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A quantitative and qualitative analysis of microbial community development during low-temperature anaerobic digestion of dairy wastewater

A thesis submitted to the National University of Ireland for the degree of

Doctor of Philosophy

by:

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Head of Microbiology/Research Supervisor: Professor Vincent O’Flaherty
Real knowledge is to know the extent of one's ignorance

Confucius
Acknowledgements

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Anaerobic digestion, from a philosophical point of view, is the “Animal (elephant) in a Darkroom,” a philosophic story written by Rumi (the great Persian poet) in his Masnavi. In this story, some Hindus were holding an elephant in a dark room, and many people gathered to see it. However, as the place was too dark to permit them to see the elephant, they all felt it with their hands to gain an idea of what it looked like. One felt its trunk and declared that the beast resembled a water-pipe; another felt its ear and said it must be a large fan; another felt its leg and thought it must be pillar; another felt its back and declared that the beast must be like a great throne. According to which part each person felt, he gave a different description of the animal. They all disagreed until they lit a candle.
Abstract

The objective of this thesis was to link microbial community structure, population dynamics and microbial activity with performance of two different bioreactor configurations during low-temperature anaerobic digestion of dairy wastewater.

Firstly, the efficiency of the process was assessed in two different bioreactor configurations: an Expanded Granular Sludge Bed (EGSB) and an Inverted Fluidized Bed (IFB) bioreactor operated at 37°C (Chapter 2). Distinct methanogenic communities developed in the IFB and EGSB reactors reflecting step-wise reductions in the applied hydraulic retention time from 72 to 12 h. Variations in reactor performance and in-reactor VFA concentrations during the 200-day trial influenced the microbial community structure of each reactor. Non-metric multidimensional scaling (NMS) and moving window analyses, based on absolute and relative abundance quantification of the 16S rRNA gene concentration obtained by qPCR, were demonstrated to be useful tools to link methanogenic population shifts and reactor performance. The aceticlastic family Methanosarcinaceae was only detected in the IFB and the order Methanomicrobiales was also much more abundant in this reactor, while the aceticlastic family Methanosaetaceae was more abundant in the EGSB. The hydrogenotrophic order, Methanobacteriales, predominated in both reactors under all applied operational conditions.

Subsequently, IFB and EGSB reactors were employed to investigate performance efficiency and methanogenic community structure and population dynamics, during operating temperature transitions from 37°C to 25°C, and from 25°C to 15°C, over a 430-day trial (Chapter 3). A comparable level of performance was recorded for both systems at 37 and 25°C, but a more dynamic and diverse microbial community in the IFB reactors supported better stability and adaptive capacity towards low-temperature operation. The emergence and maintenance of particular bacterial genotypes (phyla Firmicutes and Bacteroidetes) was possibly correlated with efficient protein hydrolysis in the IFB, while protein hydrolysis was inefficient in the EGSB. A significant community shift towards hydrogenotrophic methanogens of the
order Methanomicrobiales, and specifically, Methanocorpusculum-like organisms, was demonstrated during operation at 15°C in both reactor configurations.

Finally the reactors were deployed to explore the feasibility of low-temperature anaerobic biotreatment at 10°C (Chapters 4 & 5). Stable and efficient biotreatment of complex dairy based wastewaters at 10°C was found to be feasible in the EGSB bioreactor at applied organic loading rates (OLR) of 0.5-2 kg COD m⁻³ d⁻¹ with mean chemical oxygen demand removal efficiency (COD RE) >85% (Chapter 4). The process was dependent on the OLR applied and values >2 kg COD m⁻³ d⁻¹ resulted in process deterioration. OLR also influenced microbial community dynamics. An increased abundance of hydrogenotrophic methanogenic groups (Methanomicrobiales and Methanobacteriales) was recorded, using quantitative polymerase chain reaction (qPCR) analysis, by the end of the 335 day trial. Despite the perturbations in OLR applied during the 10°C bioreactor trial, the aceticlastic Methanoseta spp. were maintained at stable levels.

On the other hand, the IFB reactor displayed poor performance throughout the whole 10°C trial with mean COD RE of 54±17% at applied OLRs of 0.5-5 kg COD m⁻³ d⁻¹ (Chapter 5). The applied OLR above 2 kg COD m⁻³ d⁻¹ influenced the microbial composition and dynamics. Hydrogenotrophic methanogens: Methanomicrobiales and Methanobacteriales were monitored via qPCR and demonstrated 16478-fold and 85-fold decrease in their, abundance, respectively. This suggests that those organisms were inhibited or washed out from the system after the OLR stress was applied and did not regrow even when the conditions were changed and stress removed. The bacterial community in the bioreactor was monitored via denaturing gradient gel electrophoresis (DGGE), and the results of this analysis also suggested an influence of OLR stress on bacterial community structure and population dynamics. Possible shortcomings in the bioreactor operation are indicated, which could be helpful in future design and optimization of fluidized reactors intended for digestion of complex industrial wastewaters during LTAD.

