration on the activity of syntrophic bacteria was assessed using ethanol as indirect substrate, while acetate and H_2/CO_2 were used as direct substrates for the methanogens. Basal media preparation followed the procedure detailed in 3.1.1, with some notable changes. In these assays, 160 mL bottles were used with a final working volume of 55 mL. No reducing agent was used so extra measures were adopted to minimize O_2 diffusion to the media in each step of media dispensing: the degranulated sludge was diluted in a 1 to 1 ratio with N_2-flushed basal medium before being transferred to the vials, for a final volume of approximately 4 g/L VS. During basal media dispensing, the bottles' headspace was constantly flushed with N_2 gas, as well as the syringe which was flushed with N_2 prior to each media transfer. The media was also not autoclaved.

The assays were divided in two phases: phase-one (P1), the cultures were grown with no addition of O_2 until half of the added substrate was consumed for acetate, or totally consumed for ethanol and hydrogen; phase-two (P2), the bottles headspace was flushed and pressurized with N_2/CO_2 (assays amended with acetate or ethanol) or H_2/CO_2, which was followed by O_2 addition in increasing concentrations of 0.5%, 1%, 2.5% and 5%. The target O_2 concentrations were achieved by injection of air using a glass gas tight syringe (SGE Analytical Science, Trajan, Ringwood, Victoria, Australia). Controls were also prepared in which no O_2 was added upon phase transition. To avoid substrate limitation in P2, bottles were supplemented with half or the total initial amount of substrate in acetate assays and an equal amount of the initial substrate in ethanol and H_2/CO_2 assays. Blank assays, without substrate addition, were also launched. Bottles were incubated in horizontal position, to promote higher contact between biomass and substrate and gas diffusion in the case of the H_2/CO_2 assay, at 37 °C and 110 rpm.

At the start of the assays, the bottles were flushed with N_2/CO_2 (80/20 % v/v) and placed at 37 °C and 110 rpm overnight to remove residual substrate. In the next day, headspace flushing, and substrates

addition was performed as described in 3.2.2.2. Final concentrations for ethanol, acetate and H_2/CO_2 were 30 mM, 30 mM and 60 mM, respectively. This initiated phase P1 of the assay.

Measurements of CH_4 were done periodically, while O_2 and H_2 measurements were performed at the start and end of each phase. Pressure measurements were made at the start and end of each phase using a pressure transducer (section 3.1.1). Liquid samples were collected for volatile fatty acid (VFA) analysis: at the start and end of each phase for substrates directly converted to CH_4 (H_2/CO_2 and acetate), and at the start and end of P1 and periodically during P2 for the assays amended with ethanol. At the end of the assay, ORP, pH and VS were also determined.

3.2.4 Effects of microaeration on hexadecane conversion to methane

Basal media was prepared according to the procedure described in 3.1.1, although no reducing agent was added, and the media was not autoclaved. Hexadecane was added using a glass syringe at a final concentration of 1 mM in all conditions. The bottles were flushed with N_2/CO_2 (80/20 % v/v) and the absolute pressure adjusted to 1.2 atm. Air was injected into the bottles at the start of the assay, to a final headspace $\%O_2$ of 2.5%, 5% and 21%; the later $\%O_2$ was obtaining by preparing the bottle in the open. Initially, the final pressure was not adjusted to 1 atm, resulting in increasing pressure proportional to the amount of O_2 added. After a period of residual substrate consumption, the bottles were flushed with N_2/CO_2 (80/20 % v/v), air was then injected, and the pressure was adjusted to 1 atm. This process was repeated monthly until the end of the assay. The CH_4 produced and O_2 consumed were measured by GC. The pressure was monitored using a pressure transducer. ORP and pH measurements were performed at the start and end of the assay.

3.3 Effects of microaeration on the conversion of hexadecane to methane in bioreactors

3.3.1 Experimental set-up

Three plexiglass column reactors, with a total volume of approximately 2,2 L, were operated at a work volume of 1.8 L (Figure 3.1) at 37 °C.

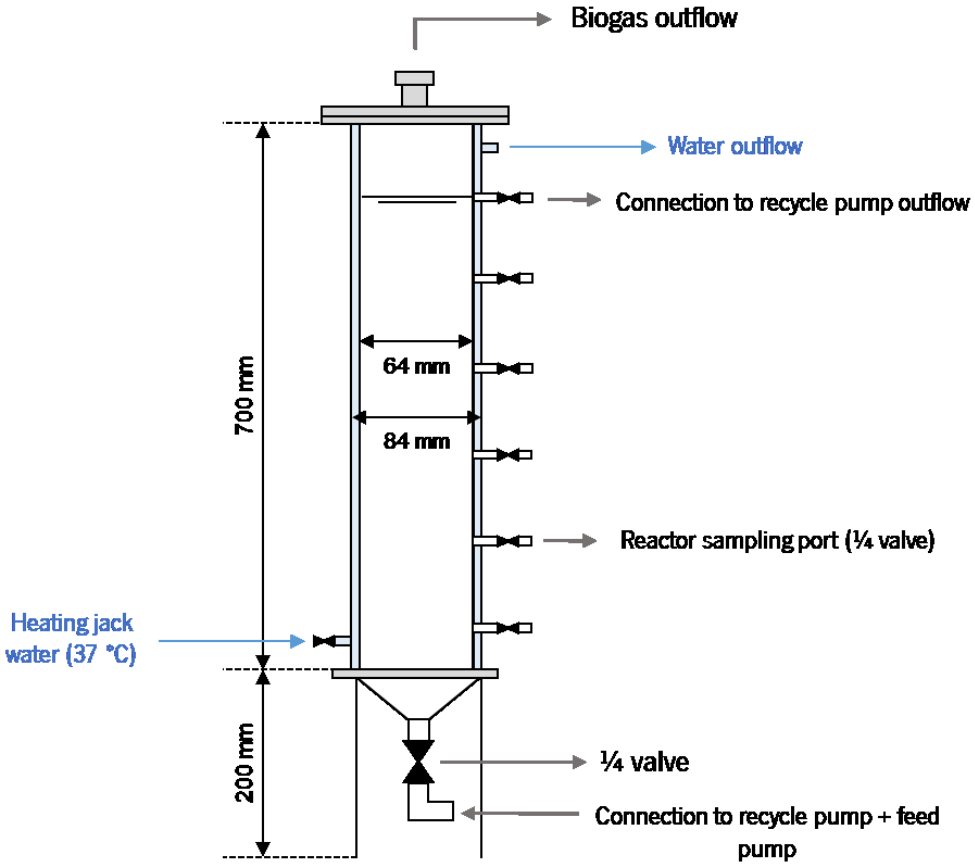

Figure 3.1 – Schematic representation of the bioreators used during the experimental procedure.

Different redox conditions were applied to each reactor: strict anaerobic (reactor AnR), anaerobic with periodic air pulses (reactor O2P) and continuous supply of vestigial concentrations of dissolved O_2 (reactors O2C). Each reactor was connected to an individual biogas Ritter Miligas counter (Dr. Ing Ritter Apparatebau GmBH Bochum Germany). A trap was also installed in the gas line, in order to avoid receding oil from the biogas counters to enter the reactors, as well as to avoid biomass or water to reach the biogas counters. The reactors were operated in continuous with recycle (to promote agitation), in the up-flow direction up to day 21. At 27 days of operation, the feeding and recycle flows was switched to down-flow direction and temporary 250 mL settlers were installed, later replaced by 700 mL settlers at 52 days of operation. Two peristaltic pumps were used during operation, one for the feeding solutions and another

for the recycle. Later, a third peristaltic pump was used to recover settled sludge from the settlers and recycle it back to the bioreactors.

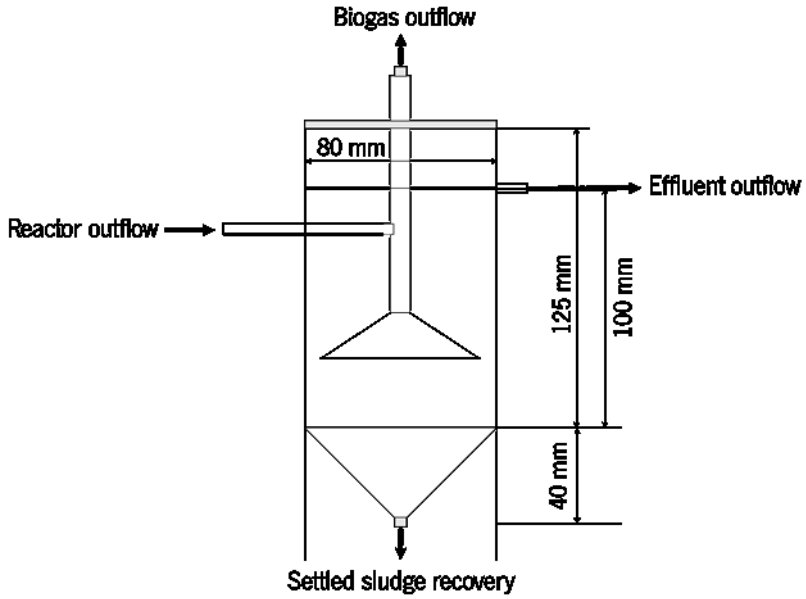

Figure 3.2 – Schematic of settlers used during stage VII.

3.3.2 Inoculum source and characterization

The inoculum used in this experiment was a 3:1 (v/v) mixture of anaerobic granular sludge (see details in sections 3.2.1 and 3.2.2) and sludge collected from a treatment plant performing *ex-situ* bioremediation of petroleum-contaminated groundwater in France. This last sludge was incubated in the laboratory under aerobic conditions at a ratio of 1:3 (v/v) in rich medium 802 containing 10 g/L peptone, 2 g/L yeast extract, 1 g/L $MgSO_4$ and additional substrates to induce selective pressure on the inoculum, *i.e.* 1 mM sodium palmitate, 20 mM sodium butyrate, 1 mM sodium octanoate and 1 mM hexadecane. The culture was incubated at room temperature (approximately 25 °C) and 160 rpm. The pH was maintained at 7.5-8.0 using phosphate buffer (K_2HPO_4/KH_2PO_4). After 1 month of incubation, the medium was centrifuged at 15000 rpm and the pellet was transferred to fresh medium. After another 1 month of incubation, the pre-inoculum was mixed with the degranulated anaerobic sludge in a 1:3 ratio (v/v) and used to inoculate the reactors.

3.3.3 Inoculum characterization

Volatile solid analysis and specific methanogenic activity was performed according to the procedures described in section 3.2.2.1 and 3.2.2.2, respectively.

3.3.4 Bioreactors operation and preparation of feeding solutions

Three pulses of hexadecane (1 mM) were made during the bioreactors operation, as detailed in the Results and Discussion section. The bioreactors were fed in continuous mode with yeast extract (0.5 g COD/L) as co-substrate, dissolved in demineralized water supplemented with micronutrients and macronutrients solutions (Table VI-2, Appendix VI). After 41 days of operation, yeast extract was removed from the feeding. The feed flow rate was set at 0.2 mL/min, hydraulic retention time (HRT) of 6 days, and the recycle flow rate to 5 mL/min. During recycle inversion, the recycle flow rate was increased to 50 mL/min to promote reactor mixing. In stage VII, the feed flow rate was decreased to 1 mL/min, 12 days HRT.

The feeding solutions for reactors O2P and AnR were heated to boiling point and deoxygenized under N_2, followed by the addition of 2 g/L sodium bicarbonate (unless stated otherwise), achieving pH 7.5. The bottles were then flushed with N_2/CO_2 and pressurized to 1.2 atm. During continuous feeding, the headspace of the feeding bottles was refilled from a bag of nitrogen. Reactor O2C feed solutions were prepared using the same procedure but were not deoxygenized, and the bottle was kept in contact with air during continuous feeding. Autoclaving of the feed solution was only employed when yeast extract was present.

3.3.5 Reactor monitoring

During the course of the operation, the reactors were monitored in several significant aspects. The stability of the operation was monitored periodically through ORP, pH, VS (see procedure at 3.2.2.1) and VFA measurements by HPLC. All measurements were performed from reactor samples. Additionally, soluble chemical oxygen demand (COD) in the reactors was measured after centrifugation at 15.000 rpm for 10 minutes. The volume of biogas produced was monitored by a biogas Ritter Miligas counter (Dr. Ing Ritter Apparatebau GmBH Bochum Germany) with a minimal threshold of 1 mL_{gas}/h, and the biogenic CH_4 production was followed by measuring the methane content ($\%CH_4$) of the biogas produced by GC. The feed bottles were periodically monitored in terms of pH, ORP and dissolved oxygen (DO), using a O_2 probe Oxi 330i/SET (WTW, Germany) for the latter. The flow rate of the feed inlet was also periodically measured.

3.4 Analytical methods

Sampling of the bottles' headspace in the batch assays and reactors was performed using a glass syringe (Trajan Scientific, Australia) under sterile conditions, by collecting 0.5 mL sample of gas from the headspace.

GC (gas chromatography) analysis, a Bruker SCION GC-486 (SCION; Goes, The Netherlands) was used, equipped with a Molsieve packed column (13x 80/100, 2 m length, 2.1 mm internal diameter) A thermal conductivity detector (TCD) was used, with argon as the carrier gases at 30 mL/min flow. Temperatures of the injector, column and detector were 100 °C, 35 °C and 130 °C, respectively. The data was analysed using the Compass CDS data system. Mixtures of H_2/CO_2 (80/20 % v/v) and $CH_4/CO_2/N_2$ (40/40/20 % v/v) were used as standards for H_2 and CH_4. Shimadzu GC-2014 (Shimadzu; Japan) was used, equipped with a Porapack Q (100–180 mesh) column, with N_2 as carrier gas at 30 mL/min and a flame ionizing detector (FID). Sampling was performed with a a glass syringe (Trajan Scientific, Australia). Temperatures of the injection port, column and detector were 110 °C, 35 °C and 220°C, respectively. Methane quantification was made by comparison of the sample peak area with the one obtained after injection of a $CH_4/CO_2/N_2$ (40/40/20 % v/v) standard.

Different GC apparatus were used during the different experiments. For the gas measurements in 4.1, the GC Bruker SCION GC-486 was used to measured H_2 and CH_4. In 4.2, GC Bruker SCION GC-486 was to measure H_2 and O_2 and the Shimadzu GC-2014 were used to measure CH_4. Shimadzu GC-2014 were used to measure CH_4 in 4.3.

For VFA and ethanol analysis (High performance liquid chromatography, HPLC), samples were centrifuged at 15,000 rpm for 10 min, the supernatant was collected and filtered using a 0.22 µm filter, followed by dispensing in a 4:1 ratio with the internal standard, crotonic acid. VFA analysis was performed by high-performance liquid chromatography (HPLC) with a Jasco AS-4050 equipped with a Jasco UV-2075 Plus and a Jasco RI-4030 detector and an Aminex 87H column (300x7.7, 8 µm particle size) from BioRad at 60 °C. Each sample was run at 0.7 mL/min using a 5 mM H_2SO_4 solution as the eluent. UV detection was performed at 210 nm.

4. Results and Discussion

4.1 Effects of hexadecane on pure methanogenic cultures

The cumulative CH_4 production and H_2 uptake profiles of *Methanobacterium formicicum* (Figure 4.1) and *Methanospirillum hungatei* (Figure 4.2) were monitored in the presence of increasing concentrations of hexadecane. The different parameters calculated by fitting the experimental CH_4 production data to the modified Gompertz equation are shown in Table 4.1.

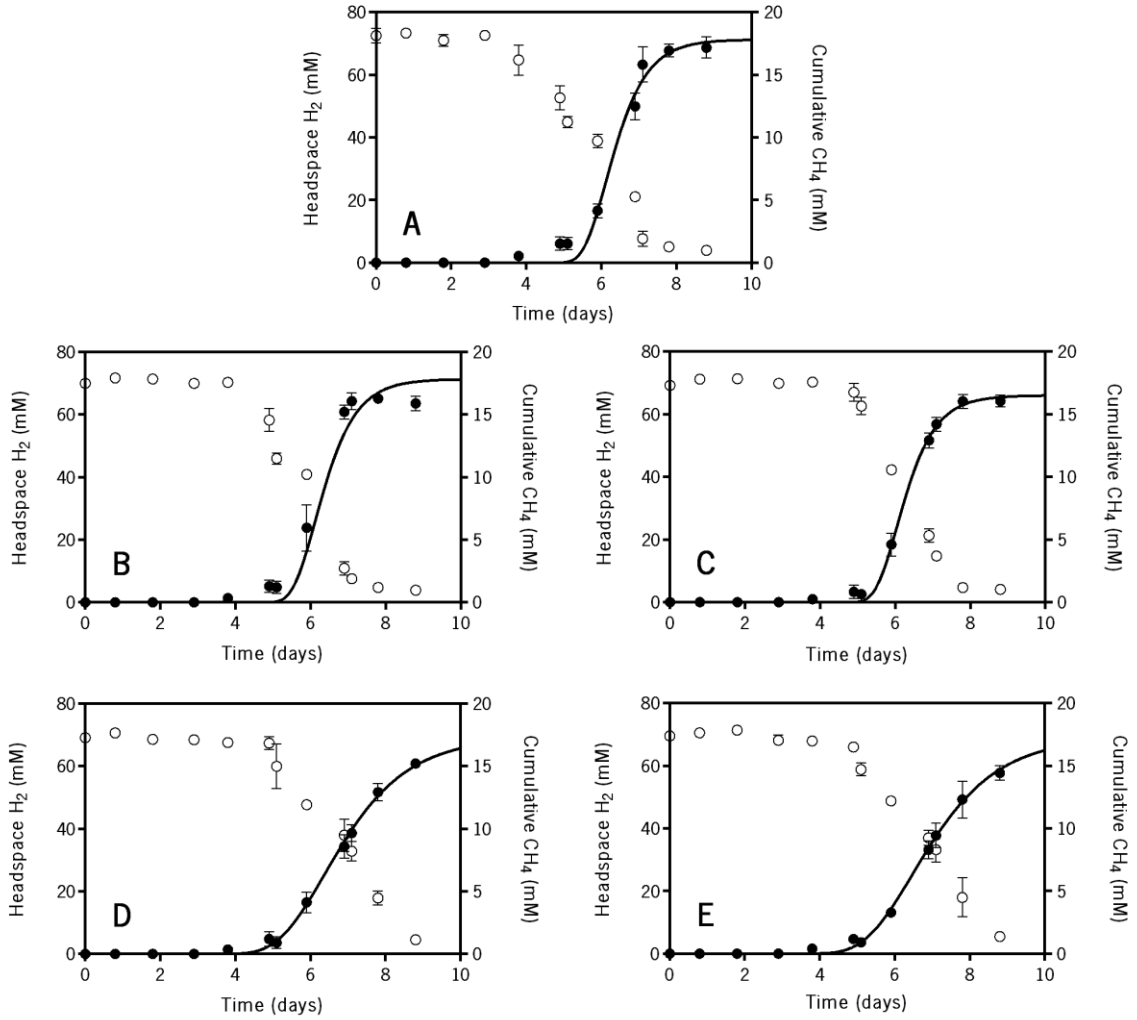

Figure 4.1 - Effects of increasing hexadecane concentration on the cumulative CH_4 production (●) and H_2 consumption (○) by *M. formicicum*. A - 0 mM hexadecane; B - 1 mM hexadecane; C - 5 mM hexadecane; D - 15 mM hexadecane; E - 30 mM hexadecane. The lines represent the fitting of the cumulative methane production data by the modified Gompertz equation (—).