This thesis provides new information on the feasibility of anaerobic digestion of dairy wastewater of two different reactor configurations, under variable operating conditions. The influence of operating temperature as well as other operating conditions (i.e. organic loading rate and hydraulic retention time) on the process
performance and community structure and population dynamics was investigated in the course of this study. The overall results and findings of this work provide a comparative insight into LTAD. Identification of significant knowledge gaps can be potentially helpful in better future reactor design and process control.
Chapter 1: Introduction

1.1 Anaerobic digestion

1.1.1 Process of anaerobic digestion

Human society generates large amounts of waste that represents a tremendous threat to the environment and human health. To protect against, and control, these risks a range of different waste treatment options and disposal methods is employed. The choice of method must always be based on maximum safety, minimum environmental impact and on valorization of the waste and final recycling of the end products. One of the main trends of today’s waste management policies is to reduce the stream of waste going to landfills and to recycle the organic material and the plant nutrients back to soil (Ahring, 2003; Holm-Nielsen et al., 2009; Nishio & Nakashimada, 2007). Waste is increasingly becoming a problem and secure recirculation is gaining more and more attention. Anaerobic digestion (AD) is one way of achieving this goal together with possibility of energy production, which is of major importance to the global environment. AD has been implemented for years as a means for the stabilization of sewage sludge but in recent years anaerobic digestion technologies have been expanded to emphasize treatment, energy recovery from many other types wastes including animal wastes (Holm-Nielsen et al., 2009; Karim et al., 2005), organic industrial wastes and industrial wastewater (Angenent et al., 2004; Chen et al., 2008) as well as food waste (Cuellar & Webber, 2010), faecal wastes (Ma et al., 2009) and domestic waste (Verstraete et al., 2009; Zeeman et al., 2008).

The process of AD involves the complete breakdown of complex organic molecules by different consortia of microorganisms, in the absence of oxygen or other respiratory electron acceptors, and it results in the production of biogas as the end product. The process occurs naturally in anaerobic ecosystems such as paddy fields, peatlands, marshes, soils and sediments (Figure 1). Schink (1997) concluded that AD is a series of the most complicated biological processes in the environment.

The microbiology of anaerobic digestion is complicated, involving several bacterial and archaeal groups, which form a complex interdependent food web. Four major
steps can be distinguished during AD: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Batstone et al., 2002; Bitton, 2005; Narihiro & Sekiguchi, 2007); Figure 2).

Figure 1. Natural environments for anaerobic digestion: paddy fields, peatlands, marshes and soils (www.wikipedia.org)
Hydrolysis is the initial stage of AD process, where the degradation of particulate or macromolecular substrate to its soluble monomers and polymers takes place i.e. proteins, carbohydrates and fats are transformed to amino acids, monosaccharides and fatty acids, respectively (Batstone et al., 2002). Hydrolysis is a chemical process in which a water molecule is added to a substance resulting in the split of that substance into two parts. One fragment of the target molecule (or parent molecule) gains a hydrogen ion (H\(^+\)) from the split water molecule. The other portion of the target molecule collects the hydroxyl group (OH\(^-\)) of the split water molecule. In effect an acid and a base are formed, resulting in the breakdown of complex organic polymer into their monomeric state. Hydrolysis of organic polymers is usually carried out by extra-cellular enzymes (hydrolases) such as cellulases, proteases, and lipases, but can also be catalyzed by physiochemical reactions. The characteristics of typical hydrolytic species and their substrates and products are shown in Table 1.
Although the hydrolysis of particulate organic material has been considered the rate-limiting step in anaerobic digestions (Pavlostathis & Giraldogomez, 1991), some authors have emphasized that the hydrolytic process still remains as the least well defined step (Gavala et al., 2003; Miron et al., 2000). The cumulative effects of the different processes taking place during hydrolysis have traditionally been simplified to a single first-order kinetics for the substrate biodegradation (Eastman & Ferguson, 1981). However, relatively high hydrolysis rates were reached in anaerobic biodegradability tests with a high inoculum-to-substrate ratio (Fernández et al., 2001), showing some degree of dependence of hydrolysis to biomass concentration or activity. Sanders et al. (2003) concluded that the first-order kinetics can only be applied when the rate-limiting factor is the surface of the particulate substrate, and bioavailability or biodegradability related phenomena do not interfere. The biodegradability, however, depends on the content of biodegradable carbohydrates (including cellulose, hemicellulose and lignin fractions), proteins and lipids (Angelidaki & Sanders, 2004) as well as the non-degradable fraction of the substrate, which can greatly fluctuate depending on the organic substrate (Kayhanian, 1995). Hydrolysis of particulate organic matter is characterized by surface phenomena (colonization by enzymes or biomass and degradation reactions) and transport phenomena (enzymes from the bulk liquid or reaction products from the surface) (Vavilin et al., 2008). Consequently, the first order kinetics may be inaccurate to describe the hydrolysis of certain complex substrates (Batstone et al., 2002). Hydrolysis efficiency in anaerobic digestion should be viewed in the context of the complex interaction between the different biomass species and intermediate products, and their relative spatial distribution (Vavilin et al., 2008).

For example the characteristics of dairy wastewaters can be very different depending on the kind of products produced by the factory. This can influence the relative proportions of fats, proteins and carbohydrates, thus significantly affecting intermediate compounds generated during the anaerobic biodegradation of dairy wastewaters and their interactions (Vidal et al., 2000). Proteins and fats have been identified as responsible for the typical digestion problems in high rate anaerobic digesters (Perle et al., 1995). Proteins (mostly casein which is the main protein in milk; 80% of total proteins) are hydrolysed by proteases into polypeptides and amino acids (Vidal et al., 2000). In anaerobic conditions hydrolysis of proteins is slower than the hydrolysis of carbohydrates (Pavlostathis & Giraldogomez, 1991) and...
depends on the acclimation of the biomass (Perle et al., 1995). Lack of a developed proteolytic enzymatic system, capable of casein solubilization, in the non-acclimated sludge, can lead to very slow rate of consumption of casein and hence low process efficiency (Perle et al., 1995). The biodegradation of soluble carbohydrates (such as lactose) is generally faster and almost total in anaerobic conditions (Pavlostathis & Giraldogomez, 1991). The biodegradation of lipids is difficult because of their low bioavailability (Petry & Lettinga, 1997). Fats in dairy wastewaters produce glycerol and LCFA during the hydrolytic step (Vidal et al., 2000). Glycerol was found to be a non-inhibitory compound (Perle et al., 1995). However, LCFA (saturated fatty acids with 12±14 carbon atoms and unsaturated fatty acids with 18 carbon atoms) are reported to inhibit various microorganisms (Rinzema et al., 1994), particularly methanogenic bacteria (Koster & Cramer, 1987).

Table 1. Products, substrates, and an application of typical species of hydrolytic bacteria (modified from Amani et al., 2010)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
<th>Typical species</th>
<th>Remark</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins, sugars</td>
<td>Amino acids, sugars</td>
<td>Clostridium sp.</td>
<td>Brewery waste treatment</td>
<td>(Kim et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteus vulgaris</td>
<td>Directed evolution of a lipase</td>
<td>(Fang et al., 2009)</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>Sugars</td>
<td>Bacillus sp.</td>
<td>Syntrophic culture with Clostridium</td>
<td>(Chang et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clostridium sp.</td>
<td>Palm oil mill effluent treatment</td>
<td>(Chong et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetivibrio cellulolyticus</td>
<td>Degradation of cellulose to CH₄</td>
<td>(Khan, 1980)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Staphylococcus sp.</td>
<td>Co-metabolism in the presence of glucose</td>
<td>(Ziagova et al., 2009)</td>
</tr>
<tr>
<td>Lipids</td>
<td>Higher fatty acids, alcohols, amino acid</td>
<td>Clostridium sp.</td>
<td>Food waste treatment</td>
<td>(Jo et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Micrococcus sp.</td>
<td>Isolation of biosurfactants from Micrococcus sp.</td>
<td>(Tuleva et al., 2009)</td>
</tr>
</tbody>
</table>

Acidogenesis (fermentation) is the next step during AD and is generally defined as an anaerobic acid-producing microbial process, without an additional electron acceptor or donor (Gujer & Zehnder, 1983). This includes the degradation of soluble sugars and amino acids by obligatory and facultative fermentative anaerobic bacteria into simpler products: volatile fatty acids (VFA; acetate, propionate, butyrate,
lactate), alcohols, aldehydes, ketones, hydrogen and carbon dioxide (Demirel & Yenigun, 2004; Fang et al., 2002). Some of the identified acidogens and their substrates and products are listed in Table 2. Fermentations can be either classified on the basis of substrate fermented or the fermentation products formed. For example many members of genus *Clostridium* ferment different carbohydrates into acetone, butanol, butyric acid, lactate, ethanol, propionate, H$_2$+CO$_2$ while others ferment amino acids with the production of acetate, ammonia, butyrate, H$_2$ and CO$_2$ (Madigan & Martinko, 2005). Characteristics of the substrate used can affect the products of the acid-phase digestion (Elefsiniotis & Oldham, 1994) but also the main organic acids produced are strongly influenced by reactor pH (Horiuchi et al., 2002). Ghosh et al. (1985) concluded that, for a given temperature, pH and mixing regime, the rate of product formation and the substrate conversion efficiency were dictated by two important operating parameters: retention time and loading. Subsequently, it was observed that anaerobic acidogenesis of dairy wastewater was greatly influenced by operational conditions and the highest amount of VFAs was produced at the shortest HRT and at the highest OLR (Demirel & Yenigun, 2004; Fang, 2000).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
<th>Typical species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>valerate, isovalerate, propionate, butyrate acetate, H$_2$, higher fatty acids</td>
<td><em>Lactobacillus</em> sp., <em>Eschericia coli</em></td>
</tr>
<tr>
<td>Sugars</td>
<td>CO$_2$, H$_2$, formate, acetate</td>
<td><em>Eschericia coli</em></td>
</tr>
<tr>
<td>Fatty acids</td>
<td>valerate, isovalerate, propionate, butyrate, acetate, H$_2$</td>
<td><em>Clostridium</em> sp.</td>
</tr>
<tr>
<td>Alcohols</td>
<td></td>
<td><em>Syntrophomonas wolfei</em></td>
</tr>
</tbody>
</table>