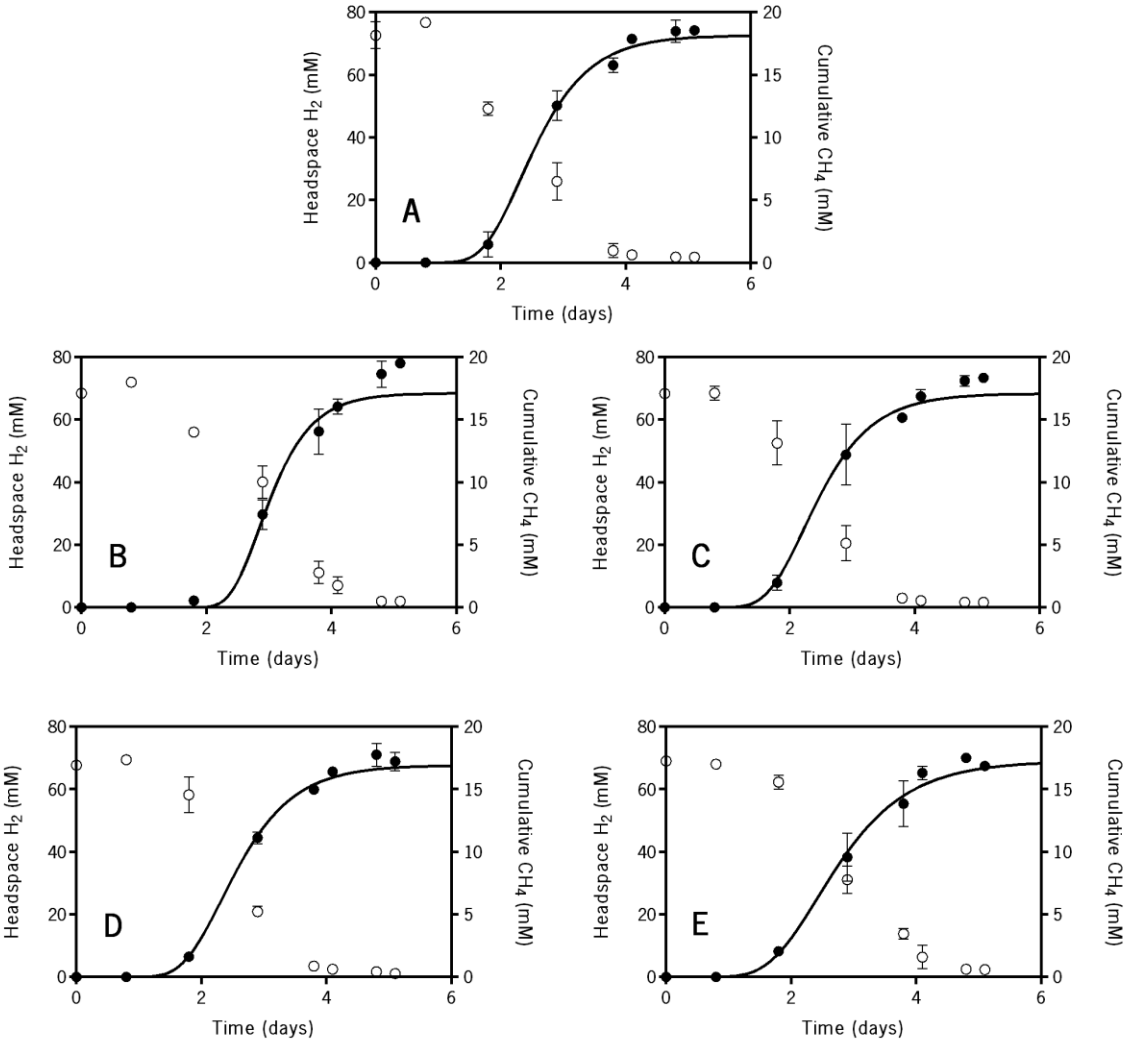

Figure 4.2 - Effects of increasing hexadecane concentration on the cumulative CH_4 production (●) and H_2 consumption (○) by *M. hungatei*. A - 0 mM hexadecane; B - 1 mM hexadecane; C - 5 mM hexadecane; D - 15 mM hexadecane; E - 30 mM hexadecane. The lines represent the fitting of the cumulative methane production data by the modified Gompertz equation (—).

Table 4.1– Methane production parameters calculated by fitting the experimental data from incubations with *M. formicicum* and *M. hungatei* to the modified Gompertz model. R^2 values for the Gompertzian exponential phase are shown. P – maximum CH_4 production (mM); Rm – maximum CH_4 production rate (mM/day); λ – lag-phase (days).

Methanogen	Hexadecane (mM)	λ	P	Rm	R^2	Inhibition (%)
M. formicicum	0	5.5 ± 0.1	17.8 ± 0.6	10.9 ± 1.0	0.984	-
	1	5.1 ± 0.1	16.7 ± 0.7	10.2 ± 0.6	0.994	6 ± 1
	5	5.5 ± 0.1	16.5 ± 0.3	10.6 ± 0.6	0.995	3 ± 0
	15	5.1 ± 0.1	17.3 ± 0.8	**5.2 ± 0.3	0.993	52 ± 6
	30	5.2 ± 0.1	17.2 ± 1.0	**5.0 ± 0.3	0.984	54 ± 6
M. hungatei	0	1.7 ± 0.1	18.2 ± 0.3	11.1 ± 0.8	0.992	-
	1	2.2 ± 0.3	17.1 ± 0.7	12.5 ± 2.6	0.979	-14 ± 3
	5	1.6 ± 0.1	17.1 ± 0.4	10.5 ± 1.1	0.987	4 ± 1
	15	1.7 ± 0.1	16.9 ± 0.3	**9.9 ± 0.7	0.994	10 ± 1
	30	1.6 ± 0.1	17.3 ± 0.6	**8.1 ± 0.8	0.987	27 ± 3

(**) represents the statistical significance, $p < 0.05$, compared to control set (0 mM hexadecane).

In the assays with *M. formicicum,* similar lag phases of approximately 5 days were observed for all the hexadecane concentrations tested. Nevertheless, the maximum methane production rate (Rm) was significantly lower ($p < 0.05$) in the incubations with 15 mM and 30 mM of hexadecane, relatively to the other hexadecane concentrations studied (Figure 4.1, Table 4.1). For these two higher hexadecane concentrations, Rm decreased approximately 52% and 54%, respectively, comparatively to the control.

For the assays with *M. hungatei* (Figure 4.2, Table 4.1), maximum cumulative CH_4 production was achieved after 5 days of incubation, with exponential phases lower than 2 days, which shows faster methane production by *M. hungatei* than by *M. formicicum*. Significant changes ($p < 0.05$) in Rm were observed for hexadecane concentrations of 15 and 30 mM, representing a decrease of 11% and 27%, respectively, in relation to the assay performed without hexadecane (control, 0 mM). These results suggest a higher tolerance of *M. hungatei* to the presence of hexadecane, compared with *M. formicicum*.

For the two methanogens, the results from the methane production were confirmed by the H_2 uptake profiles, that followed the same trends (Figures 4.1 and 4.2). Considering the H_2 concentrations measured at the start and end of the assays, and the stoichiometry of hydrogenotrophic methanogenesis ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$), the methane yields (mole of methane produced per mole of hydrogen consumed, expressed in percentage relatively to the expected value of 1:4) were calculated (Table 4.2). These results show 91% - 100% conversion of H_2 to CH_4 for *M. formicicum*, and 101% - 117% for *M. hungatei*.

Table 4.2 - Hydrogen concentrations measured at the start (T_0) and end (T_f) of the assays, hydrogen consumed, maximum cumulative methane produced and calculated methane yields for the incubations of *M. formicicum* and *M. hungatei* with increasing hexadecane (Hex.) concentrations. Concentration values for experimental measurements are shown as mean ± standard deviation from triplicates.

Methanogen	Hex. (mM)	$[H_2]_{T0}$ (mM)	$[H_2]_{Tf}$ (mM)	$[H_2]$ consumed (mM)	Max. $[CH_4]$ (mM)	CH_4 yield (%)
M. formicicum	0	72.56 ± 1.90	3.94 ± 0.16	68.62 ± 1.90	17.19 ± 0.70	100 ± 11
	1	69.95 ± 0.56	3.89 ± 0.08	66.07 ± 0.57	15.89 ± 0.47	96 ± 2
	5	69.21 ± 0.49	4.10 ± 0.10	65.11 ± 0.50	16.05 ± 0.39	99 ± 2
	15	69.31 ± 0.56	4.57 ± 0.11	64.74 ± 0.57	15.20 ± 0.11	94 ± 1
	30	69.21 ± 0.48	5.48 ± 0.90	63.73 ± 1.02	14.44 ± 0.40	91 ± 4
M. hungatei	0	72.65 ± 3.51	1.77 ± 0.03	70.89 ± 3.51	18.55 ± 0.11	105 ± 3
	1	68.45 ± 0.5	1.93 ± 0.06	66.51 ± 0.51	19.51 ± 0.03	117 ± 0
	5	68.36 ± 0.64	1.60 ± 0.01	66.76 ± 0.64	18.34 ± 0.28	110 ± 2
	15	67.71 ± 1.01	1.13 ± 0.76	66.59 ± 1.26	17.22 ± 0.01	103 ± 18
	30	69.02 ± 0.42	2.38 ± 0.20	66.64 ± 0.46	16.85 ± 0.27	101 ± 1

The pH and ORP measures (Table I-1 and I-2, Appendix I) showed slight increases for both parameters, increasing in average from 7.1 to 7.6 and -283 mV to -240 mV, respectively, for *M. hungatei* and 7.2 to 8.0 and -275 mV to -270 mV, respectively, for *M. formicicum*.

In the literature, no studies were found on the toxicity of hexadecane (or other long-chain alkanes) to hydrogenotrophic methanogens in pure culture. Most studies focus on the inhibition of methanogenesis in mixed cultures, either in anaerobic bioreactors or in batch assays. Short-chain linear hydrocarbons (Sherry et al., 2014), polyaromatics (PAH) and BTEX (Chen et al., 2008; Dou et al., 2008; Rodriguez et al., 2008) have all been shown to inhibit methanogenesis, which raises the question of possible direct inhibition of methanogens or indirect inhibition by disrupting the microbial community relationships necessary for the anaerobic biodegradation of hydrocarbons. The inhibitory effect is attributed to their higher water solubility compared to n-alkanes, rendering them more bioavailable for biodegradation but also more toxic, since excessive accumulation of these compounds can cause membrane instability. Other compounds such as halogenated aliphatic are also very toxic due their highly reactive halogen group (mainly Cl and Br) (Colleran et al., 1992; Hongtang & Mith, 2000; Pries et al., 1994; Sanz et al.,

1997). Biodegradation of these compounds was reported to occur at slow rates and it is suggested that prolonged acclimation may help the communities to adapt (Leahy & Colwell, 1990; Sikkema et al., 1995; Weber & Bont, 1996).

In terms of adverse effects to microorganisms, n-alkanes have been shown to interfere with cytoplasmic membranes as detailed in several reviews (Sikkema et al., 1995; Weber & Bont, 1996). Lipophilic compounds, such as n-alkanes, accumulate between the hydrophobic hydrocarbon chains of the membrane phospholipids, near the centre of the bilayer. This accumulation causes an increase in thickness and consequently affects the activity of transmembrane carrier proteins. Studies in bilayer models (McIntosh et al., 1980; Pope et al, 1989) show that due to their size, long chain n-alkanes such as hexadecane have limited solubility (unlike short-chain alkanes) up to a maximum amount of one hexadecane molecule per 6-10 lipid molecules. This limited solubility may explain the similar inhibition percentages on the methane production rate (Rm) in the assays with *M. formicicum* and hexadecane concentration of 15 mM and 30 mM (Figure 4.1 and Table 4.1). The total density of the microbial culture will influence the apparent toxicity observed since for the same concentration of hexadecane, a denser community can exhibit less harmful effects. Likewise, the greater the concentration of hexadecane the higher the effects on individual microbial cells. Methanogens are not capable of biodegrading alkanes therefore this accumulation can be irreversible. These studies have focused mainly on bilayer models and don't consider the presence of the cell wall or the hydrophilic parts of the outer membrane layer.

N-alkanes, such as hexadecane and other long-chain alkanes, are non-polar and chemically stable molecules, virtually insoluble in water. Even though the organic carbon-water partition coefficient (log K_{oc}) of hexadecane reveals high affinity towards organic carbon, direct interaction between lipophilic (hydrophobic) molecules and hydrophilic phospholipids heads at the outer membrane layer is considered unfavourable (Sikkema et al., 1995). Studies have shown that two different strains of the same bacteria mixed with hexadecane, one can adhere poorly while the other shows good adherence (Busscher et al., 1995; Rosenberg & Rosenberg, 1981), so questions remained regarding hexadecane interactions with the methanogenic cultures.

The dissolution rate of a compound is the critical for its bioavailability and thus toxicity, since it governs the transfer of the compound into the microorganisms (Sikkema et al., 1995). Due to long-chain n-alkane low aqueous solubility in standard conditions, experimental data is scarce but studies have attempted to provide insight, through both experimental data and computational modelling (Ferguson, Debenedetti, & Panagiotopoulos, 2009; Pereda et al., 2009; Trinh et al., 2016; Veja et al., 2009). Overall, their findings suggest that solubility decreases exponentially with the number of carbons on the chain,

with solubility molar fraction (M) in the magnitude 10^{-7} mol/L and lower for C15 and above n-alkane chains. Experimental measurements (Coates et al., 1985; Tolls et al., 2002) showed that vigorous mixing and alkane concentration increase leads to the formation of clusters/aggregates in the water phase, thus enhancing the mass transfer between the alkane-water phase and consequently increasing the concentration of n-alkanes in the water phase.

This is in alignment with our results as the initial concentrations of hexadecane, 1 mM and 5 mM, were most likely too low for a reasonable level of aggregation to form as the solubility decreases exponentially with increasing number of carbon atoms. Reported stirring rates were substantially greater than our experiment's apparatus, which was perhaps compensated by increasing hexadecane concentration to achieve considerable n-alkane aggregate/microdroplet formation. Other factors such as solubility and partition coefficients of H_2 could had been responsible for the lower H_2 uptake and consequently CH_4 production, namely in the assays with the higher hexadecane concentrations. The presence of a hydrocarbon floating layer in the water-gas interface, or a hydrocarbon layer adsorbed to the microbial cells, may induce mass transfer limitations, making more difficult the H_2 uptake or the release of the produced CH_4.

Nonetheless, the increased hexadecane mass transfer and microdroplets formation combined with higher bioavailability promoted by moderate shaking during incubation supports the hypothesis that hexadecane was indeed responsible for the decrease in MPR as its low water solubility and unfavourable lipophilic-hydrophilic interactions may not completely hinder its interaction with the microbial suspension under the experimental conditions employed.

The differences in sensitivity to hexadecane between the two methanogens studied might be linked to differences in cell wall structure and membrane lipid composition. *M. hungatei* is a rod-shaped cell and exists as a filamentous chain enclosed in a tubular proteinaceous sheath. This sheath encloses the cell-chain community and not the individual cells (Albers & Meyer, 2011) and it differs from other protein S-layers found in Archaea cell walls in that it exhibits very low porosity. The sheath is so impermeable that only small molecules such as H_2, CO_2 and CH_4 can penetrate it, whereas larger molecules can only diffuse inward from the more porous terminal ends (Beveridge & Graham, 1991). *M. formicicum* possesses no protein sheath or S-layer, but an amorphous peptidoglycan layer known as pseudomurein. As such, hexadecane would have a much higher difficulty affecting, or even accessing, the *M. hungatei* cells, and as a result showed greater tolerance to hexadecane.

A study by Sousa and Salvador (Sousa et al., 2013) explored the toxicity of long-chain fatty acid (LCFA) oleate (C18:1), stearate (C18) and palmitate (C16), in pure cultures of *M. formicicum* and *M.*

hungatei. Although the study doesn't focus on long-chain n-alkanes, LCFA can be considered a parallel for data comparison, as they are thought to be intermediaries of alkanes biodegradation. The authors observed that *M. hungatei* presented significantly lower IC_{50} compared to *M. formicicum* in all tested LCFA. These results are the opposite to our results with hexadecane. Cell structure, particularly the membrane composition, was the determining factor for LCFA vulnerability. The cytoplasmatic membranes do in fact present significant differences. *M. formicicum* has three different phospholipids (inositol, ethanolamine, serine) compared to only two in *M. hungatei* (glycerol and aminopentanetetrol) (Koga & Morii, 2005; Koga et al., 1998; Koga et al., 1993). The different compositions may indeed translate to different levels of sensitivity or give clues to the cell's mechanisms, or lack thereof, to counteract the disruptions caused by lipophilic compounds such as LCFA or alkanes to the lipid membrane stability. It also demonstrates the impact of the more reactive carboxylic group in LCFA compared to the simple n-alkane chains.

4.2 Effects of microaeration on the conversion of hexadecane to methane in batch assays

4.2.1 Effects of microaeration on syntrophic and methanogenic communities

The anaerobic sludge used as inoculum in these experiments exhibited good SMA in the presence of H_2/CO_2 and ethanol (878 ± 79 and 671 ± 60 mL CH_4@STP/gVS.day), respectively), but low activity with acetate (24 ± 1 mL CH_4@STP/gVS.day). To test the effects of microaeration, the sludge was inoculated with these substrates in the absence of oxygen (phase-1, P1), after which air and additional substrate were added (phase-2, P2). To establish when to transition to P2, the amount of CH_4 measured was compared with the amount expected to be produced from the bioconversion of H_2/CO_2, acetate and ethanol according to the stoichiometric equations in Table 4.3.

Table 4.3 Stoichiometry for reactions involved in syntrophic degradation coupled to CH_4 production.