Acetogenic bacteria, convert VFAs and alcohols into acetate (Ac), H$_2$, formate, and CO$_2$, which are used by the methanogens later. In view of microbial growth, generally, acetogenic bacteria ($\mu_{max} = 1$ h$^{-1}$) grow much faster than methanogens ($\mu_{max} = 0.04$ h$^{-1}$) (Shigehisa & Takane, 1994). Some of the identified acetogens and their substrates and products are listed in Table 3. The interaction of acetogens and
methanogens is critical to the performance of an anaerobic digester; this relationship is known as syntrophic interaction (Schink & Stams, 2006). Failure to maintain the balance between these two groups of microorganisms is the primary cause of reactor instability (Wang et al., 2009). Therefore, the products (acetate, formate, H₂ and CO₂) of acetogenesis must be removed efficiently by methanogens (Ahring, 2003). The most difficult step in this degradation is the conversion of short-chain fatty acids such as propionate and butyrate. Under standard conditions (P_H₂ of 1 atm, substrate and product concentrations of 1M, temperature 298K), propionate and butyrate oxidation to H₂, formate and acetate are endergonic (Müller et al., 2010). In anoxic environments, methanogenic Archaea maintain low H₂, formate, and acetate concentrations which make propionate and butyrate degradation feasible (Stams & Plugge, 2009). Anaerobic butyrate degraders known to date belong to only two groups of bacteria, the genus Syntrophomonas within the phylum Firmicutes and the genus Syntrophus within the order Syntrophobacterales of the phylum Proteobacteria. Fermentation of butyrate to acetate and hydrogen is endergonic and occurs only at very low hydrogen partial pressures, e.g. in the presence of methanogenic archaea (Schink, 1997). All currently identified syntrophic propionate-oxidizing bacteria are affiliated with the class of Deltaproteobacteria within the phylum of Proteobacteria (McInerney et al., 2005), or the low G+C Gram-positive bacteria in the class Clostridia within the phylum Firmicutes (de Bok et al., 2005; Imachi et al., 2002; Plugge et al., 2002). Some of the Syntrophobacter sp. are able to use sulfate as the electron acceptor for propionate oxidation (McInerney et al., 2005) and can grow in pure culture by propionate oxidation with sulfate.

It is generally accepted that hydrogen is an interspecies electron carrier, although evidence that formate also plays an important role increased (de Bok et al., 2003; Stams & Dong, 1995). Work of Worm (2010) showed that both hydrogenases and dehydrogenases are involved in each electron generating step of the methylmalonyl-CoA pathway of propionate conversion, indicating that hydrogen and formate are both widely used interspecies electron carriers in syntrophic associations.
Table 3. Substrates and products of some identified acetogenic bacteria
(modified from Amani et al., 2010).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Products</th>
<th>Typical species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyrate</td>
<td>Acetate</td>
<td><em>Syntrophobacter wolinii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>S. fumaroxidans</em></td>
</tr>
<tr>
<td>Propionate</td>
<td>H₂/CO₂, Formate</td>
<td><em>Syntrophomonas wolfei,</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pelotomaculum thermopropionicum,</em> P. Schinkii</td>
</tr>
<tr>
<td>H₂, CO₂</td>
<td>Butyrate, acetate</td>
<td><em>Smithella propionica</em></td>
</tr>
<tr>
<td>CO₂</td>
<td>Acetate</td>
<td><em>Clostridium aceticum</em></td>
</tr>
</tbody>
</table>

Methanogenesis is the final step in the decomposition of organic materials with concurrent production of methane by methanogenic *Archaea. Archaea* exist in a broad range of habitats, and as a major part of global ecosystems (DeLong, 1998) may contribute up to 20% of earth's biomass (DeLong & Pace, 2001). The first-discovered archaeons were extremophiles (Valentine, 2007). Indeed, some archaea survive high temperatures, often above 100°C, as found in geysers, black smokers, and oil wells. Other common habitats include very cold habitats and highly saline, acidic, or alkaline water. However, archaea include also mesophiles that grow in mild conditions, in marshland, sewage, the oceans, and soils (Valentine, 2007). Despite such abundance and resilience to different habitats, methanogenic archaea can only use a limited number of organic compounds as a source of carbon and energy supporting their growth, such as: H₂/CO₂, acetate, formate, methanol, methylated amines and monoxide (Boone et al., 2001; Colleran et al., 1992; Vogels, 1988).

Methanogens are classified into five orders within the domain *Archaea* which can be grouped into two guilds, aceticlastic and hydrogenotrophic methanogens, determined by methane production pathways. Aceticlastic methanogens include only *Methanosarcinales* which comprises two families, *Methanosaetaceae* utilizing only acetate and *Methanosarcinaceae* utilizing acetate as well as various methyl compounds and hydrogen (Boone et al., 2001). The hydrogenotrophic methanogens comprises the remaining four orders, i.e., *Methanobacteriales, Methanococcales, Methanomicrobiales,* and *Methanopyrales*, which utilize only H₂ and CO₂ or formate to produce methane (Boone et al., 2001). *Methanopyrales* members are not likely to
be present in anaerobic processes due to their extremely high growth temperature (>80°C) (Boone et al., 2001).