		$\Delta G°'$/reaction
Methane-producing reactions		
Hydrogenotrophic (H_2/CO_2)	$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$	-131 kJ[a]
Acetoclastic (Acetate)	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-75 kJ[a]
Syntrophic oxidation to acetate and H_2		
Ethanol	$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + H^+ + 2H_2$	+9.8 kJ[b]
Butyrate	$CH_3CH_2CH_2CO^- + 2H_2O \rightarrow 2CH_3COO^- + H^+ + 2H_2$	+48 kJ[a]
Propionate	$CH_3CH_2CO^- + 2\ H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2$	+72 kJ[a]
Hexadecane	$C_{16}H_{34} + 16H_2O \rightarrow 8CH_3COO^- + 8H^+ + 17H_2$	+470,8 kJ[c]
Syntrophic oxidation to acetate and methane		
Ethanol	$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COO^- + 2H^+ + CH_4$	-116,3 kJ[d]
Total oxidation to CH_4		
Ethanol	$2CH_3CH_2OH + H_2O \rightarrow 3CH_4 + HCO_3^- + H^+$	-179 kJ[e]
Hexadecane	$C_{16}H_{34} + 7.5H_2O \rightarrow 12.25\ CH_4 + 3.75\ CO_2$	-371,8 kJ[c]

[a](Stams & Plugge, 2009);[b](Wu et al., 1991);[c](Dolfing et al., 2008);[d](Zinder, 1993);[e](Bernhard Schink, 1985a)

4.2.1.1 Methane production

The cumulative CH_4 production recorded in P1 and P2, during the bioconversion of H_2/CO_2, acetate and ethanol is shown in Figures 4.3, 4.4 and 4.5, respectively. The CH_4 production rates (MPR) were determined by calculating the initial slope of the cumulative methane production curves in P1 and P2 and are shown in Table 4.4. The ratio between the MPR in P2 and in P1, slope ratio (S_r) was calculated for each incubation condition, to correct for changes observed upon phase transition in the control (Silva et al., 2016). Additionally, the S_r for increasing $\%O_2$ incubation conditions was then compared to the control to determine the inhibitory effect (%).

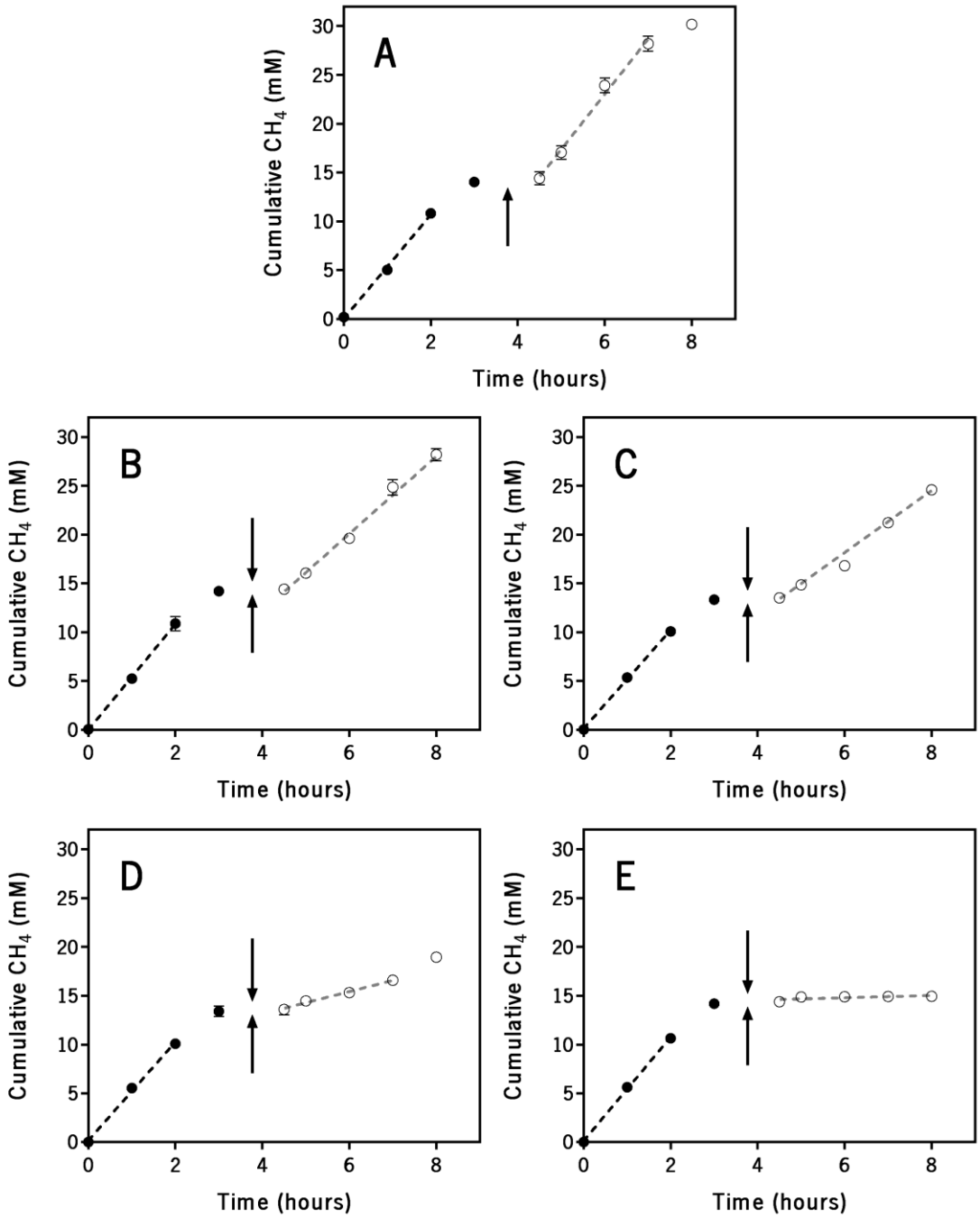

Figure 4.3 - Effects of microaerobic conditions on CH_4 production from bioconversion of H_2/CO_2 by an anaerobic sludge at different $\%O_2$. Increasing $\%O_2$ were tested, 0% O_2 (A), 0,5% O_2 (B), 1% O_2 (C), 2,5% O_2 (D) and 5% O_2 (E). Cumulative methane production before O_2 addition, P1, (●) and after O_2 addition, P2, (○); dashed lines show the data points used to calculate the methane production rate in P1 (---) and P2 (---), respectively; (↓) indicates addition of air pulse and (↑) indicates H_2/CO_2 replenishment (100% of initial concentration, 60 mM). Each data point is presented as the mean of triplicates ± standard deviations.

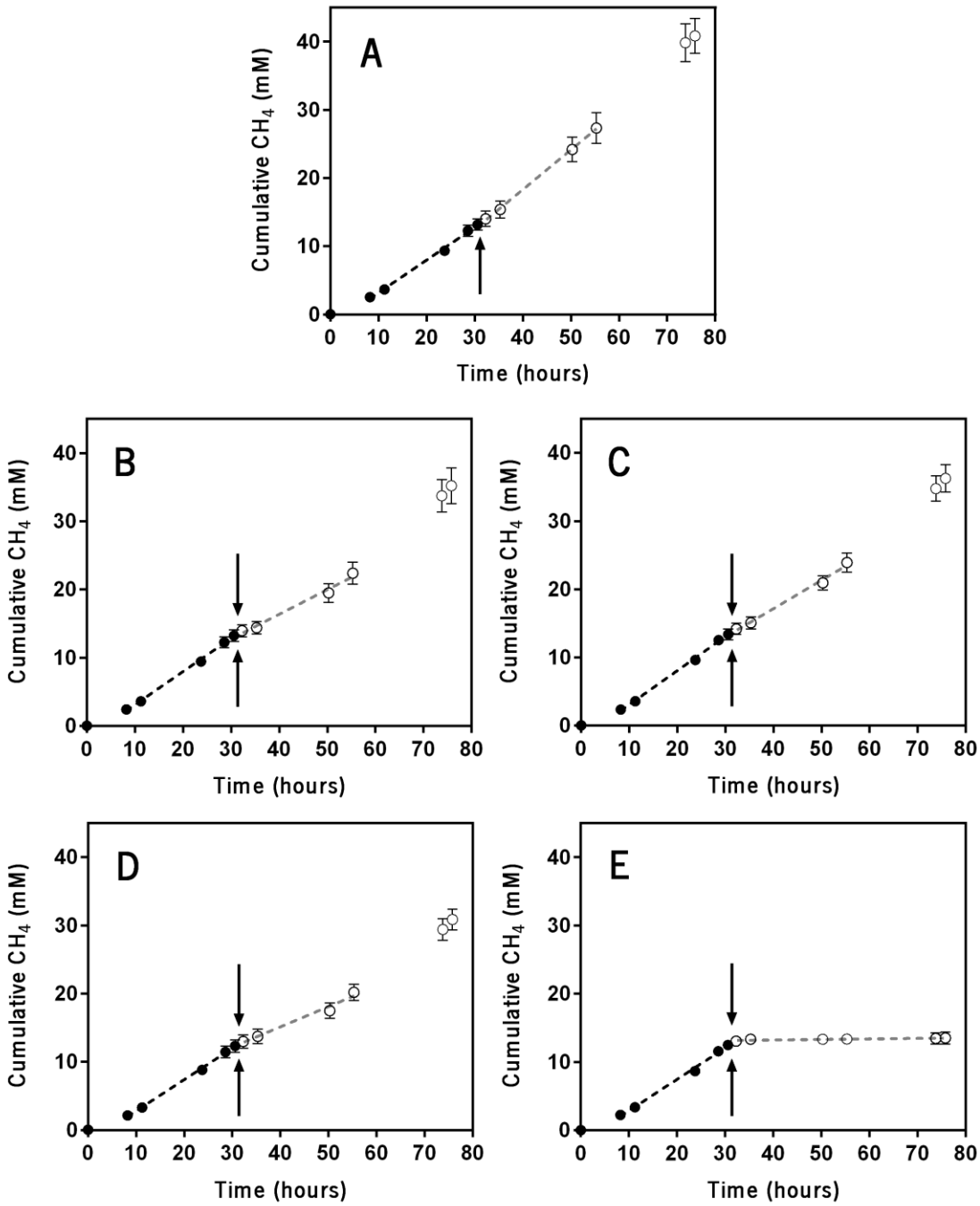

Figure 4.4 - Effects of microaerobic conditions on CH_4 production from bioconversion of acetate by an anaerobic sludge at different $\%O_2$. Increasing $\%O_2$ were tested, 0% O_2 (A), 0,5% O_2 (B), 1% O_2 (C), 2,5% O_2 (D) and 5% O_2 (E). Cumulative methane production before O_2 addition, P1, (●) and after O_2 addition, P2, (○); dashed lines show the data points used to calculate the methane production rate in P1 (---) and P2 (---), respectively; (↓) indicates addition of air pulse and (↑) indicates acetate replenishment (50% of initial concentration, 30 mM). Each data point is presented as the mean of triplicates ± standard deviations.

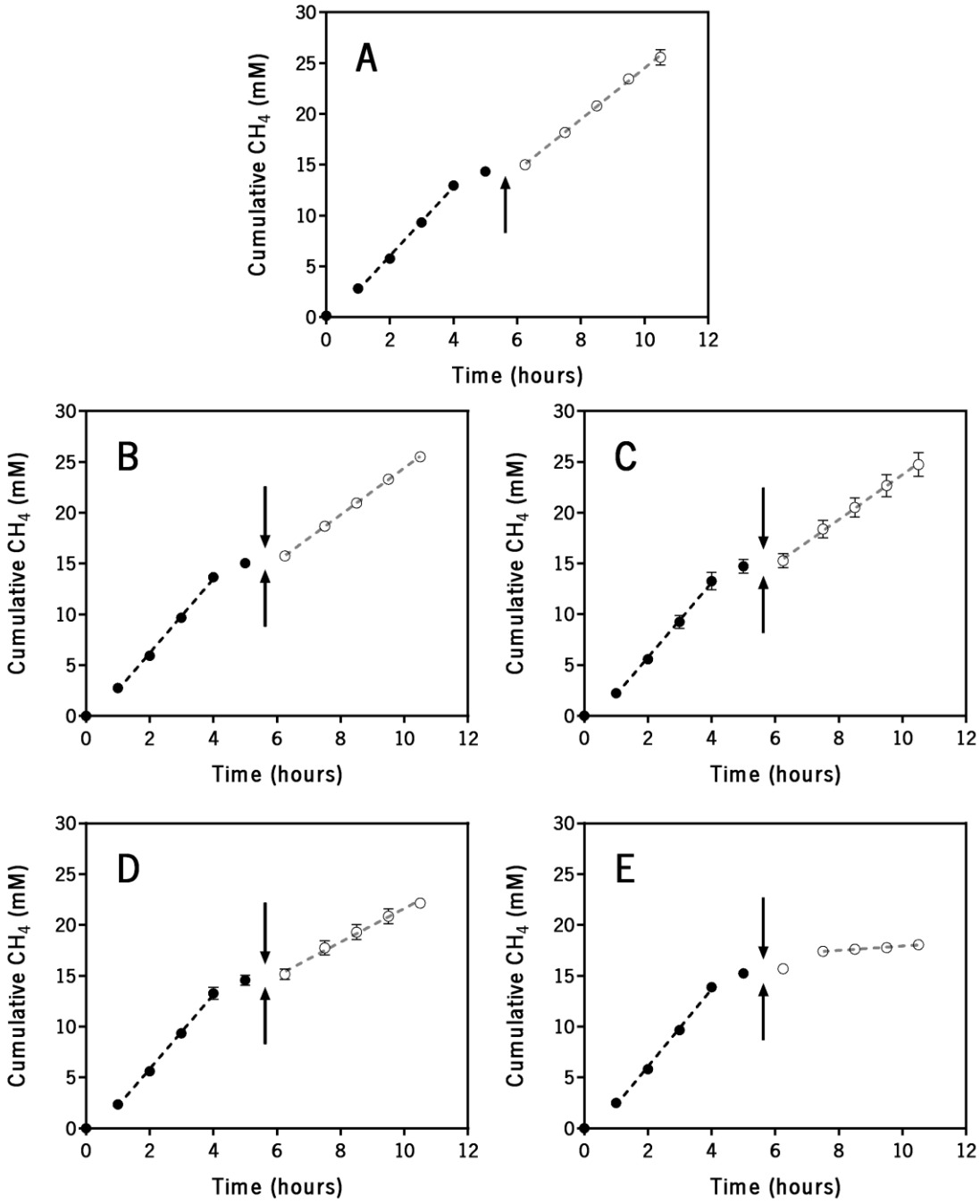

Figure 4.5 - Effects of microaerobic conditions on CH_4 production from bioconversion of ethanol by an anaerobic sludge at different $\%O_2$. Increasing $\%O_2$ were tested, 0% O_2 (A), 0,5% O_2 (B), 1% O_2 (C), 2,5% O_2 (D) and 5% O_2 (E). Cumulative methane production before O_2 addition, P1, (●) and after O_2 addition, P2, (○); dashed lines show the data points used to calculate the methane production rate in P1 (---) and P2 (---), respectively; (↓) indicates addition of air pulse and (↑) indicates ethanol replenishment (100% of initial concentration, 30 mM). Each data point is presented as the mean of triplicates ± standard deviations.

Table 4.4 - CH_4 production rate (MPR) determined for unamended (blank) cultures and H_2/CO_2, acetate and ethanol bioconversion by an anaerobic sludge at increasing headspace $\%O_2$. P1 – phase one, pre-O_2 addition growth; P2 – phase two, post-O_2 addition growth; S_r – MPR_{P2}/MPR_{P1} ratio. Data shown for P1 and P2 derives from the mean values ± standard deviation of triplicate bottles.

Substrate	O_2 (%)	MPR (mM/h)		S_r	Inhibition (%) [a]
		P1	P2		
H_2/CO_2	0	5.32 ± 0.12	5.66 ± 0.09	1.07 ± 0.03	-
	0.5	5.41 ± 0.31	**3.98 ± 0.03	0.74 ± 0.04	31 ± 5
	1	5.03 ± 0.07	**3.18 ± 0.05	0.63 ± 0.01	41 ± 3
	2.5	5.03 ± 0.05	**1.14 ± 0.08	0.23 ± 0.02	79 ± 4
	5	5.31 ± 0.12	**0.02 ± 0.01	0.03 ± 0.00	98 ± 4
Acetate	0	0.48 ± 0.02	0.58 ± 0.04	1.22 ± 0.09	-
	0.5	0.49 ± 0.03	**0.36 ± 0.02	0.74 ± 0.06	39 ± 10
	1	0.50 ± 0.02	**0.42 ± 0.02	0.83 ± 0.05	31 ± 9
	2.5	0.46 ± 0.03	**0.30 ± 0.00	0.65 ± 0.04	46 ± 9
	5	0.46 ± 0.02	**0.01 ± 0.00	0.01 ± 0.00	99 ± 11
Ethanol	0	3.39 ± 0.08	2.52 ± 0.12	0.74 ± 0.04	-
	0.5	3.65 ± 0.14	2.30 ± 0.08	0.63 ± 0.03	15 ± 7
	1	3.67 ± 0.20	2.22 ± 0.11	0.60 ± 0.04	19 ± 8
	2.5	3.65 ± 0.15	**1.73 ± 0.06	0.47 ± 0.03	36 ± 7
	5	3.80 ± 0.09	**0.22 ± 0.05	0.06 ± 0.01	92 ± 8
Blank	0	0.06 ± 0.00	0.04 ± 0.00	0.70 ± 0.02	
(no added substrate)	0.5	0.06 ± 0.00	0.02 ± 0.00	0.26 ± 0.01	63 ± 4
	1.0	0.06 ± 0.00	0.01 ± 0.00	0.16 ± 0.00	77 ± 4
	2.5	0.06 ± 0.00	0.00 ± 0.00	0.05 ± 0.02	93 ± 4
	5	0.06 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	96 ± 4

(**) represents statistical significances with $p < 0.05$, compared to the corresponding assay control set (0% O_2).
(a) Inhibition (%) was calculated by comparing the S_r obtained from the O_2 supplemented assays (S_rO_2) with the S_r obtained from the control assays (S_rC), according to the equation: Inhibition (%) = ((S_rC - S_rO_2)/S_r C)*100).

In the assays with H_2/CO_2 (Figure 4.3, Table 4.4), MPR was high before O_2 addition (P1), in agreement with the results from the specific methanogenic activity (SMA) test. Upon O_2 addition (P2), the MPR evidently decreases with increasing O_2 concentration, with substantially low MPR at 2,5% O_2 and a complete inhibition of CH_4 production at 5% O_2. In the control bottles (0% O_2), a significant increase in

MPR ($p < 0.05$) was observed upon transition to P2 (Table 4.4), possibly due to culture acclimation or biomass growth. The MPR achieved during P1 presents no significant differences ($p > 0.05$) when comparing each condition (%O_2) with all others within the respective assays, regardless of the substrate being tested, thus we can assume that all conditions triplicates transitioned to P2 at identical stages of culture acclimation, and that changes observed in the control conditions are most likely present in all other conditions.

Figure 4.4 shows CH_4 production during the assays supplemented with acetate. Acetoclastic methanogenic activity of the anaerobic sludge was low, hence in P1 approximately 30 hours of incubation were necessary for the bioconversion of half of the initially added acetate (30 mM) to CH_4. In P2, MPR increased significantly ($p < 0.05$) in the control condition upon phase transition (Table 4.4), meaning that as the sludge was continually amended with acetate at optimal growth conditions, the activity of the acetoclastic community was enhanced. For the assays performed under microaerobic conditions, MPR decreased with addition of O_2. Similar MPR values were observed at 0.5%, 1% and 2.5% O_2. At 5% O_2 the CH_4 production was neglectable, as complete inhibition was observed.

Figure 4.5 shows CH_4 production during ethanol bioconversion. In the SMA test using ethanol as the substrate, a faster methane production in the first 4-5 hours (corresponding to the SMA value of 671 ± 60 $mLCH_{4STP}$/gVS.day), was followed by a second slower phase of methane production (MPR of 17 ± 4 $mLCH_{4STP}$/gVS.day for the remaining of the test) (Figure II-1, Appendix II). The occurrence of multiphasic profiles of CH_4 evolution during SMA tests has been previously observed in different sludges of diverse age (Colleran et al., 1992). Such patterns were not observed with either acetate or H_2/CO_2 (direct methanogenic substrates) but rather ethanol or butyrate (indirect methanogenic substrates) and were indicative of imbalance between the various subpopulations needed to convert indirect substrates to CH_4 and $C0_2$. The high CH_4 production rate in the first hours of incubation is likely linked to the initial bioconversion of H_2 (high hydrogenotrophic methanogenic activity) to CH_4 as the ethanol substrate is rapidly consumed, whereas the accumulated acetate requires longer periods for complete bioconversion (low acetoclastic methanogenic activity), essentially creating a profile with two distinct CH_4 production rates (Liu et al., 2013; Wu et al., 1991). Figure 4.5 will mostly represent the bioconversion of ethanol to H_2 and further to CH_4, with acetate accumulating in the medium. As the %O_2 increases, noticeable decreases in CH_4 production rate can be observed at 2.5% O_2 and 5% O_2, with a substantial decrease in the latter (Table 4.4). Contrary to what was verified in the assays with H_2/CO_2 and acetate, for the ethanol assay a significant decrease ($p < 0.05$) in MPR was observed in the controls after P2 transition, most likely due to the accumulation of acetate and associate decrease in pH which can inhibit methanogenesis

(Steinbusch et al., 2009). Measurements of pH at the end of the assays (Appendix I-B) show low levels for assay with ethanol, averaging 5.7 at 0%, 0.5%, 1% and 2.5% O_2 but increasing to 6.5 at 5% O_2. In comparison, a slightly higher pH was detected in the H_2/CO_2 assay, decreasing from 8 to 7.5 with increasing $\%O_2$ in the headspace. In the assays with acetate, pH levels were steady at approximately 7.5, regardless of the condition. Since only residual H_2 accumulation was observed for 0%, 0.5% and 1% O_2, the decrease in pH is likely the most significant factor in lower MPR, and not $\%O_2$, which could explain the decrease in overall MPR upon P2 transition, even at 0% O_2 (Table 4.4). ORP measurements at the end of the incubations presented lower values in the conditions with highest $\%O_2$ in the headspace, which could be related to increased biological activity at the lower $\%O_2$ (lower or absent inhibition). Overall, ORP measurements (Appendix I-B) show lower potentials in the blank assay, H_2/CO_2 assay (-232 ± 12 mV) followed by acetate (-199 ± 20 mV) and ethanol (-180 ± 23 mV). For optimal methanogenic activity, an anoxic and reducing media (-300 mV) is usually preferred (Jasso-Chávez et al., 2015) but these differences may not have had a significant effect on the MPR, as in ethanol it is substantially high. Additionally, VS analysis (Appendix I-B) shows that for all assays (except ethanol), the biomass didn't deviate significantly from the estimated 4 g/L during assay preparation, with a slight increase at 5% O_2. Due to experimental problems, the VS values in the assays with ethanol could not be accurately determined.

The results show that O_2 exposure had a significant effect ($p < 0.05$) in all tested $\%O_2$ (0.5%, 1% 2.5% and 5%) for the H_2/CO_2 and acetate amended cultures, while in the ethanol amended cultures, a significant effect was observed only at 2.5 and 5% O_2. At 5% O_2, methanogenic activity was negligible on the assays amended with H_2/CO_2 and acetate, presenting MPR similar to those in blank assays (Table 4.4), while in the ethanol assays a considerable CH_4 production rate was obtained, *i.e.* 0.22 ± 0.05 mM/h (ethanol) vs. 0.02 ± 0.01 mM/h (H_2/CO_2) and 0.01 ± 0.00 mM/h (acetate). These results indicate that the presence of ethanol stimulated the activity of the overall community, including facultative and aerobic populations, thus preventing complete inhibition of the methanogenic activity by O_2 exposure.

In the blank assays, a decrease of the MPR upon phase transition was observed (Figure III-A in Appendix III and Table 4.4), but this could be expected as, with no substrate available, the CH_4 production generally tends to become very low (resulting mainly from self-consumption of the biomass), or even ceases. The maximum CH_4 accumulated 2.74 ± 0.04 mM was observed in the 1% condition after 47 hours of incubation, though no significant differences ($p > 0.05$) were found between 0%, 0.5% and 1% O_2. Additionally, the MPR values calculated for these assays indicate that the background CH_4 production

rate is substantially lower than the MPR from the substrates added. Therefore, the contribution of the background CH_4 production can be considered negligible.

4.2.1.2 Substrate consumption and intermediary products formation

HPLC analysis (for acetate and ethanol concentrations) and GC (for H_2/CO_2 quantification) were employed to monitored substrate uptake and intermediary products formation. Table 4.5 shows the substrate evolution during both P1 and P2 in the assays with H_2/CO_2 or acetate. Total substrate uptake decreased with increasing $\%O_2$ for both assays, corroborating the CH_4 production results.

Table 4.5 – Quantification of H_2 and acetate, at the start and end of P1 and P2, in H_2/CO_2 and acetate bioconversion assays, respectively, and total substrate uptake (calculated by the difference between the initial substrate concentration in the specific phase and the final concentration in said phase).

Substrate	Condition ($\%O_2$)	P1		P2	
		Initial [substrate] (mM)	Total uptake (mM)	Initial [substrate] (mM)	Total uptake (mM)
H_2/CO_2	0	55.6 ± 0.6	48.6 ± 2.2	53.2 ± 1.2	49.3 ± 1.4
	0.5	53.5 ± 0.5	48.2 ± 1.5	51.6 ± 0.5	43.5 ± 1.5
	1	53.4 ± 0.3	44.9 ± 0.7	48.5 ± 2.3	32.8 ± 2.6
	2.5	53.2 ± 0.3	46 ± 1.1	46.1 ± 0.6	12.9 ± 1.1
	5	53.5 ± 0.5	48.3 ± 3.6	42.2 ± 0.2	-6.4 ± 0.4
Acetate	0	24.3 ± 0.6	12.1 ± 1.5	25.4 ± 2.1	24 ± 2.6
	0.5	25.2 ± 0.3	12.6 ± 0.7	25.9 ± 0.5	17.6 ± 1.5
	1	23.7 ± 2.0	11.4 ± 2.0	25.2 ± 0.6	19.3 ± 0.7
	2.5	24.0 ± 0.8	10.6 ± 1.2	25.9 ± 0.7	16.0 ± 1.3
	5	24.7 ± 0.9	11.7 ± 1.3	26.6 ± 1.1	0.4 ± 1.9

Differences between the H_2 concentrations at the start of P2 in the different conditions studied are the results of O_2 addition, which decreases the total amount of H_2 in the headspace after depressurization of the bottles to 1 atm. Another result that presents some discrepancy is the case of 5% O_2 that suggests that H_2 was produced rather than consumed. As the O_2 is depleted from the headspace, its inhibitory effect on the hydrogenotrophs leads to a complete halt in H_2 uptake, thus an apparent H_2 production is observed as the concentration of the gases in the headspace changes overtime.

In the assays with acetate, other metabolites were detected such as formate and butyrate. Formate was only detected at 5% O_2 at the end of P2, in one of the triplicates that accumulated 0.92 mM of formate. Accumulation of formate is normally associated with the inhibition of hydrogenotrophic methanogenesis (Steinbusch et al., 2009), which leads to a switch from H_2 to CH_4 conversion to formate production instead. Butyrate was also present during P2 in the assays amended with 5% O_2, with 0.35 ± 0.01 mM butyrate detected at the start and 0.42 ± 0.05 mM measured at the end. These results suggest that O_2 might stimulate the production of butyrate, most likely by inhibiting methanogenesis. Still, the butyrate produced had a significantly low yield (26.6 ± 1.1 mM acetate accumulated in 5% O_2, P2). Production of butyrate was previously achieved from H_2 and acetate (Steinbusch et al., 2009). The authors used low pH (< 6) and thermal treatments to inhibit methanogens and observed both ethanol and butyrate production from acetate reduction, with butyrate production becoming more favourable as the ethanol accumulated in the media. Another study revealed both acetate and butyrate accumulation during ethanol oxidation (Metje & Frenzel, 2005), with butyrate synthesis accounting for the largest fraction of acetate uptake. This was not observed in the ethanol assays of this work, as no significant butyrate was formed even though the cultures suffered a steep decrease in pH (5.5) and were exposed to O_2. The butyrate detected was mostly residual (< 0.06 mM) and no accumulation was observed overtime. There is no certainty regarding the origin of the butyrate formed at 5% O_2 during acetate oxidation.

In ethanol bioconversion, ethanol and acetate concentrations were measured periodically throughout P1 and P2 (Figure 4.6), and the respective uptake and production is shown in Table 4.6. This substrate was completely consumed in all conditions during P1, and the produced acetate presents no significant differences between conditions (Table 4.6), thus all triplicates transitioned to P2 at identical metabolite levels. This shows that indeed CH_4 production measured during P1 can be mostly attributed to the H_2 produced being converted to CH_4, since a substantial amount of acetate, on average 93 ± 1% of predicted conversion, is still present in the culture media at the end of P1. This is further reinforced by the fact that acetate consumption averaged 1.7 ± 0.5 mM in all conditions during this period (Table IV-1, Appendix IV) (no significant differences between conditions), and that the experimental methane production exceeded the theoretical expected value from hydrogenotrophic activity in 1.7 ± 0.5 mM CH_4 (Table IV-2, Appendix IV). Larger fractions of CH_4 originating from hydrogenotrophic activity rather than from acetoclastic activity is commonly observed during ethanol oxidation (Y. Liu et al., 2013; Metje & Frenzel, 2005; W. Wu et al., 1991).

Table 4.6 – Ethanol uptake and acetate production in ethanol oxidation by an anaerobic sludge at increasing % O_2. Initial and final ethanol and acetate concentrations are shown for P1 (pre-O_2 addition) and for P2 (post-O_2 addition); ethanol uptake and acetate production rates are shown for P2. The inhibition percentages were calculated compared to the control set.

Phase	Condition (% O_2)	$[Eth]_{T0}$ (mM)	$[Eth]_{Tf}$ (mM)	$[Ac]_{T0}$ (mM)	$[Ac]_{Tf}$ (mM)	Ethanol uptake		Acetate production	
						Rate (mM/h)	Inhibition (%)	Rate (mM/h)	Inhibition (%)
P1	0	26.6 ± 0.0	0	0.1 ± 0.0	24.9 ± 0.2	n.d.	n.d.	n.d.	n.d.
	0.5	26.1 ± 0.4	0	0.1 ± 0.0	24.6 ± 0.4	n.d.	n.d.	n.d.	n.d.
	1	26.1 ± 0.4	0	0.1 ± 0.0	24.4 ± 0.1	n.d.	n.d.	n.d.	n.d.
	2.5	25.7 ± 0.6	0	0.1 ± 0.0	24.2 ± 0.5	n.d.	n.d.	n.d.	n.d.
	5	26.2 ± 0.3	0	0.1 ± 0.0	24.3 ± 0.2	n.d.	n.d.	n.d.	n.d.
P2	0	27.7 ± 0.8	7.3 ± 1.1	24.3 ± 0.3	42.8 ± 1.2	4.3 ± 0.3	-	4.0 ± 0.3	-
	0.5	27.4 ± 0.7	8.3 ± 0.8	23.9 ± 0.6	41.7 ± 0.9	4.1 ± 0.1	2 ± 0	3.9 ± 0.2	7 ± 1
	1	26.9 ± 0.4	8.1 ± 0.7	23.7 ± 0.1	41.3 ± 0.5	4.1 ± 0.4	8 ± 1	3.7 ± 0.2	7 ± 0
	2.5	27.4 ± 1.1	7.8 ± 1.2	23.6 ± 0.6	26.1 ± 0.8	**3.2 ± 0.2	18 ± 2	**2.9 ± 0.1	20 ± 1
	5	28.2 ± 0.0	22.3 ± 0.4	24.0 ± 0.1	29.6 ± 0.7	**0.9 ± 0.1	73 ± 10	**1.1 ± 0.1	79 ± 6

(**) represents statistical significance, $p < 0.05$, compared to the respective control set (0% O_2); n.d. – not determine.

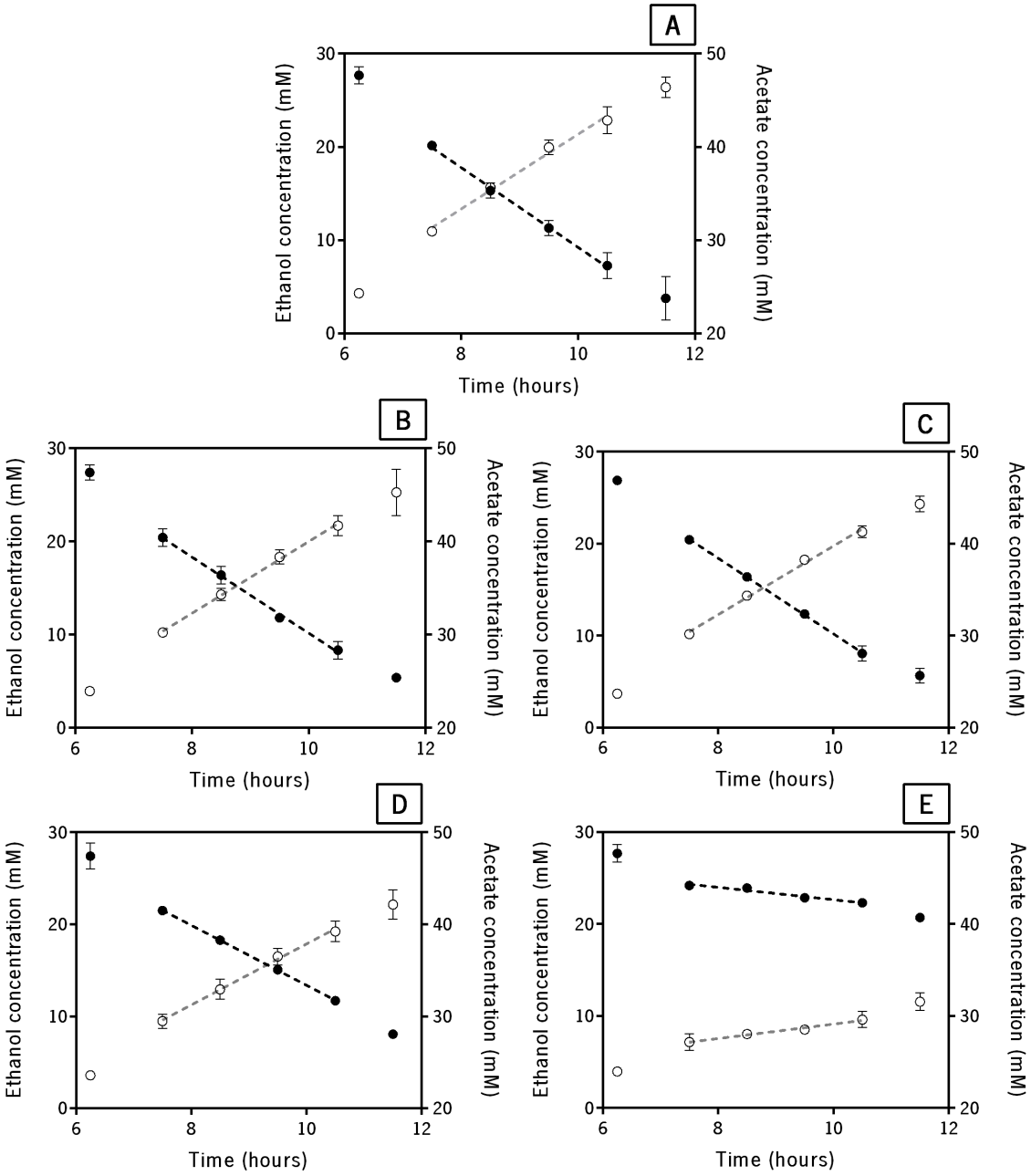

Figure 4.6 –Ethanol (●) and acetate (○) concentrations during P2 of ethanol bioconversion assays by an anaerobic sludge at different headspace $\%O_2$. Effects of increasing concentrations of O_2, 0% (A), 0.5% (B), 1% (C), 2.5% (D) and 5% (E) can be observed. Dashed lines show the data points used to calculate the ethanol uptake rate (- - -) and acetate production rate (- - -). Each data point is representative of the mean of triplicates and respective standard deviations. The initial acetate concentrations (>20 mM) are the result of accumulation during P1.

As previously observed, hydrogenotrophs and acetotrophs were sensitive to O_2 exposure thus at higher $\%O_2$, a decrease was expected in ethanol oxidation and subsequent acetate accumulation.

Significant differences ($p < 0.05$) on ethanol uptake and acetate production were observed at 2.5% and 5% O_2 in P2 (Table 4.6). Also, in this period, higher uptake and production rates were observed in the first hour of incubation, for all the $\%O_2$ concentrations tested (Figure 4.6). During P2, a decrease in the acetate consumption was observed with increase $\%O_2$ (Table IV-3, Appendix IV), revealing a complete inhibition of acetoclastic activity at 2.5% and 5% O_2. These results suggest that hydrogenotrophic activity is the main source of CH_4 production, as no significant acetate uptake was reported during P2.