1.1.2 Low-temperature (psychrophilic) adaptation of anaerobic digestion

Microorganisms are classified into ‘temperature classes’ on the basis of the optimum temperature and the temperature span in which the species are able to grow and metabolize. The optimum growth temperature ranges can overlap (Figure 3), therefore there is no clear boundary between psychrophilic, mesophilic and thermophilic microorganisms (Lettinga et al., 2001). Thermophilic and mesophilic methanogenesis is well documented but psychrophilic methanogenesis is relatively understudied, although occurring in most terrestrial and aquatic environments (Madigan et al., 2000; Schleper et al., 2005). The majority (~75%) of the Earth’s biosphere is cold, and psychrophilic Archaea can be found in permanently, seasonally and artificially cold environments (Cavicchioli, 2006). It has become clear that cold-adapted archaea and bacteria contribute to global energy cycles through the processing of organic and inorganic carbon and nitrogen compounds (Cavicchioli, 2006); therefore it is important to understand the diverse coping strategies of those microorganisms for acclimation to low-temperature conditions. Cold conditions affect nearly all cellular processes including membrane fluidity, nutrient/waste transport, rates of transcription, translation and cell division and protein folding (D'Amico et al., 2006). Cold-adapted microorganisms overcome these challenges through genotypic and phenotypic changes. Such strategies include structural changes to proteins (such as more flexible structural and conformation changes), increasing membrane fluidity (through changes in the membrane lipid composition), synthesis of „antifreeze” glycoproteins and peptides, and synthesis of „cold-shock” proteins to sustain normal protein synthesis (Giaquinto et al., 2007; Pikuta et al., 2007).
Temperature decrease influences the rate and path of carbon flow during methanogenesis by affecting the activity of particular microbial groups and the structure of consortia (Glissman et al., 2004), although the microbial interactions and biochemical pathways involved are not well understood. The initial, hydrolytic stage is viewed as the rate-limiting step at low/ambient temperatures. Reduced temperature may limit the energy gain from syntrophic volatile fatty acid (VFA) degradation and the critical importance of acetogens, as providers of methanogenic substrates, may thus increase in cold anoxic environments (Kotsyurbenko, 2005). In natural, low-temperature environments, acetate has been reported as the main methanogenic precursor (Kotsyurbenko, 2005). Similar findings have been observed within some engineered ecosystems operated <20°C (Akila & Chandra, 2007; Enright et al., 2009). It has been proposed that homoacetogenesis is an important biochemical pathway during low-temperature methanogenesis and, that enhanced acetoclastic activity under conditions of low-temperature may arise from elevated autotrophic acetogenesis (Kotsyurbenko, 2005). In a balanced system with low prevailing H₂-partial pressures, however, hydrogenotrophic methanogens may out-compete homoacetogens, due to their higher affinity for hydrogen. Methanogenesis may thus proceed mainly through the hydrogenotrophic pathway in both natural
(Kotsyurbenko et al., 2007; Metje & Frenzel, 2007) and engineered systems (Enright et al., 2009; Madden et al., 2010; McKeown et al., 2009a; McKeown et al., 2009b; O'Reilly et al., 2009b; Syutsubo et al., 2008). The precise reasons for the observed discrepancies in the carbon flow during low-temperature methanogenesis are unresolved and represent a key biological knowledge gap with practical implications. For example, acetoclastic Methanoseta species are regarded as being important for the formation and maintenance of strong granular sludge, which is a requirement for the operation of granular sludge-based bioreactor such as the Upflow Anaerobic Sludge Blanket (UASB) (McHugh et al., 2005). A study by O'Reilly et al. (2010), however, provides evidence that wastewater composition can influence the granulation rate.

The majority of full-scale anaerobic digestion reactors are operated under mesophilic temperature conditions (>18°C) to ensure optimal microbial activity, although significant input of energy is required to heat bioreactors to the treatment temperature (Lettinga et al., 2001). Therefore, efficient full-scale operation of bioreactors under ambient or low-temperature conditions (<18°C) would reduce the treatment costs associated with waste streams discharged at sub-ambient temperatures, making low-temperature AD (LTAD) an attractive option for the wastewater treatment (McKeown et al., 2009b).