At the end of the ethanol assay, H_2 was detected at increasing concentrations with $\%O_2$, albeit the concentrations peaked at 0.16 ± 0.03 mM and 0.15 ± 0.02 mM (4×10^{-3} atm H_2 partial pressure) in 2.5% and 5% O_2, respectively, a small fraction of the total gas. H_2 concentrations at 0%, 0.5% and 1% O_2 were 0.03 ± 0.01 mM, 0.05 ± 0.00 mM, 0.07 ± 0.01 mM, respectively. No H_2 was detected at the start of P2. The predicted H_2 generation (Table 4.3) from the reported ethanol uptake rate (Table 4.6) is approximately 6.24 ± 0.11 mM/h, a rate which would generate H_2 accumulation values far above the reported 0.16 ± 0.03 mM for 2.5% O_2 at the end of the assay (after 5 hours of incubation), if inhibition of hydrogenotrophic activity was occurring. Similarly, at 5% O_2, the predicted H_2 generation rate also exceeds the accumulated H_2 at the end of the assay, 2.08 ± 0.13 mM/h and 0.15 ± 0.02 mM H_2, respectively. The differences observed between predicted and experimental CH_4 production from H_2 (Figure IV-4, Appendix IV) shows a significant difference ($p < 0.05$) at 2.5% and 5% O_2, with 2.3 ± 0.1 mM CH_4 not being produced as predicted in the latter. Therefore, despite not being accumulating in the headspace, it is also likely not being generated at the maximum predicted rate shown above, indicating that ethanol dissimilation at this $\%O_2$ may not be entirely syntrophic. This CH_4 deficit cannot be explained by acetotrophic activity since it was completely inhibited at both 2.5% and 5% O_2, as previously shown. Besides butyrate, formate and propionate were also present. Propionate was detected in all conditions at the end of P1 at similar concentrations in all conditions, averaging 0.80 ± 0.2 mM. During P2, by the end of the assay, propionate concentrations decreased with increasing $\%O_2$, with 1.21 ± 0.11 mM at 0% O_2 and 0.35 mM at 5% O_2. Conversion of ethanol to propionate has been reported in several bacteria such as *Pelobacter propionicus* (B Schink, Kremer, & Hansen, 1987), *Clostridium neopropionicum* (Tholozan & Touzel, 1992) and *Desulfobulbus propionicus* (Tasaki & Kamagata, 1992). These are anaerobic bacteria, which may justify the decrease in accumulate propionate with increased $\%O_2$.

4.2.1.3 O_2 consumption

At the end of the assays, most of the O_2 present in the headspace had been consumed (Table 4.7).

Table 4.7 - O_2 consumption in H_2/CO_2, acetate and ethanol bioconversion by an anaerobic sludge, as well as in unamended (blank assay) cultures. $P2_i$ – start of phase two; $P2_f$ – end of the assay. All data points are the result of the mean of triplicates bottles. Measured values for O_2 indicate headspace percentage/concentration.

Substrate	O_2 (%)	Measured O_2 (%)		Measured O_2 (mM)	
		$P2_i$	$P2_f$	$P2_i$	$P2_f$
H_2/CO_2	0	-	-	-	-
	0.5	0.2	n.d.	0.04 ± 0.32	n.d.
	1	1.7	n.d.	0.62 ± 0.25	n.d.
	2.5	2.3	n.d.	0.87 ± 0.58	n.d.
	5	3.9	n.d.	1.50 ± 0.25	n.d.
Acetate	0	-	-	-	-
	0.5	0.6	0.0	0.22 ± 0.10	0.32 ± 0.15
	1	1.2	0.8	0.47 ± 0.08	0.12 ± 0.31
	2.5	2.7	0.3	1.06 ± 0.43	0.22 ± 0.14
	5	5.1	0.5	2.01 ± 0.10	0.08 ± 0.24
Ethanol	0	-	-	-	-
	0.5	0.6	0.1	0.23 ± 0.02	0.06 ± 0.00
	1	1.0	0.2	0.41 ± 0.03	0.07 ± 0.02
	2.5	2.6	0.6	1.02 ± 0.05	0.14 ± 0.02
	5	4.9	1.0	1.93 ± 0.04	0.40 ± 0.09
Blank	0	-	-	-	-
(no added substrate)	0.5	0.5	0.0	0.19 ± 0.05	0.02 ± 0.01
	1	1.0	0.1	0.38 ± 0.06	0.04 ± 0.01
	2.5	2.8	0.1	1.09 ± 0.13	0.03 ± 0.01
	5	4.9	0.1	1.94 ± 0.02	0.05 ± 0.01

n.d. – not determined.

The O_2 concentrations determined in the H_2/CO_2 assays are not in accordance with the expected values at the start of P2, which is probably due to peak overlapping between H_2 and O_2 during GC analysis. Ethanol assays showed residual O_2 in the headspace, except for the 2.5% and 5% conditions which still show considerable amounts of O_2, specially at 5%. This indicates that in these two conditions, the cultures were exposed to considerable amounts of O_2 throughout the entire assay, while at 0.5% and 1%, the O2 was readily consumed.

The O_2 uptake was studied in detail for the unamended cultures (Figure 4.7). The O_2 was rapidly depleted in the first 3 hours of incubation for 0.5% and 1% conditions, and substantially reduced to 20 ±

2% and 34 ± 2% of the initial concentration in the 2.5% and 5% conditions, respectively, after 7 hours of incubation. At 24 hours, O_2 was detected in residual amounts in all conditions. Innate O_2 uptake rate by the culture is quite significant.

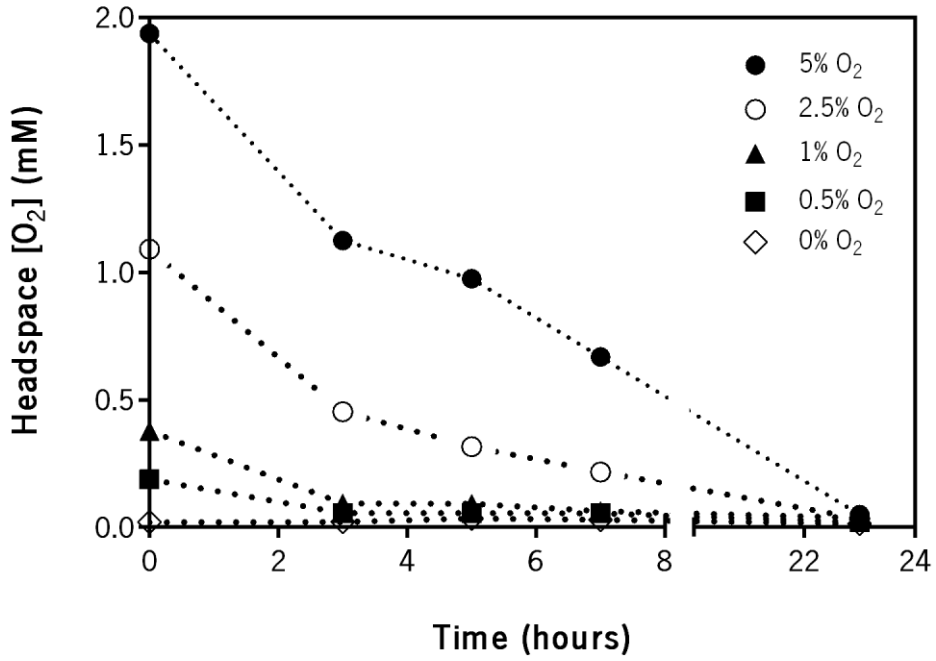

Figure 4.7 – O_2 uptake in unamended cultures (blank assay) by an anaerobic sludge at various headspace $\%O_2$. The evolution of O_2 concentration (mM) in the headspace during P2 (post-O_2 addition) of the blank assay where no additional substrate was added is shown. Data is represented as the mean of triplicates ± standard deviation.

These results demonstrate that, within the conditions established in these assays, longer incubations times can result in an apparent higher resistance to O_2 exposure. During P2 of H_2/CO_2 (Figure 4.3) the incubation lasted less than 4 hours meaning that, even at lower $\%O_2$ such as 0.5% and 1%, there was considerable O_2 exposure throughout the entire phase. On the contrary, P2 in the acetate assays (Figure 4.4) lasted over 30 hours meaning that at lower $\%O_2$ the microorganisms might have been able to adapt after the initial exposure to O_2 in the first hours of incubation thus appearing to present lower sensitivity to O_2 exposure. This can be observed on the CH_4 production observed in the blank assay (Figure III-1, Appendix III), where after 24 hours of incubation, the 0.5% and 1% O_2 conditions recovered from the adverse effects caused by O_2 exposure and achieved similar CH_4 levels as the controls. At 5% O_2, complete inhibition of the methanogenic communities, with a low chance of full recovery, was verified in both acetate and H_2/CO_2 assays, as the MPR observed remained lower than the background MPR obtained in

the blank assays (Table 4.4.) over the period of incubation. Even though P2 of the ethanol assays lasted less than 5 hours, the cultures were able to maintain their methanogenic communities active (Table 4.4), compared to the assays with the other substrate, albeit O_2 exposure was significant throughout this phase. O_2 uptake after 4-5 hours of incubation was 58 ± 3 % higher in the assays with ethanol than in the blanks, *i.e.* O_2 uptake of 1.52 ± 0.06 mM and 0.96 ± 0.03 mM, respectively, revealing considerable stimulation of aerobic metabolism. Increase in O_2 uptake was also observed in the 2.5% O_2 condition.

Studies have reported so called "shielding effects" towards O_2 exposure by anaerobic sludges (Botheju & Bakke, 2011), although most of these studies were performed using granular sludges. Facultative or aerobic microorganisms that colonize the surface of granules may shield the methanogens living deep inside with diffusion barriers which stop the full penetration by O_2 (Kato, Field, & Lettinga, 1993b, 1993a). Yet, studies with suspended cultures, as is the case of the sludge used for this work, have shown that shielding against O_2 is also observed, and reported a high correlation between O_2 uptake by the facultative biomass and sludge O_2 tolerance (Zitomer & Shrout, 1998).

Kato and coworkers (Kato et al., 1993a) concluded that the presence of substrate was the most significant factor in O_2 tolerance, and reported that ethanol provided a substantially higher O_2 tolerance than acetate to acetoclastic methanogens. At dissolved O_2 concentrations of up to 12.4 mg/L, these authors still observed considerable methanogenic activity and the O_2 IC_{50} varied between 0.05 mg/L and 6.10 mg/L for five different sludges. This shows that the diversity in microbial communities can be a factor in O_2 tolerance as different sludge sources can exhibit significantly higher O_2 tolerances. The differences between suspended or granular sludges weren't significant when tested in tolerant sludges. In our assays, the dissolved O_2 (DO), determined by Henry's Law, was approximately 0.2 mg/L, 0.4 mg/L, 1 mg/L and 2 mg/L for 0.5%, 1%, 2.5% and 5% O_2 respectively. The dissolved O_2 IC_{50} for our anaerobic sludge is between 1 mg/L - 2 mg/L for ethanol, approximately 1 mg/L for acetate, and 0.4 mg/L - 1 mg/L for H_2/CO_2 assays, considering the MPR shown in Table 4.4.

Ethanol oxidation under anaerobic conditions has been observed in a few selected group of microorganisms and much is still left to be learned about the metabolic pathways and interactions in these partnerships (Sieber et al., 2012). Direct utilization of ethanol by methanogens is quite unusual, shown to occur only in *Methanogenium organophilum* (Metje & Frenzel, 2005), with syntrophic ethanol oxidation to acetate and H_2 being a more common process (Bernhard Schink, 1985b; Thiele & Zeikus, 1988). In anaerobic conditions, syntrophic oxidation to acetate and H_2 is an endergonic reaction (Table 4.3), but under low H_2 partial pressures, achieved by the presence of a H_2 scavenger methanogenic partner, the overall metabolic process becomes exergonic (Schink, 1997; Seitz & Schink, 1990; A. J. M.

Stams & Plugge, 2009). Hydrogenotrophic activity is thus vital for syntrophic ethanol oxidation. As previously mentioned (section 2.1.1), the first identified syntrophic partnership was the ethanol oxidation in the *Methanobacillus omelianskii* and *Methanobacterium bryantii* strain MoH co-culture. Eventually the culture was lost but other syntrophic ethanol oxidizing partnerships have been identified, *e.g. Pelobacter acetylenicus* and *Methanospirillum hungatei* or *Methanobacterium bryantii* (Schmidt et al., 2014; Seitz et al., 1990), *Pelobacter venetianus* and *Methanospirillum hungatei* (Bernhard Schink & Stieb, 2007), *Pelobacter carbinolicus* and *Methanospirillum hungatei* (Butler et al., 2009; Schmidt & et. al, 2014), and *Desulfovibrio gigas* and *Methanospirillum hungatei* (Kremer, Nienhuis-Kuiper, & Hansen, 1988), though in the latter significant growth was not reported for the fermenter partner. Ethanol syntrophic oxidation has also been reported with *Geobacter* and *Methanosaeta* species partnerships in brewery wastewaters, although direct interspecies electron transfer was required (Morita et al., 2011; Shrestha & Rotaru et al., 2014).

Increase in $\%O_2$ resulted in higher H_2 accumulation at 2.5% and 5% O_2, revealing a reduction of hydrogenotrophic activity which can affect the ethanol oxidation rate. Direct inhibition of the fermenting partner is also a strong possibility as most exhibit a strict anaerobic lifestyle, such as the genus *Pelobacter* (Schmidt & et. al, 2014). However, reported partial pressures were insufficient to justify a complete inhibition of hydrogenotrophic activity and ethanol syntrophic oxidation, since the accumulated H_2 was far below the predicted H_2 generation by ethanol oxidation, meaning that a substantial fraction of H_2 was consumed by the culture in both conditions, despite the presence of O_2. Additionally, higher thresholds for H_2 partial pressure build-up are to be expected for syntrophic ethanol oxidation as its lower free Gibbs energy ($\Delta G°$) makes it more thermodynamically favourable compared to other syntrophic oxidations (Table 4.3). For example, syntrophic ethanol oxidation by *P. acetylenicus* is possible under H_2 partial pressures as high as 3200 Pa (approximately 0.03 atm), 10^2 greater, on average, when compared to other syntrophic oxidations, such as those of propionate (10- 40 Pa) and butyrate (30-300 Pa) (Stams et al., 1994). Furthermore, in all conditions during P2, the initial ethanol uptake rate in the first 1-2 hours of incubation, is considerably higher than the remainder of the phase, as previously mentioned. This could be the result of combination of factors such as possible the initial accumulation of H_2 until a thermodynamic threshold is reached, the initial high O_2 diffusion to the media which hindered the culture syntrophic capability for the remainder of the incubation or the increased accumulation of acetate that lead to a pH decrease.

Syntrophic oxidation of ethanol is dependent on interspecies electron transfer (Stams et al., 1994), with both microorganisms usually being strict anaerobes. The increased O_2 uptake during ethanol

oxidation allowed for continued proliferation of methanogenic populations in the culture as the O_2 was being removed from the culture media at a sufficiently higher rate compared to the innate O_2 uptake capacity of the inoculum (blank assay O_2 uptake *i.e* background O_2 uptake). In the latter, MPR (methane production rate) for H_2/CO_2 and acetate assays (direct methanogenic substrates) presented significant decreases even at low percentages of O_2, resulting in substantial loss of methanogenic activity. Background O_2 uptake was insufficient to shield the methanogen populations in the highest $\%O_2$ (5%) and severely affected the methanogenic capacity of the cultures (approximate IC_{50}) in the remaining $\%O_2$, even though eventually a recovery was observed in some conditions. This is assuming that addition of direct methanogenic substrates (H_2/CO_2 and acetate) did not significantly stimulated O_2 uptake by the sludge, thus O_2 uptake in these assays should be identical to that of the blank assay. In a study with co-cultures of *M. omelianskii* and *M. bryantii* (Barker & Johns, 1960), ethanol oxidation was completely inhibited by the presence of O_2 in the headspace, meaning that the fermenting bacteria partner in syntrophic ethanol oxidation is not capable of shielding the methanogen and is directly affected, as previously mentioned. Therefore, the addition of ethanol most likely stimulated the activity of specific microbial populations capable of rapid consumption of dissolved O_2 in the media thus protecting the syntrophic populations.

The stoichiometric imbalances observed between the maximum predicted CH_4 production from H_2 generated through syntrophic ethanol oxidation and experimental CH_4 production at 2.5% and 5% O_2 (mainly at 5%) (Appendix IV), which lead to the possibility of ethanol oxidative fermentation. Since acetoclastic methanogens were completely inhibited during P2 at both these conditions, CH_4 produced can be attributed to hydrogenotrophic activity, indicating that ethanol oxidation may not have been entirely syntrophic, reducing the amount of H_2 generated and consequent hydrogenotrophic CH_4 production. The acetic acid bacteria (AAB) are obligate aerobic bacteria that naturally produce acetic acid from ethanol (Saichana et al., 2015). Several genera have been identified, the most common being *Acetobacter*, *Acidomonas*, *Gluconobacter*, and *Komagataeibacter* (formally known as *Gluconacetobacter*) (Gullo et al., 2014; Saichana et al., 2015; Yamada & Yukphan, 2008). They are known to possess a unique fermentation ability, oxidative fermentation. In this process an incomplete oxidation occurs where the ethanol is oxidized to acetaldehyde which is then converted to acetic acid, by membrane-bound dehydrogenases, and is then released to the surrounding environment. The dehydrogenases are strictly bound to the respiratory chain and the electrons generated by the reactions are transferred by an ubiquinone to O_2, which acts as the final electron acceptor (Gullo et al., 2014; Mamlouk & Gullo, 2013; Wang et al., 2015). For each mole of ethanol oxidized to acetate, one mole of O_2 is required.

In the present work, assuming a complete uptake of O_2 by AAB, the maximum ethanol oxidation would be 1.70 ± 0.11 mM and 2.87 ± 0.14 mM for 2.5% and 5% O_2, although due to background O_2 uptake (Figure 4.7), other microorganisms could be competing with AAB for the available O_2. Even though significant differences ($p < 0.05$) in MPR and ethanol uptake were observed only at these two conditions, it can be inferred that AAB activity contributed in shielding methanogenic communities, as O_2 uptake was substantially higher with the addition of ethanol and inhibition by O_2 exposure required increased $\%O_2$ compared to H_2/CO_2 and acetate assays. Some genera of AAB, namely *Acetobacter* and *Komagataeibacter*, present a diauxic growth when incubated with ethanol. This is due to an overoxidation phenomenon that leads to oxidation of the accumulated acetate to CO_2 and H_2O once other carbon sources (namely ethanol) have been depleted (Akihiko, 1997; Gullo et al., 2014; Sakurai et al., 2012). This acetate oxidation phase was not reported for the acetate assays of this work at higher $\%O_2$, as no significant acetate uptake was observed throughout P2 at 5% O_2 and in the 2.5% O_2 the acetate was oxidized mostly to CH_4. However, the lag phase until acetate oxidation phase is observed quite is extensive, especially when grown directly on acetate (120-170 hours) but substantially reduced when ethanol is introduced (Akihiko, 1997). Furthermore, most these studies utilize steady aeration rates, unlike those of the present work where the O_2 decreases rapidly after the O_2 pulse is performed, thus it is unlikely that acetate oxidation was a viable metabolic pathway in the conditions set for the acetate assays.

4.2.2 Effects of microaeration on hexadecane conversion to methane

In this assay, an attempt was made to reduce the activation time of hexadecane by introducing small amounts of O_2 to the cultures and stimulating facultative anaerobe populations. By taking advantage of their fast and effective aerobic activation mechanisms, the products of hexadecane activation would then be directed towards further degradation using anaerobic pathways, leading to the production of CH_4. Hexadecane-amended cultures were prepared with a mixture of two different sludges as inoculum. To avoid any potential inhibitory effects, a concentration of 1 mM hexadecane was chosen, based on the results from section 4.1. CH_4 production was monitored for 203 days incubation at increasing $\%O_2$ (Figure 4.8).

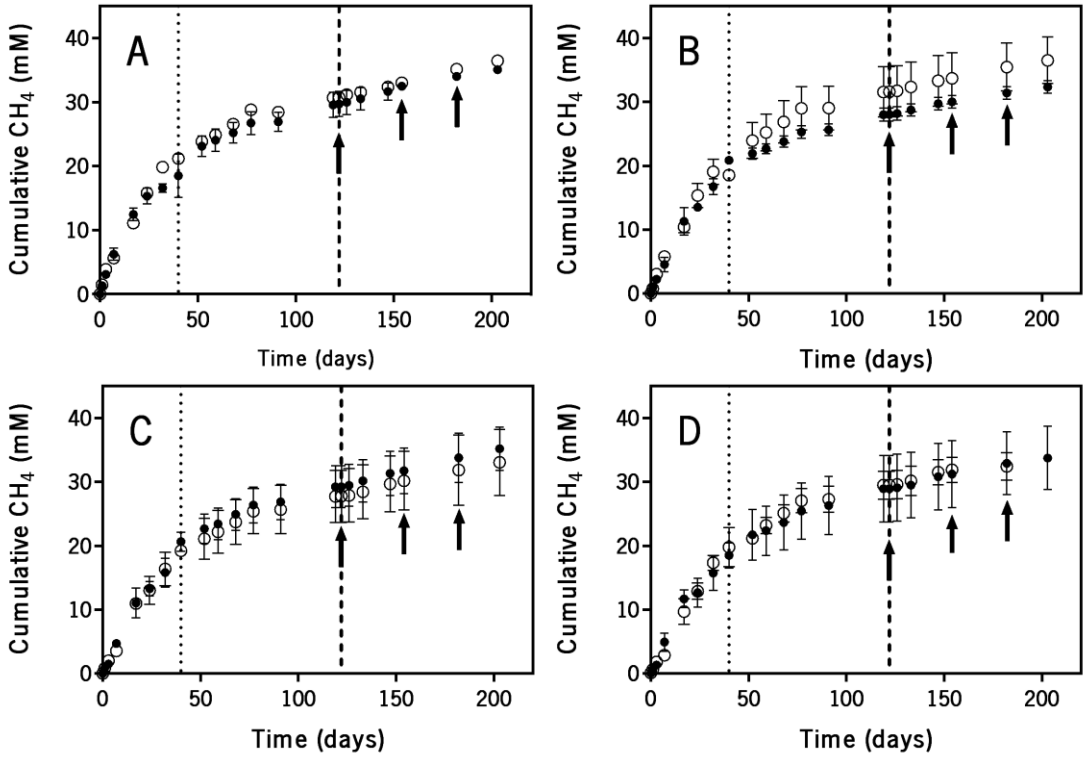

Figure 4.8 – CH_4 production in hexadecane-amended cultures at increasing $\%O_2$. Hexadecane was added at 0 days at a concentration of 1 mM in all conditions, along with the first O_2 addition: A – 0% O_2, B – 2.5% O_2, C – 5% O_2 and D – 21% O_2. CH_4 production in hexadecane amended cultures (●) and respective blanks (○) are shown. Dotted line (·) at 40 days of incubation marks the beginning of a deceleration; dashed line (-) at 122 days of incubation indicate the first periodic renewal of headspace and O_2 pulses, represented as (↑), with 2 more renewals at 154 and 182 days of incubation. The data points are shown as the mean of duplicates (blank) and triplicates (hexadecane amended) ± standard deviation.

The results show that the blank assays achieved a similar CH_4 production to the respective hexadecane-amended assays, with no significant differences ($p > 0.05$) being observable between these cultures. This high methane production in the blanks was not expected and highlighted the presence of important amounts of residual substrate in the inoculum. Considering the results obtained in section 4.2.1, this residual amount was possibly associated with the sludge collected from the underground water treatment plant.

Based on the theoretical stoichiometric equation for the complete syntrophic conversion of hexadecane to CH_4 (Table 4.3) (Siddique et al, 2011), 1 mM of hexadecane should be converted to 12.25 mM CH_4. The similarity in the total cumulative CH_4 produced by the hexadecane amended cultures and respective blanks suggests that the added hexadecane has most likely not degraded at this stage of incubation. A recent study faced similar issues regarding residual substrate (Fowler & Gieg, 2016) – in hexadecane amended cultures, following a long lag phase, an exponential increase in CH_4 production was observed. However, none of the replicas showed methane production above that of the non-amended control. Therefore, it is important to guarantee that the inoculum used for experimental procedures has exhausted its original substrate.

Addition of O_2 at 0 days of incubation didn't show a significant effect on hexadecane biodegradation to methane, as seen by the CH_4 production and, surprisingly, even in the 21% O_2 condition CH_4 production was similar to that of the controls (0% O_2). Hydrocarbons are particularly recalcitrant compounds, so their biodegradation is substantially more difficult compared to other substrates. More than 300 days were reported before for the onset of methane production from hexadecane by enriched cultures in batch incubations (Zengler et al., 1999; Embree et al., 2014). Thus, the incubation time in this experiment is still not sufficient to allow the withdrawal of conclusion. Nevertheless, as an attempt to accelerate this biological process, after 122 days of incubation three additional O_2 pulses were performed monthly at 122 days, 154 days and 182 days of incubation, and the O_2 was measured weekly between 122 days and 154 days of incubation to follow O_2 evolution (**Table 4.8**) No significant changes ($P > 0.05$) in CH_4 production were observed between different $\%O_2$ conditions.

Table 4.8 – Periodic GC measurements for O_2 evolution monitoring in hexadecane-amended cultures after initial residual substrate exhaustion. O_2 percentages and concentration (mM) are relative to the headspace.

Substrate	O_2 (%)	Measured O_2 (%)			Measured O_2 (mM)		
		[a]122 days	[b]126 days	[c]154 days	[a]122 days	[b]126 days	[c]154 days
Blank (no added substrate)	0	-	-	-	-	-	-
	2.5	2.3 ± 0.3	0.1 ± 0.5	3.0 ± 0.7	1.04 ± 0.22	0.15 ± 0.04	2.09 ± 0.17
	5	4.2 ± 0.3	0.6 ± 0.0	5.7 ± 0.7	1.90 ± 0.14	0.09 ± 0.07	2.95 ± 0.04
	21	11.9 ± 1.3	0.3 ± 0.6	19.3 ± 0.2	5.42 ± 0.91	0.04 ±0.04	9.49 ± 0.12
Hexadecane	0	-	-	-	-	-	-
	2.5	2.1 ± 0.2	0.3 ± 0.2	3.4 ± 0.4	0.94 ± 0.12	0.14 ± 0.15	1.34 ± 0.07
	5	4.2 ± 0.2	0.3 ± 0.3	6.1 ± 0.7	1.89 ± 0.11	0.01 ± 0.04	2.53 ± 0.35
	21	12.2 ± 3.5	0.0 ± 0.3	19.9 ± 1.0	6.76 ± 1.07	0.07 ± 0.08	8.43 ± 0.40

([a]) First pulse, t = 0; ([b]) First pulse, t = 4 days; ([c]) Third pulse, t = 0.

The results show that O_2 was detected in residual amounts after four days of incubation, following the O_2 pulse, in all conditions. GC analysis also shows that the $\%O_2$ in the 21% conditions the headspace didn't reach 21%, however it was considered representative of an aerobic environment for the purposes of this work, as 10–15% O_2 in the headspace results in initial dissolved O_2 concentrations between approximately 4.0-5.5 mg/L O_2, as determined through Henry's Law. For comparison, the O_2 concentrations obtained in 2.5% and 5% O_2 were approximately 1 mg/L and 2 mg/L, respectively, and as discussed previously in section 4.2.1, these concentrations are considered viable for aerobic growth.

Comparative studies were not found on the literature regarding the effects of O_2 addition on n-alkane methanogenic activation/biodegradation. Exposure of the anaerobic sludge to microaeration conditions appears to have no significant effect on the methanogenic biodegradation of hexadecane in batch assays. Nevertheless, the proposed hypothesis assumed a supply of trace amounts of O_2, to trigger hydrocarbons activation without significant deviation of carbon from the methanogenic route, which is difficult to achieve through periodic pulses in batch conditions. The O_2 amounts were probably too high for the proposed objective, thus favoring aerobic biodegradation.

The O_2 uptake in the assays with H_2/CO_2 and acetate (direct substrates for the methanogens), described in section 4.2.1.3 (Table 4.7), shows that O_2 is readly consumed, almost completely, towards the end of the incubation. Additionally, blank assays show similar results (Figure 4.7), with total O_2 uptake on the 0.5%, and 1% O_2 conditions after 5 hours of incubation, and 2.5% and 5% between 7-24 hours of incubation. Since acetate and H_2/CO_2 are direct methanogenic substrates, the O_2 uptake is likely similar

to that of unamended cultures. This showed that even when the cultures were incubated in optimal conditions with no added substrate they still presented substantial O_2 uptake, revealing significant O_2 scanvanging likely related to background bacterial activity (such as facultative anaerobes). As the inoculum used for the hexadecane assay was composed of 3 out of 4 parts of brewery sludge (used in 4.2.1 assays), we can deduce that similar effects occured during the hexadecane assays. As only one O_2 pulse was performed in the initial stages of incubation (0-122 days) at 0 days of incubation, O_2 was probably consumed after the first day of incubation, resulting in a shift to anaerobic conditions until the second O_2 addition at 122 days of incubation. This can only be inferred for the 2.5% and 5% O_2 conditions as 21% O_2 was not tested in H_2/CO_2 and acetate assays, although O_2 measurements performed to the hexadecane assays at 122 days of incubation detected residual amounts of O_2 in the headspace 4 days after O_2 pulse in all conditions, 2.5%, 5% and 21% O_2.

The cultures were thus subjected to "spikes" of O_2 for a short period (less than 24 hours for 2.5% and 5% O_2, over 24 hours for 21% O_2), much lower than the total incubation period before subsequent O_2 additions (mimimum 28 days period after the second addition at 122 days). This explains why even at 21% O_2 we observe similar CH_4 production profiles compared to the controls instead of aerobic degradation of residual substrates since the aerobic conditions achieved at the moment of the O_2 pulse likely shifted to anaerobic/microaerophilic in a matter of days. On a time microscale, aerobic/microaeration conditions may have been present, but on a macroscale the assay was conducted mostly in anaerobic conditions.

Alkanes comprise an abundant fraction of many crude oils and are able to produce higher CH_4 production rates compared to other types of hydrocarbons (Berdugo-Clavijo & Gieg, 2014b; Jiménez et al., 2016) thus their biodegradation under anaerobic conditions is of practical relevance to biotechnological applications in hydrocarbon-contaminated sites. There remains much to be learned regarding the interactions amongst organisms that methanogenically metabolize hydrocarbons. Long lag phases are typically observed in methanogenic hexadecane degradation assays. These lag phases range from 100-150 days (Berdugo-Clavijo & Gieg, 2014b; L. Y. Wang et al., 2012), to 150-250 days (Fowler & Gieg, 2016), 200-250 days (Siddique et al., 2011). Complex hydrocarbon mixtures such as crude oil or simpler artificial alkane mixtures also present extended lag phases (Siddique et al., 2011; Townsend et al, 2003) and curiously lag-phases are shorter. An argument could be made that using a specific hydrocarbon for growth may exert a selective pressure that ultimately hinders the growth capability of the hydrocarbon syntrophic consortia. In these studies, a steep increase in CH_4 production is observed when

hexadecane/hydrocarbons uptake begins (including blank controls with residual substrate) which was not yet observed in this experiment.

4.3 Effects of microaeration on the conversion of hexadecane to methane in bioreactors

Three reactors were operated at different aeration conditions to assess the influence of O_2 in hexadecane activation time and biodegradation rate: reactor O2C was operated with continuous supply of trace amounts of dissolved O_2, reactor O2P with periodic air pulses and reactor AnR in strict anaerobic conditions. Measured specific methanogenic activity shows low specific acetoclastic methanogenic activity compared to the hydrogenotrophic methanogenic activity, 27 ± 1 $mLCH_{4STP}$/gVS.day and 792 ± 8 $mLCH_{4STP}$/gVS.day, respectively. During the operation, several experimental problems could be identified, and the system was improved in accordance. Considering these upgrades and periodic changes to the operating conditions, 7 different stages were defined, as shown in Table 4.9.

Table 4.9 – Stage delineation from 70 days of operation of the three reactors at different aeration conditions, continuous supply of dissolved O_2 (O2C), periodic air pulses (O2P) and anaerobic conditions (AnR).

Stage	Operation time (days)	Description	Observations
I	0-14	Operation in continuous mode with yeast extract (YE) as the sole carbon source	-
II	14-21	First hexadecane pulse (1,3 mL - 0.5 mg/L) and start of O2 pulses in O2P (20 mL daily)	t = 17 days - No bicarbonate added on subsequent feed solutions
III	21-27	Batch mode with inversion of the recycle/feed flow (up-flow to down-flow) and YE pulse	
IV	27-41	Continuous mode with inversion and temporary settlers	
V	41-45	Second hexadecane pulse with daily air pulses in O2P (20 mL) and no YE on the feed	-
VI	45-52	Batch mode and assembly of new settlers	t = 48 days - Recovery of all sludge trapped in the settlers for new settler implementation
VII	52-70	Third hexadecane pulse and operation in continuous mode	t = 52 days – increase of the HRT to 10 days (0.1 mL/min feed flow rate)

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Figure 4.9 shows the cumulative CH_4 evolution and CH_4 percentage variations (%CH_4) for approximately 70 days of operation, after a two week period in batch for residual substrate exhaustion.

After this initial batch period, pH and ORP in the three reactors presented values around 8-8.5 and -250 to -300 mV, respectively, and no substantial amounts of VFA were detected by the start of the operation in continuous mode (0 days operation).

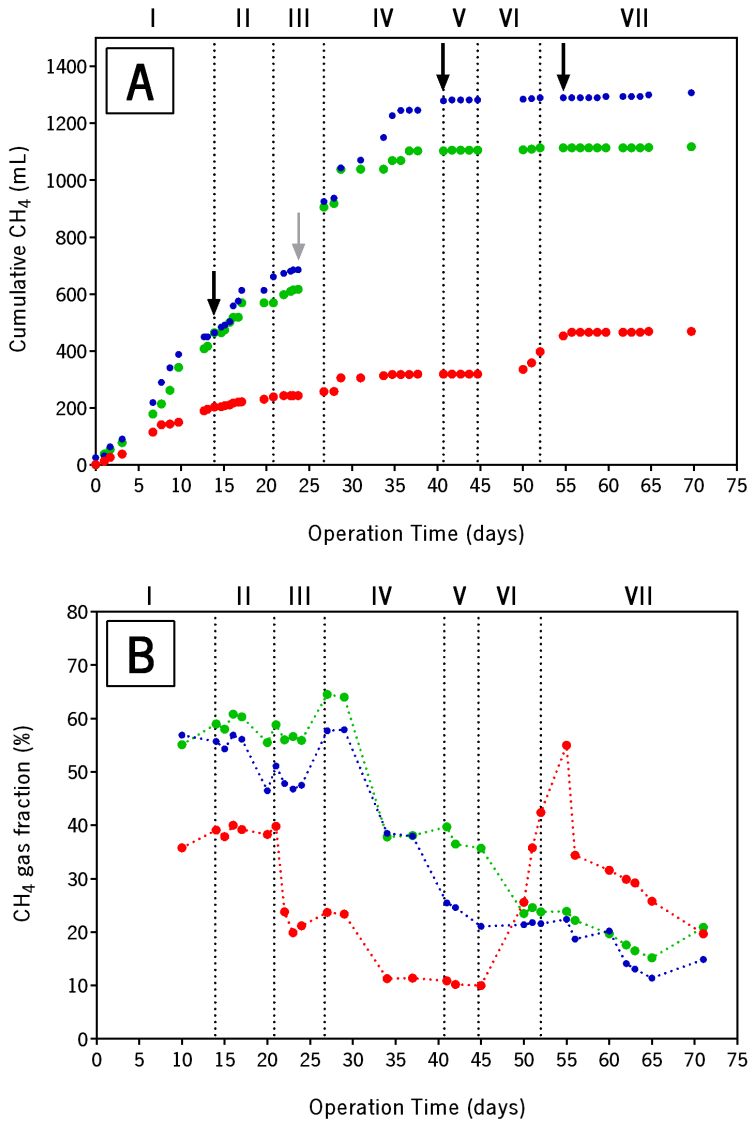

Figure 4.9 – Cumulative CH_4 production (A) and %CH_4 in the biogas (B) in the three reactors at different aeration conditions: O2C (●), O2P (●) and AnR (●), during 70 days of operation. Different operation phases are highlighted according to significant changes in the operating conditions: **I** – Operation in continuous mode with yeast extract (YE) as the sole carbon source; **II** –First hexadecane pulse (↓) (1.3 mL - 0.5 mg/L) and start of O_2 pulses in O2P (20 mL daily); **III** – Batch mode with inversion of the recycle/feed flow (up-flow to down-flow) and YE pulse (↓); **IV** – Continuous mode and temporary settlers; **V** – Second hexadecane pulse with daily air pulses in O2P (20 mL) and no YE on the feed; **VI** – Batch mode and assembly of new settlers; **VII** – Third hexadecane pulse and operation in continuous mode.

During stage I, the biogas production was steady for all reactors, with no significant operation issues observed. Reactors O2P and AnR presented the highest CH_4 production and $\%CH_4$ (only measured by day 10 of operation), with 57% and 55% of the total biogas produced, respectively. Reactor O2C presented the lowest $\%CH_4$, with 36% by the start of the measurements. Given that the feed was maintained at aerobic conditions, with dissolved O_2 exceeding 6 mg/L in the media and ORP of +100 mV, and that during this period yeast extract was the only carbon source, this decrease of CH_4 yield was expected. In stage II, upon hexadecane pulse, the hexadecane was seen traveling to the surface, which raised concerns regarding substrate/biomass contact as the sludge blanket was at the bottom of the reactors. The biogas continued to accumulate, although the reactors volume was not entirely stable, especially reactor O2P and AnR. A decision was then made to reverse the recycle flow downwards in an attempt to increase contact between the biomass and hexadecane.

In stage III, the biogas production stagnated and decreases in $\%CH_4$ were observed for in all reactors, particularly reactor O2C, followed by a recovery of the $\%CH_4$ upon yeast extract addition in reactors O2P and AnR but not a significant one in O2C. At this stage, problems associated with pressure drops in the gas line (reactor O2C) and reactor working volume were observed (reactors O2P and AnR) which interferes with accurate readings of biogas produced. During stage IV and onward in the operation, problems associated with pressure drops, working volume variations, gas leaks from the settlers (that exacerbate pressure variations in the gas line) contributed to a constant instability of the operation. The accumulated volume registered by the gas counters was mostly derived from working volume variations (up to 300 mL in some instances), and a decrease in biogenic CH_4 production is shown in the $\%CH_4$, with a continuous decrease overtime. Reactors O2C and O2P were the most affected.