A temperature reduction has a strong effect on the maximum substrate utilization rates of microorganisms (Kettunen & Rintala, 1997; Lettinga et al., 1999; Rebac et al., 1995; Wu et al., 1993). Generally, lowering the operational temperature leads to a decrease in the maximum specific growth and substrate utilization rates but it might also increase biomass yield of methanogenic populations (van den Berg, 1977). A drop in temperature is also accompanied with a change of the physical and chemical properties of the wastewater, which can considerably affect design and operation of the treatment system. During high-rate AD under low-temperature conditions the following parameters should be taken into consideration: (i) increase in the solubility of gaseous compounds such as methane, hydrogen and CO₂ as temperature decreases below 20°C which can lower reactor pH under psychrophilic conditions (Rebac, 1998); (ii) increase in liquid viscosity that in consequence changes the hydraulic
shearing force on the particles (Mahmoud et al., 2003) and thus more energy is required for mixing in low-temperature bioreactors; (iii) particles will settle more slowly because of a decreased liquid–solid separation; (iv) diffusion of soluble compounds will drop (Perry, 1984). Despite those disadvantages, a number of successful low-temperature treatments have been demonstrated at laboratory- and pilot-scale (Connaughton et al., 2006b; Enright et al., 2007; Enright et al., 2005; McKeown et al., 2009b; Rebac et al., 1995; Rebac et al., 1999; Siggins et al., 2011c). Successful acclimation of mesophilic biomass to low-temperature conditions within engineered systems has been achieved through successive changes in the community structure (Enright et al., 2009; Madden et al., 2010; O'Reilly et al., 2009b; Syutsubo et al., 2008) indicating the selective enrichment of psychroactive microorganisms during the acclimation process. Several authors have demonstrated that cold acclimation results in enhanced methanogenic activity, calculated based on substrate conversion rates, under low-temperature conditions (Collins et al., 2006b; Connaughton et al., 2006b; Lettinga et al., 2001; O'Reilly et al., 2009a). Prolonged cultivation of mesophilic inocula under low-temperature conditions has suggested the emergence of truly psychrophilic homologues in the methanogenic activity profiles (McKeown et al., 2009a).

1.1.3 Characteristics of dairy wastewater during AD

Increased demand for milk and milk products caused enormous growth of dairy industries in most countries of the world. It has been estimated that achieving the target of a 50% increase in milk production by 2020 in Ireland requires a growth in milk deliveries from an average of 5.1 billion liters over the 2007 to 2009 period, to 7.66 billion liters in 2020 (Dillon, 2011). Consequently, the amount of wastewater generated and discharged from milk processing industries will also increase (Kushwaha et al., 2011). The dairy industry wastewaters are primarily generated from the cleaning and washing operations in the milk processing plants. It is estimated that about 2% of the total milk processed is wasted into drains (Munavalli & Saler, 2009). Dairy wastewaters are characterized by high biological-oxygen demand (BOD) and chemical oxygen demand (COD) concentrations (80–95 000 mg COD l\(^{-1}\); (Kushwaha et al., 2011)), and generally contain carbohydrates, proteins and fats, as well as detergents and sanitizing agents (Perle et al., 1995). Nutrients lead to
eutrophication of receiving waters, and detergents affect the aquatic life. Due to the high pollution load of dairy wastewater, the milk-processing industries discharging untreated/partially treated wastewater cause serious environmental problems (Milani et al., 2011).

Dairy wastewaters are treated using mainly biological processes since physico-chemical treatment processes involve high reagent costs and poor removal of soluble COD (Vidal et al., 2000). Among biological treatment processes, anaerobic digestion (AD) is often an ideal treatment option (Wheatley, 1990). AD is an advantageous treatment option for several reasons including: (1) no requirement for aeration, (2) low volumes of excess sludge, and (3) smaller footprints and lower land requirements than aerobic treatment processes (Demirel et al., 2005).

The characteristics of the effluents arising from the production of various dairy products, such as milk, butter, yoghurt, ice-cream, desserts and cheese, are variable depending on production systems, methods and operations used (Vidal et al., 2000). The diversity and complexity of dairy waste streams implies that different anaerobic treatment applications are required to optimally enhance the process efficiency and economic feasibility of AD treatment. Engineering optimized reactor configurations is among the most widely studied approach to improve AD of dairy wastewaters (Alvarado-Lassman et al., 2008).

1.1.4 Bioreactor design

Wastewater treatment processes are subject to variations in one or more parameters that affect or define the reactor performance, i.e. flow rate, influent type and concentration, sludge retention time (SRT), nutrient availability, temperature, pH, the presence of xenobiotics, and others (Chen et al., 2008; Leitao et al., 2006). Some of these variations can be predicted and controlled, and the reactor can be designed to accommodate them.

Dairy wastewater defined as a complex type of substrate (Angelidaki et al., 1999; Fang, 2000) and high-rate AD can often be problematic, even at mesophilic
temperatures (Demirel et al., 2005). It is, therefore, important to investigate different reactor configurations to promote enhanced efficiency of AD process. Additionally, since environmental parameters such as temperature might influence the process performance and microbial composition and dynamics during AD (Bergamo et al., 2009), bioreactors intended for low-temperature applications should compensate negative effects of low-temperature during AD. One of the parameters that would facilitate the high-rate anaerobic wastewater treatment of cold wastewater is increased contact between retained sludge and wastewater to utilize all the available capacity within the bioreactor. Progress into optimizing the contact of sludge and wastewater in upflow anaerobic sludge bed (UASB) reactors, led to the development of an advanced reactor design, the expanded granular sludge bed (EGSB), internal circulation (IC) reactor, which is a modification of the EGSB, and anaerobic fluidized bed (FB). The most common configuration for the full-scale anaerobic digestion is the UASB and EGSB. However, a granule disintegration observed during LTAD (McKeown et al., 2009b) may evoke failure of the bioreactors to retain granular sludge, which can lead to severe hydraulic wash-out of psychroactive sludge (Lettinga et al., 1999). In this context, to assess the influence of granule- and non-granule-based systems during LTAD, two different laboratory scale reactor configurations (i.e., an Expanded Granular Sludge Bed (EGSB) and an Inverted Fluidized Bed (IFB) bioreactor) were investigated and discussed in detail.

1.1.4.1 Expanded Granular Sludge Bed (EGSB) bioreactor

The use of effluent recirculation combined with tall reactors (or a high height/diameter ratio), resulted in the expanded granular sludge bed (EGSB) reactor (Figure 4a; (Kato et al., 1994)). The EGSB is a variant of the up-flow anaerobic sludge blanket (UASB) concept, and in this reactor type, the up-flow liquid velocity (usually recommended to be >4 m h⁻¹) is the key operating feature, which expands the sludge bed and maximizes sludge-wastewater contact (Seghezzo et al., 1998). Soluble pollutants are efficiently treated in EGSB reactors but suspended solids are not substantially removed from the wastewater stream due to the high upflow velocities applied. Recirculation of the effluent dilutes the influent concentration, but
it was extensively proven that low strength wastewater can efficiently be treated in EGSB reactors (Kato et al., 1994). Influent dilution may also allow the treatment of toxic compounds in these reactors. Compared to UASB reactors, higher organic loading rates (up to 40 kg COD m\(^{-3}\) d\(^{-1}\)) can be accommodated in EGSB systems (Seghezzo et al., 1998).

1.1.4.2 Inverted Fluidized Bioreactor (IFB)

The IFB (Figure 4b) bioreactor has been identified as a promising design for AD (García-Bernet et al., 1998). The novelty of this configuration arises from the use of floatable particles with a specific density lower than the liquid, such that the particles are fluidized downward (García-Calderon et al., 1998). Extendosphere\textsuperscript{TM} (Sphere One, Chattanooga, Tennessee, USA), a light mineral material composed mostly of silica and traces of aluminum, density of 0.69 g cm\(^{-3}\), was used as a carrier material for the IFB reactor (Figure 5). Due to the large specific area of support particles available for biomass retention, this technology offers advantages in the treatment of high-strength effluents by using reduced spaces and shorter hydraulic retention times (Alvarado-Lassman et al., 2008). The liquid and the produced biogas are flowing in opposite directions, which help for bed expansion (Arnaiz et al., 2003). Therefore, the down-flow (or inverse) configuration reduces energy requirements, because of the low fluidization velocities (García-Calderon et al., 1998) when compared to up-flow systems. Although many advantages, there are some crucial parameters that restrict a widespread application of this technology such as: support material selection, establishment of reactor operating conditions, and start-up duration (Punal et al., 2000), that depends on the biofilm formation and stabilization and this can range from 90-160 days (Castilla et al., 2000; Michaud et al., 2002). Recent studies using pre-colonized support media significantly shortened start-up times (Alvarado-Lassman et al., 2010; Arnaiz et al., 2005).
Figure 4. Diagram of reactor configuration of A: EGSB and B: IFB bioreactors.

Figure 5. Carrier particles (Extendosphere™) used for the IFB bioreactor.
1.2 Microbial Analysis

1.2.1 Physiological Analysis: Specific Methanogenic Activity (SMA) Assay

Physiological characterizations of the seed sludge and bioreactor samples at specific sampling times have been determined by specific methanogenic activity (SMA) testing (Enright et al., 2005; McKeown et al., 2009b; Siggins et al., 2011a). SMA tests assess the activity of the various trophic groups found within the biomass. Biomass populations are investigated against a variety of soluble substrates (ethanol, acetate, propionate and butyrate) and a gaseous substrate (H$_2$/CO$_2$) according to method described by (Coates et al., 1996; Colleran et al., 1992).

The substrates used in SMA test are generally intermediates of the anaerobic digestion process and, thus, supplying each of these on an individual basis to the biomass allows the assessment of the activity of each trophic group. Acetate and H$_2$/CO$_2$ are the direct precursors of methane, via conversion by methanogens. Two main groups of methanogens have been identified as being involved in anaerobic digestion; acetoclastic methanogens and hydrogenotrophic methanogens. Acetoclastic methanogens produce methane by the decarboxylation of acetate while hydrogenotrophic methanogens produce CH$_4$ by the reduction of CO$_2$ with hydrogen. Propionate, butyrate and ethanol are indirect methanogenic substrates, converted to methanogenic substrates by acetogenic bacteria. In this method, therefore, change in pressure within a sealed batch vial was monitored in response to substrate degradation and corresponding methane production, and specific methanogenic activity was determined as ml CH$_4$ gVSS$^{-1}$ day$^{-1}$ (Colleran et al., 1992).