Beyond stage V no yeast extract was added. Once the yeast extract was removed from the feed, no substantial biogas accumulation was detected in reactors O2P and AnR until the end of the operation. A significant increase in biogas production and $\%CH_4$ was observed for reactor O2C during VI while in batch mode, however once the operation switched to continuous mode (stage VII), a stagnation of biogas production and decrease of $\%CH_4$ was followed. Small variations on the reactors work volume and constant pressure drops in the gas lines, particularly reactor O2C, didn't allow for a continuous stable operation.

The pH, ORP and COD monitoring is depicted in Figure 4.10. A clear decrease in pH is observed overtime after bicarbonate was removed from the feed solutions at stage II. ORP levels increased overtime, being notoriously unstable after inversion of the recycle flow though eventually stabilized close to the pre-inversion levels after the reactors had time to adapt to the new conditions. The ORP was lower,

in general, in reactor O2C, followed by O2P and AnR. Still, the ORP values are quite similar despite the different aeration conditions imposed on the reactors. The decrease in the COD removal efficiency observed in stage IV can be explained by the yeast extract pulse applied during batch mode in stage III. %COD removal was higher on reactors O2P and AnR compared to reactor O2C, with feed solutions with and without added yeast extract.

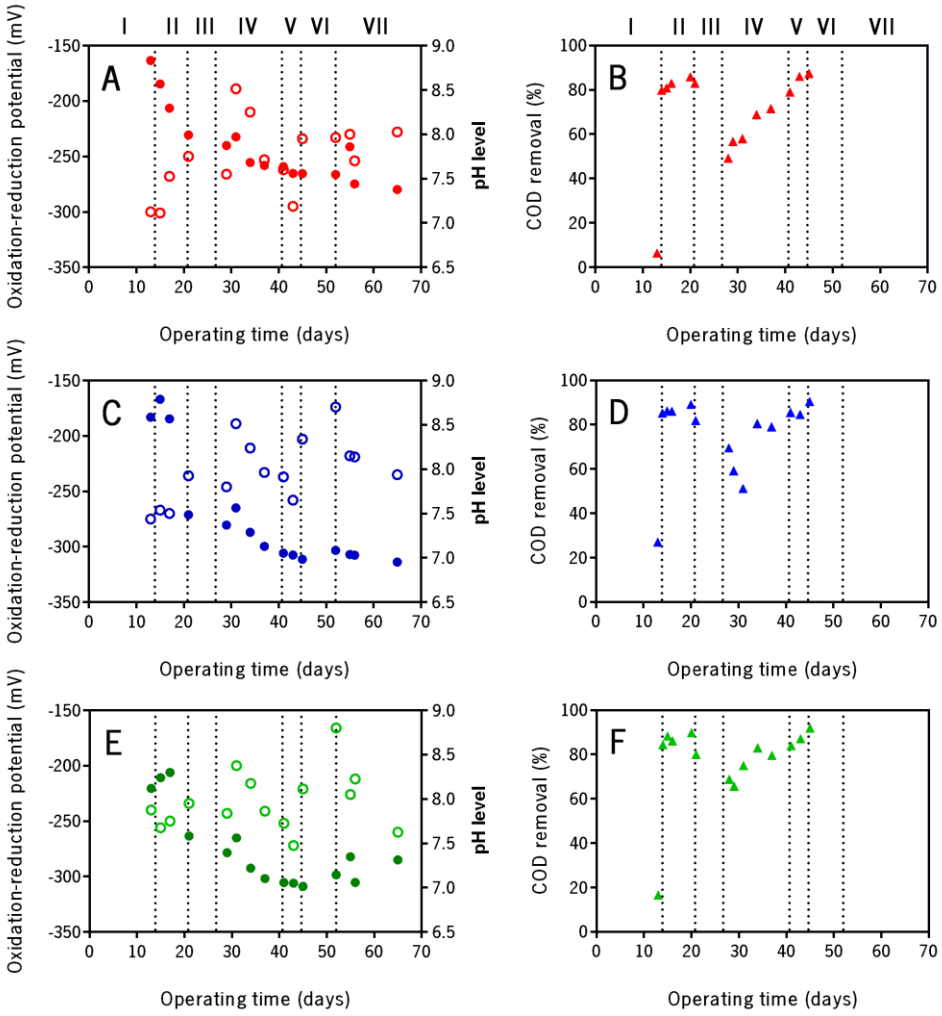

Figure 4.10 – Monitoring of pH (●), ORP (○) and soluble COD (▲) in the three reactors at different aeration conditions: O2C (A, B), O2P (C, D) and AnR (E, F), during 70 days of operation. Different operation phases are highlighted according to significant changes in the operating conditions: I – Operation in continuous mode with yeast extract (YE, 0.5 gCOD/L) as the sole carbon source; II –First hexadecane pulse (1.3 mL - 0.5 mg/L) and start of O2 pulses in O2P (20 mL daily); III – Batch mode with inversion of the recycle/feed flow (up-flow to down-flow); IV – Continuous mode and temporary settlers; V – Second hexadecane pulse with daily air pulses in O2P (20 mL) and no YE on the feed; VI –Batch mode and assembly of new settlers; VII – Third hexadecane pulse and operation in continuous mode.

VS analysis (Figure 4.11) shows a loss of 0.65 ± 0.08 g/L, 0.55 ± 0.08 g/L and 0.51 ± 0.02 g/L for reactors O2C, O2P and AnR, respectively, at 13 days of continuous mode operation. This VS loss was even increased once the recycle flow was inverted, due to the higher degree of mixing thus achieved, to 1.47 ± 0.02 g/L, 2.55 ± 0.09 g/L and, 24 ± 0.03 g/L for reactors O2C, O2P and AnR, respectively, at 32 days of operation. Subsequent analysis maintained high VS loss values at 34 and 38 days of operation, 1.66 ± 0.02 g/L, 1.36 ± 0.00 g/L, 1,61 ± 0.07 g/L and 0.97 ± 0.05 g/L, 1.93 ± 0.02 g/L, 111 ± 0.04 g/L, for reactors O2C, O2P and AnR, respectively.

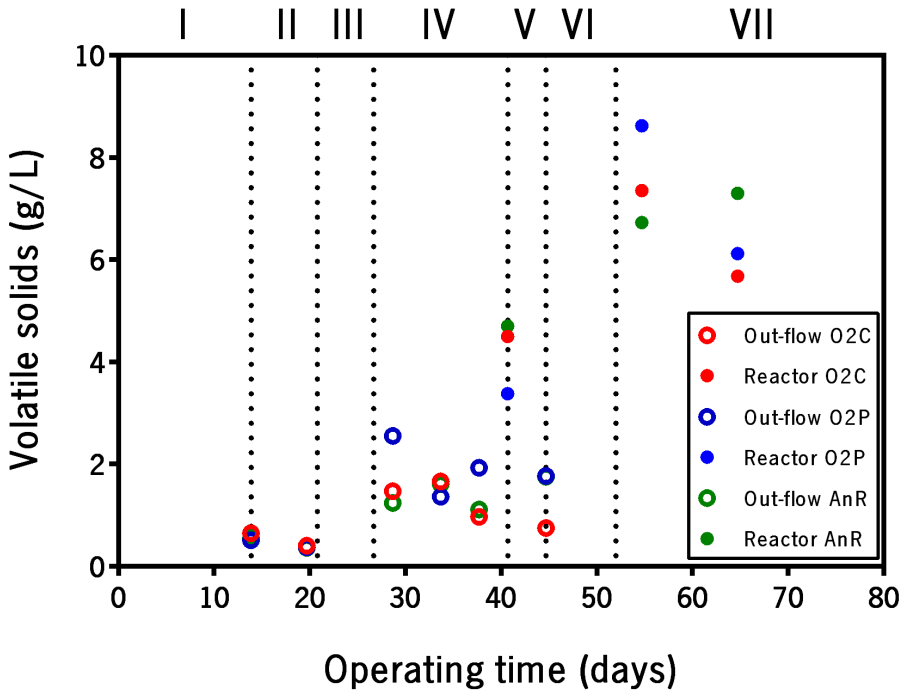

Figure 4.11 – Volatile solid analysis from the outlet and inside the reactors. The values from the outlet were obtained when the feed solution/effluent bottles were renewed (3-4 days periods).

The recovery system set to reintroduce the reactor content trapped in the temporary settlers, although necessary, was not as effective in retaining sludge content as expected, and a considerable amount of VS was lost during its use. The reactor VS content was substantial lower compared to the initial 15 g/L, 4.83 ± 1.29 g/L, 3.38 ± 0.10 g/L and 4.7 ± 0.19 g/L for reactors O2C, O2P and AnR, respectively. Once the temporary settlers were replaced with new settlers in stage VI, and all the sludge was reintroduced into the reactors, the VS content analysis during stage VII revealed extensive permanent loss in all reactors, with total VS reaching around 50% of the initial VS value, *i.e.* 7.35 ± 0.25 g/L, 8.62 ± 0.05 g/L, and 6.73 ± 0.37 g/L for reactors O2C, O2P and AnR, respectively.

The HPLC analysis showed some residual VFA accumulation during operation, particularly after recycle/feed inversion. The only significant VFA accumulation occurred at the end of the assay, with 81.91 mg/L and 533.88 mg/L butyrate accumulation in reactors O2C and AnR, respectively, which correlates well with the increase in soluble COD measured at the end of stage VII, 98,4 mgCOD/L and 340 mgCOD/L, respectively.

The adaptation of anaerobic biomass to specific conditions and maintenance of a stable operation are key points for the success of a continuous reactor operation. Therefore, the reactor's design and operation should be carefully planned and further validated with preliminary assays, that will allow the identification of unforeseen operation problems and underpin the application of correction measures. Here, the instability of the operation in continuous mode prevented a confident assessment of the research hypothesis. Nevertheless, several operational problems were detected, and the system was changed in accordance. A downflow operation was adopted to improve the sludge/hexadecane contact but this approach induced a high VS loss. Although settlers were prepared and installed, a significant sludge loss occurred that may have compromised this experiment. Recycling of the settled sludge was performed once or twice a day for a specific time period. Thus, continuous recycling of the settled sludge may potentially contribute to improve the performance of these systems. Moreover, several pressure drops were reported during continuous operation on all three conditions, especially after the recycle inversion in stage III (Table 4.9), generally associated with variations of the reactors working volume. Even with supposed increases in biogas production, the $\%CH_4$ in the biogas decreased continuously until the end of the operation. Besides the aforementioned variations of volume which can lead to apparent increases in biogas production, receding oil from the gas sensors due to pressure variations had to be recovered which meant opening the gas line, leading to disruption of the difference gas % that compose the biogas. Moreover, the %COD removal indicates biodegradation thus biogas production is occurring during these stages. However, it is difficult to assess whether aerobic or anaerobic biodegradation is the most prevalent. Once yeast extract was removed from the feed solutions, no considerable biogas production was detected, as small volume variations lead to constant pressure drops that prevent biogas to be measured in the sensors. The only exception to this trend was observed in reactor O2C during stage VI, when the reactors were temporarily operated in batch mode, with reactors O2P and AnR showing small increases quickly countered by pressure drops. Biogas production increased considerably as well as the $\%CH_4$, reaching higher yields compared to those obtained in stage I-II, peaking during continuous mode in stage VII. This shows that anaerobic biodegradation is present in the reactors, but the instability of the continuous operation mode is affecting biogas monitoring. During stage VII, in general, biogas

production was negligible. This is possibly related with the fact that hexadecane is a much more recalcitrant substrate compared to yeast extract so pressure build-up from biogas production was likely considerably lower, which may further exacerbate the negative effects of operation instability.

In anaerobic reactors, around 70% of all CH4 produced derives from acetoclastic methanogens, while the other 30% from hydrogenotrophs (Demirel et al., 2014; Jain et al., 2015). The recycle inversion appears to have resulted in considerable fluctuations of ORP, although these are most likely related to the increased biological activity, as shown by the increasing %COD removal in the reactor, after the addition of a high concentration yeast extract pulse during stage III. Curiously, the reactor with the lowest overall ORP was reactor O2C. This suggests that the addition of trace amounts of dissolved O_2 stimulates the growth of facultative anaerobes which kept the O_2 and ORP much lower compared to the anaerobic (AnR) or air pulse (O2P) reactors.

5. Conclusions and Future perspectives

The objective of the work carried out during the present dissertation was to enhance the biodegradation of hexadecane to methane by means of microaeration. Several experiments were designed to study the effects of hexadecane and oxygen exposure on the methanogenic and syntrophic microbial communities. The main conclusions of this work are as follow:

1. The first task shed light on the toxicity of hexadecane to hydrogenotrophic methanogenic archaeon. The results showed that *Methanobacterium formicicum* is substantially more sensitive to hexadecane than *Methanospirillum hungatei*. A hexadecane IC_{50} was reached between 5 mM and 15 mM of hexadecane for *M. formicicum* while in *M. hungatei* the IC_{50} was not reached, even after exposure to 30 mM hexadecane, with only a 27 ± 3 % decrease in methane production rate observed at this concentration.

2. In the second task, the effects of oxygen exposure were studied on anaerobic sludge when amended with direct methanogenic substrates, H_2/CO_2 and acetate, and more complex substrates, ethanol and hexadecane. The results indicate that ethanol addition may have stimulated the diversity of microbial communities in the culture and provided increased shielding against oxygen exposure to methanogenic populations, as shown by the overall methane production rate in all tested conditions, particularly the methane production rate at the highest O_2 concentration (5% O_2 headspace), *i.e.* 0.22 ± 0.05 mM/h (ethanol), contrary to the complete inhibition in H_2/CO_2 and acetate, 0.02 ± 0.01 mM/h and 0.01 ± 0.00 mM/h, respectively. Stoichiometric imbalances between predicted H_2 production and experimental methane production revealed possible sharing of ethanol substrate by two distinct microbial populations, syntrophic and oxidative fermenting bacteria. Microaeration conditions applied in batch did not have a significant effect on methane production and hexadecane biodegradation was not evident, although these conclusions require further validation by extended cultures incubation.

3. Three bioreactors were operated, under different aeration conditions: anaerobic (AnR), anaerobic with periodic air pulses (O2P) and continuous supply of dissolved oxygen (O2C). Hexadecane biodegradation could not be confirmed due to instability of the operation, but several experimental problems were identified. Limited hexadecane/sludge contact promoted the transitioning of the operation set-up from upflow to downflow. The low efficiency of the sludge settling, and recirculation led to great loss of reactor biomass content, which combined with multiple pressure

drops and volume variations during the 70 days of operation, prevented a sufficiently stable process to be performed. The O2C reactor showed higher capacity for O_2 scavenging.

For future work, studies on hexadecane effects on the membrane should be considered to assess the mechanisms by which hexadecane affects the membrane integrity and transmembrane protein function. Microscopy-based studies to visualize the formation of microdroplets and how their interaction with the microorganisms occurs, as well as fluorescence and biophysical membrane studies are some examples of possible additional work. Furthermore, similar studies to the ones performed in this work with hydrogenotrophs, should be considered for acetoclastic methanogens given their importance in methane production from anaerobic digestion processes (70% of total methane produced) and the difference in the physical state of their substrate, acetate, relative to hydrogenotrophs (hydrogen), which could introduce other constrains in term of hexadecane toxicity such as substrate mass transfer. Given the potential influence of the growth morphology (*e.g.* filamentous growth), a wide variety of methanogens should also be considered.

Other acidogenesis products, such as butyrate and propionate should be considered for future work regarding oxygen exposure to anaerobic sludges. Perhaps experimenting with continuous supply of trace oxygen amounts, rather than a single pulse could be interesting to analyze both the effects and possible adaptability of the microbial communities to oxygen exposure. In the hexadecane-amended batch assays, a pre-incubation should be considered to remove any traces of residual substrate and perhaps even acclimate the cultures with downstream products such as LCFA before transitioning to hydrocarbon. Mixtures of different n-alkanes could be tested to avoid any selective pressure imposed by hexadecane and to more closely resemble a real petroleum-contaminated water, while still allowing a degree of control to be maintained regarding n-alkane concentrations. Alternatively, if the batch setup is to be maintained in hexadecane-amended cultures, the air pulses should be introduced more frequently and in lower volumes to attempt to create a continuous microaeration environment. Microbial biology techniques, such as 16S RNA analysis, could be employed to monitor the evolution of the microbial communities at the set conditions.

The bioreactors should first and foremost be adapted for the degradation of hexadecane, taking into account its low solubility. The reactor and the entire operation apparatus should be carefully design beforehand taking into account two aspects: a near-perfect mixture of the reactor content is most likely to be employed and excessive loss of reactor content should be avoided. For this, settlers should be set with a recovery system which operates at similar flow-rate to the feed flow to avoid accumulation and loss

of reactor content. Another idea would be to attempt to solubilize the hexadecane/n-alkane mixture with more soluble hydrocarbons, such as BTEX or other volatile compounds (short-chain n-alkanes). This mixture could then be fed to the reactor with an USAB set-up, minimizing the loss of reactor sludge content. Alternatively, microscopic solid sorbents, such as cork or micro-scaled polymers, could be introduced to function as hexadecane/n-alkane traps and scaffolds for microbial communities to grow. The hydrocarbon/support system could be pre-prepared and introduced continuously through the feed, which would closely resemble an industrial-scale operation setup. The mechanisms by which pulses of oxygen are introduced should also be reworked by introducing a dispersion system for higher contact between the oxygen and the sludge.

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APPENDIX I – pH, ORP AND VS RESULTS

A – Effects of hexadecane exposure to pure methanogenic cultures

Table I-1 – pH and OPR measurements of *Methanospirillum hungatei* and *Methanobacterium formicicum* at increasing hexadecane concentrations. The values represent the mean of triplicates ± standard deviation. ORP – oxidation-reduction potential.

Methanogen	Hexadecane (mM)	Start of the batch assay		End of the batch assay	
		pH	ORP	pH	ORP
M. hungatei	0	7.11 ± 0.07	-288 ± 18	7.66 ± 0.17	-236 ± 10
	1	7.10 ± 0.05	-278 ± 5	7.61 ± 0.08	-209 ± 29
	5	7.11 ± 0.03	-283 ± 10	7.65 ± 0.03	-233 ± 8
	15	7.12 ± 0.01	-277 ± 5	7.64 ± 0.05	-234 ± 8
	30	7.11 ± 0.00	-280 ± 6	7.62 ± 0.09	-228 ± 7
M. formicicum	0	7.17 ± 0.02	-266 ± 2	8.05 ± 0.06	-264 ± 5
	1	7.18 ± 0.02	-270 ± 3	8.07 ± 0.01	-264 ± 8
	5	7.20 ± 0.01	-274 ± 6	8.06 ± 0.01	-269 ± 4
	15	7.18 ± 0.01	-274 ± 9	8.00 ± 0.03	-275 ± 4
	30	7.15 ± 0.01	-273 ± 5	8.07 ± 0.01	-276 ± 5

B - Effects of microaerobic conditions on the bioconversion of ethanol and anaerobic metabolic intermediaries to methane

Table I-2 – pH, OPR and VS measurements of unamended (blank) and H_2/CO_2, acetate and ethanol amended anaerobic sludge at increasing headspace $\%O_2$. The values represent the mean of triplicates ± standard deviation. ORP – oxidation-reduction potential; VS – volatile solids.

Substrate	O_2%	pH	ORP (mV)	VS (g/L)
H_2/CO_2	0	7.97 ± 0.04	-222 ± 11	3.86 ± 0.04
	0.5	7.93 ± 0.03	-237 ± 4	3.89 ± 0.06
	1	7.82 ± 0.01	-233 ± 2	3.88 ± 0.05
	2.5	7.61 ± 0.03	-221 ± 11	4.11 ± 0.13
	5	7.50 ± 0.02	-247 ± 2	4.25 ± 0.10
Acetate	0	7.68 ± 0.03	-169 ± 19	3.84 ± 0.06
	0.5	7.61 ± 0.00	-203 ± 4	3.95 ± 0.18
	1	7.57 ± 0.00	-213 ± 6	3.77 ± 0.03
	2.5	7.58 ± 0.01	-195 ± 6	3.90 ± 0.06
	5	7.46 ± 0.01	-214 ± 15	4.39 ± 0.02
Ethanol	0	5.66 ± 0.12	-166 ± 1	n.d.
	0.5	5.70 ± 0.02	-175 ± 7	n.d.
	1	5.86 ± 0.04	-185 ± 2	n.d.
	2.5	5.87 ± 0.02	-154 ± 5	n.d.
	5	6.47 ± 0.02	-212 ± 28	n.d.
Blank	0	6.96 ± 0.04	-259 ± 14	4.10 ± 0.12
	0.5	7.01 ± 0.02	-251 ± 12	4.04 ± 0.35
	1	7.03 ± 0.01	-267 ± 9	3.95 ± 0.07
	2.5	7.04 ± 0.01	-263 ± 6	4.06 ± 0.17
	5	7.06 ± 0.01	-240 ± 7	4.20 ± 0.08

n.d. – not determined.

APPENDIX II – SPECIFIC METHANOGENIC ACTIVITY (SMA) FOR ETHANOL

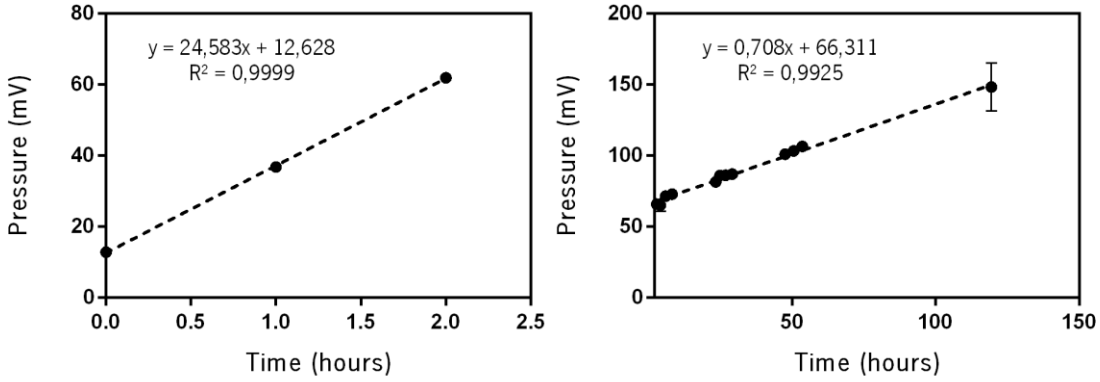

Figure II-1 – Pressure monitoring during specific methanogenic activity tests with ethanol as substrate; left – pressure build-up in the first 2 hours of incubation; right – pressure build-up for the remainder of the test. Data points are presented as mean of triplicates ± standard deviation. Linear regression and R^2 value are shown in each individual graph. Biogas accumulation (pressure) is indicated by the slope values (mV/h).

APPENDIX III – METHANE PRODUCTION RATE: BLANK ASSAYS

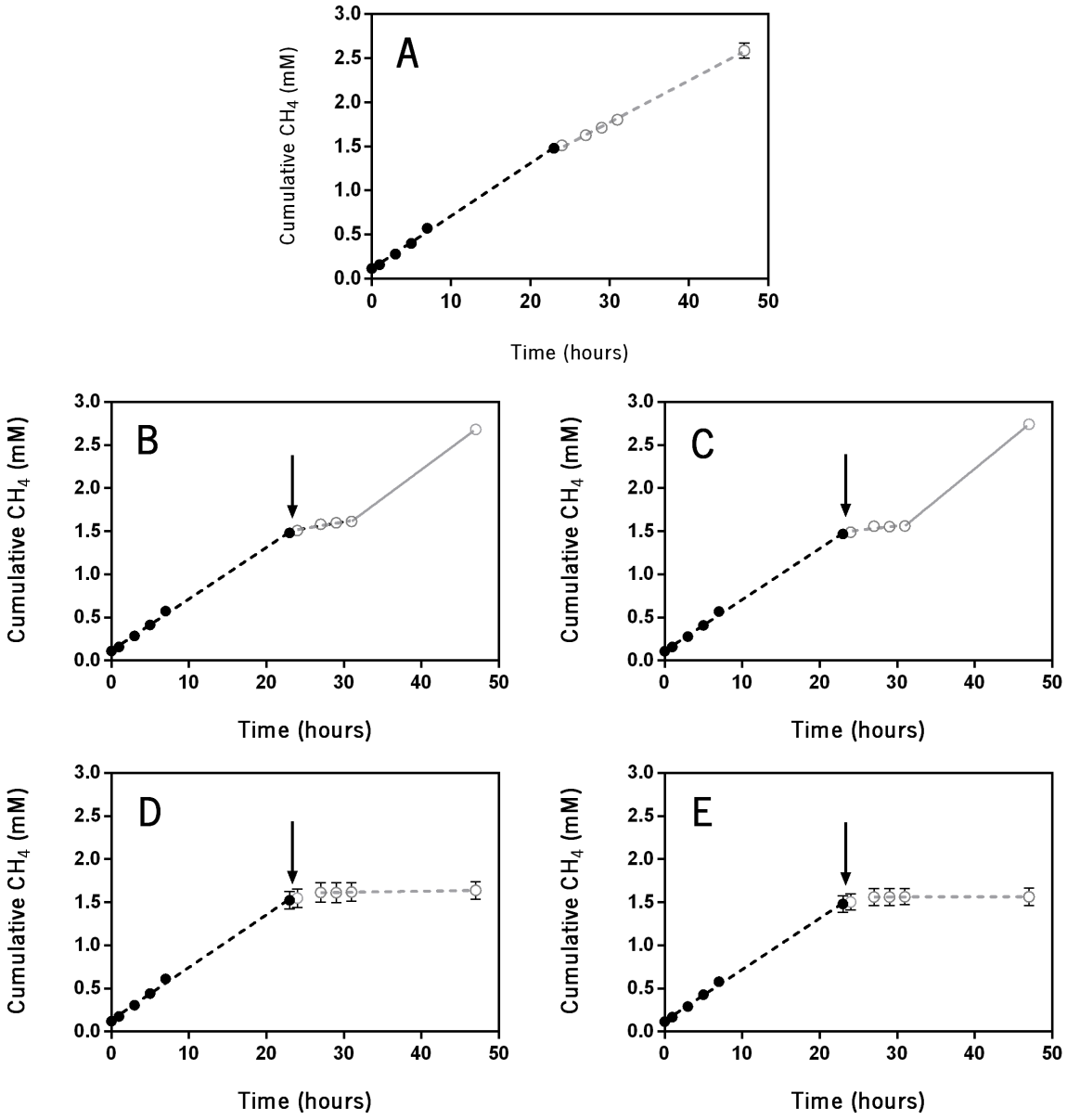

Figure III-1 - Effects of microaerobic conditions on methane production from unamended anaerobic sludge at different %O_2. Increasing %O_2 were tested, 0% O_2 (A), 0,5% O_2 (B), 1% O_2 (C), 2,5% O_2 (D) and 5% O_2 (E). Cumulative methane production before O_2 addition, P1, (●) and after O_2 addition, P2, (○); dashed lines show the data points used to calculate the methane production rate in P1 (---) and P2 (---), respectively; (↓) indicates addition of air pulse. Straight line (—) represents methanogenic community recovery. Each data point is presented as the mean of triplicates ± standard deviations.

APPENDIX IV – CARBON BALANCE IN ETHANOL-AMENDED CULTURE ASSAYS

Table IV-1 – Carbon balances for acetate production through syntrophic oxidation of ethanol during P1 (pre-O_2 injection). Predicted values are based on the stoichiometry balance presented in Table 4.3, section 4.2.1. SD stands for standard deviation. Ethanol and acetate values were obtained though HPLC analysis.

O_2(%)	Total ethanol consumed (mM)			Predicted acetate production (mM)			Experimental acetate (mM)			Differences between predicted and experimental (mM)				
	1	2	3	1	2	3	1	2	3	1	2	3	Mean	SD
0	26.6	26.6	26.6	26.6	26.6	26.6	25.1	24.7	24.6	1.5	1.9	2.0	1.8	0.2
0.5	25.8	25.8	26.6	25.8	25.8	26.6	23.9	24.9	24.8	1.9	0.9	1.8	1.5	0.4
1	26.6	25.8	25.8	26.6	25.8	25.8	24.3	24.4	24.2	2.3	1.4	1.5	1.7	0.4
2.5	25.7	26.4	25.0	25.7	26.4	25.0	23.8	24.9	23.7	1.9	1.5	1.3	1.6	0.2
5	25.8	26.1	26.5	25.8	26.1	26.5	24.3	24.5	24.0	1.5	1.7	2.5	1.9	0.4
												Average	1.7	0.5

Table IV-2 - Carbon balances for hydrogen (H_2) production through syntrophic oxidation of ethanol during P1 (pre-O_2 injection) and corresponding methane production. Predicted values are based on the stoichiometry balance presented in Table 4.3, section 4.2.1. Predicted H_2 production is based on the total ethanol consumed during P1 (seen in Table IV-1). Mean value is represented as absolute. SD stands for standard deviation.

O_2(%)	Predicted H_2 production (mM)			Maximum predicted methane (mM)			Experimental methane (mM)			Differences between predicted and experimental methane (mM)				
	1	2	3	1	2	3	1	2	3	1	2	3	Mean	SD
0	53.2	53.2	53.2	13.3	13.3	13.3	14.3	14.2	14.3	-1.1	-0.9	-1.0	1.0	0.0
0.5	51.6	51.6	53.2	12.9	12.9	13.3	15.3	14.5	15.3	-2.4	-1.6	-2.0	2.0	0.4
1	53.2	51.6	51.6	13.3	12.9	12.9	14.0	15.3	14.9	-0.7	-2.4	-2.0	1.7	0.7
2.5	51.3	52.8	50.0	12.8	13.2	12.5	14.2	14.4	15.1	-1.3	-1.2	-2.6	1.7	0.1
5	51.7	52.3	53.0	12.9	13.1	13.2	15.1	15.2	15.3	-2.2	-2.2	-2.1	2.2	0.1
												Average	1.7	0.5

Table IV-3 - Carbon balances for acetate production through syntrophic oxidation of ethanol during P2 (post-O_2 injection). Predicted values are based on the stoichiometry balance presented in Table 4.3, section 4.2.1. SD stands for standard deviation. Ethanol and acetate values were obtained though HPLC analysis.

O_2(%)	Total ethanol consumed (mM)			Predicted acetate production (mM)			Experimental acetate (mM)			Differences between predicted and experimental (mM)				
	1	2	3	1	2	3	1	2	3	1	2	3	Mean	SD
0	21.8	19.3	20.2	21.8	19.3	20.2	19.7	17.4	18.5	2.0	2.0	1.6	1.9	0.2
0.5	18.5	19.4	19.3	18.5	19.4	19.3	17.4	18.5	17.4	1.1	0.9	1.9	1.3	0.4
1	18.5	20.2	17.7	18.5	20.2	17.7	17.6	18.2	16.9	0.9	1.9	0.8	1.2	0.5
2.5	15.3	15.3	n.d.	15.3	15.3	n.d.	15.2	15.5	n.d.	0.1	-0.2	n.d.	0.1	0.1
5	5.6	5.6	6.5	5.6	5.6	6.5	5.1	5.3	6.5	0.5	0.4	-0.1	0.3	0.3

n.d. – not determined.

Table IV-4 - Carbon balances for hydrogen (H_2) production through syntrophic oxidation of ethanol during P2 (post-O_2 injection) and corresponding methane production. Predicted values are based on the stoichiometry balance presented in Table 4.3, section 4.2.1. Predicted H_2 production is based on the total ethanol consumed during P1 (seen in Table IV-3). Mean value is represented as absolute. SD stands for standard deviation. Methane analysis was performed by GC.

O_2(%)	Predicted H_2 production (mM)			Maximum predicted methane (mM)			Experimental methane (mM)			Differences between predicted and experimental methane (mM)				
	1	2	3	1	2	3	1	2	3	1	2	3	Mean	SD
0	43.5	38.7	40.3	10.9	9.7	10.1	11.1	9.9	10.7	-0.3	-0.2	-0.6	-0.4	0.2
0.5	37.1	38.7	38.7	9.3	9.7	9.7	10.3	9.4	9.6	-1.0	0.3	0.0	-0.2	0.6
1	37.1	40.3	35.5	9.3	10.1	8.9	8.9	9.5	10.0	0.4	0.5	-1.2	-0.1	0.8
2.5	30.6	30.6	n.d.	7.7	7.7	n.d.	7.3	7.4	n.d.	0.4	0.3	n.d.	0.4	0.0
5	11.3	11.3	12.9	2.8	2.8	3.2	0.6	0.5	0.9	2.2	2.3	2.4	2.3	0.1

n.d. – not determined.

APPENDIX V – CALIBRATION CURVES FOR VFAS AND ETHANOL

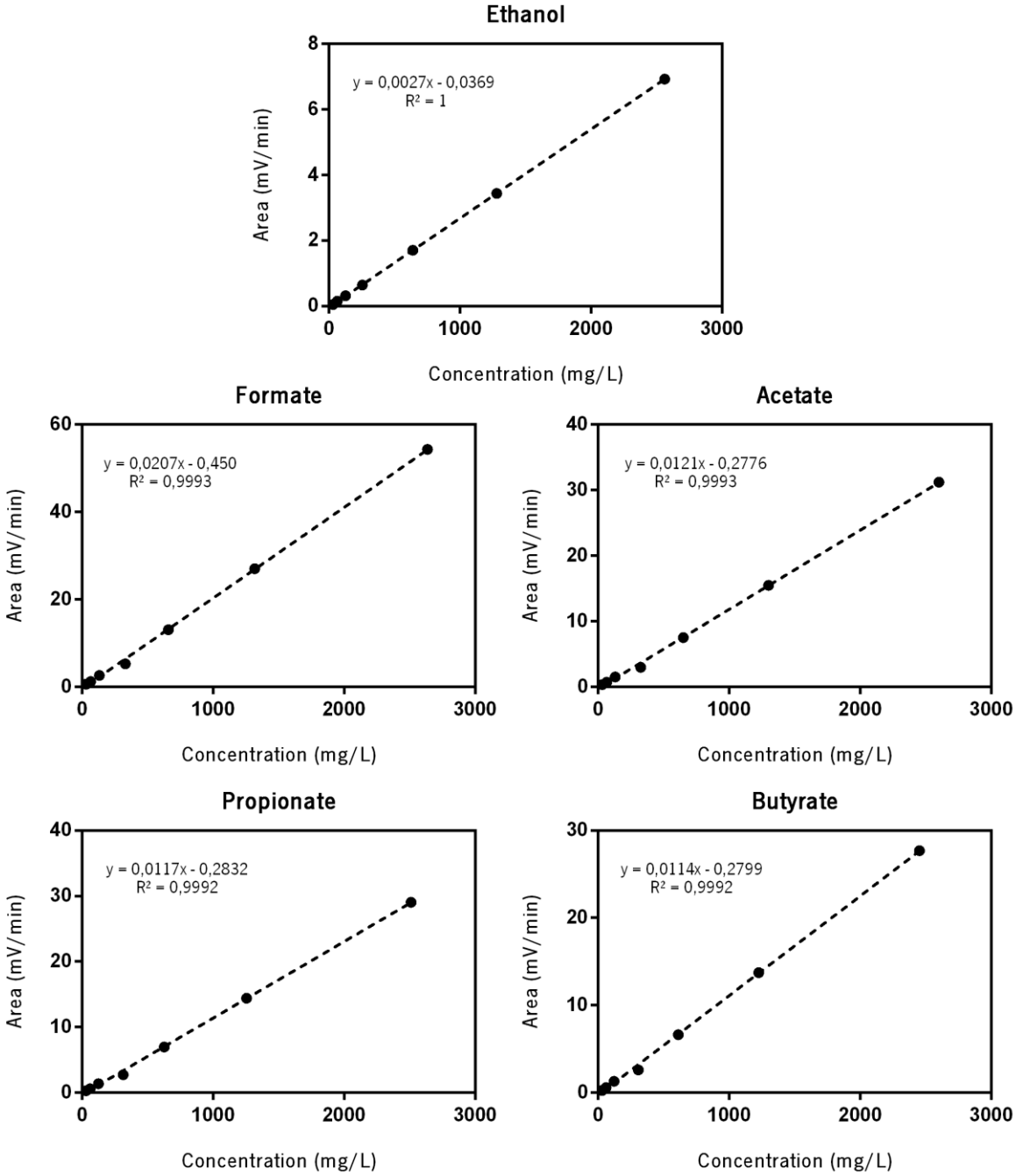

Figure V-1 – Calibration curves used to extrapolate ethanol and VFA (volatile fatty acids) concentrations (mg/L) in culture media samples. Calibration curves were built with the internal standard (crotonic acid).

APPENDIX VI – MEDIA PREPARATION SOLUTIONS

Table VI-1 – Basal media composition used for batch assays

Compound		Concentration
900 mL of deionized water		
15 mL of KH_2PO_4 solution		27.2 g/L
15 mL of $Na_2HPO_4.H_2O$ solution		36 g/L
1 mL of acidic trace elements, H^+ solution		
	HCl	50 mM
	H_3BO_3	1 mM
	$MnCl_2 \cdot 4H_2O$	0.5 mM
	$CoCl_2 \cdot 6H_2O$	0.5 mM
	$FeCl_2 \cdot 4H_2O$	7.5 mM
	$NiCl_2 \cdot 6H_2O$	0.5 mM
	$ZnCl_2$	0.5 mM
1 mL of alkaline trace elements, OH^- solution		
	NaOH	10 mM
	Na_2SeO_3	0.1 mM
	Na_2WO_4	0.1 mM
	Na_2MoO_4	0.1 mM
1 mL of resazurin solution		0.5 g/L

Table VI-2 – Media composition for micronutrients and macronutrients solutions used in bioreator feed solutions.

	Compound	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
	$(NH_4)_6Mo_7O2_4.4H_2O$	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
	NH4Cl	170