1.2.2 16S rRNA gene analysis of community structure

The genes encoding ribosomal RNAs are ancient. They are functionally constant, universally distributed and comprise highly conserved sequence domains interspersed with more variable regions (Woese, 1987). In prokaryotes there are three ribosomal RNA molecules, which have the sizes: 5S, 16S and 23S (Berg et al., 2002). Initially, the analysis of the diversity of natural microbial populations relied on direct extraction, purification, and sequencing of 5S rRNA molecules from environmental samples (Lane et al., 1985; Stahl et al., 1985), but the information from the 120 nucleotide-long 5S rRNA is relatively limited.
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(Amann et al., 1995; Olsen et al., 1986). An average bacterial 16S rRNA molecule has a length of approximately 1,500 nucleotides, the bacterial 23S rRNA has 2,900 nucleotides, and thus the two contain considerably more information than 5S rRNA (Olsen et al., 1986). 16S rRNA is more experimentally manageable than 23S rRNA and it has been used extensively to develop the phylogeny of both prokaryotes and eukaryotes. Even though 23S rRNA is larger, the currently available complete sequences are rather poor compared to the coding sequence for 16S rRNA (Ludwig & Schleifer, 1994). The conserved regions of the 16S rRNA are important for classification of higher taxa, while the variable regions can be used for differentiation between closely related species.

The first step in 16S rRNA gene-based microbial community analyses is to purify DNA from all bacteria present in a sample, while minimizing the introduction of bias due to e.g. differential lysis or recovery (Gabor et al., 2003; Mumy & Findlay, 2004; Stach et al., 2001). Previously, a method based on mechanical lysis by mechanical grinding in combination with magnetic bead-based DNA purification in order to purify DNA from microbial communities (Magtration System 12 GC, PSS, Chiba, Japan; Figure 6) allowed to obtain both robust and high throughput DNA purification (Lee et al., 2009; Lee et al., 2008). All bacterial 16S rRNA genes in the samples are subsequently amplified using primers targeting generally conserved regions in the 16S rRNA gene. The ratio between 16S rRNA gene copies for different bacteria is (in theory) conserved during the amplification due to the use of a universally conserved primer pair (Rudi, 2003). The final step in the analysis is to detect the 16S rRNA genes from the different bacteria present in the mixed amplification product. This can be done using probe based methods (Rudi et al., 2002), cloning and DNA sequencing (Rudi et al., 2004), or electrophoresis-based methods such as TRFLP (Wang et al., 2004) and denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) (Ercolini, 2004; Muyzer & Smalla, 1998). The detection methods have different fields of application. Probe-based methods like real-time PCR are used if one knows which bacteria to search for, while DGGE is used for screening purposes and pattern recognition (Theron & Cloete, 2000). A schematic outline of the process is shown in Figure 7.
1.2.2.1 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing Gradient Gel Electrophoresis is a molecular fingerprinting tool employed for the separation of PCR products based on decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a gradient of DNA denaturants (Muyzer & Smalla, 1998). DGGE analyses are employed for the separation of double-stranded DNA fragments that are identical in
length, but differ in sequence. In practice, this refers to the separation of DNA fragments produced via PCR amplification. The technique exploits (among other factors) the difference in stability of G-C pairing (3 hydrogen bonds per pairing) as opposed to A-T pairing (2 hydrogen bonds). Therefore, a mixture of DNA fragments of different sequence is electrophoresed in an acrylamide gel containing a gradient of increasing DNA denaturants (Muyzer et al., 1993). DNA fragments richer in GC will be more stable and remain double-stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate better in the acrylamide gel, while denatured DNA molecules become effectively larger and slow down or stop in the gel. In this manner, DNA fragments of differing sequence, which can be attributed to sequence variation between different species can be separated in an acrylamide gel. Each of the resulting bands on the gel theoretically represents a different microbial population present in the community. The addition of a GC clamp to one end of the DNA fragment allows the optimization of sensitivity of this technique, allowing the detection of almost 100% of the sequence variants, by preventing the two DNA strands from complete dissociation into single strands (Sheffield et al., 1989).

DGGE has been used widely in environmental microbiology to study diversity and populations’ relative abundance shifts (Muyzer, 1999) in complex systems, including manure (Leung & Topp, 2001) and anaerobic bioreactors (Connaughton et al., 2006a; Lee et al., 2008; Madden et al., 2010). Obtaining sequence information by excising DGGE DNA bands has been claimed to be very convenient and attractive (Muyzer et al., 1993; Muyzer & Smalla, 1998), by comparison to other fingerprinting methods like T-RFLP and length heterogeneity PCR (LH-PCR) that require cloning before identifying bands of interest. Another advantage is that the DGGE electrophoresis system is less expensive than the automated sequencer required for other fingerprinting methods like LH-PCR, T-RFLP and automated RISA (ARISA) methods. However, in complex microbial communities, DNA sequence information from excised gel bands requires cloning (Kisand & Wikner, 2003; Pereira et al., 2002) because of bands co-migration or poor separation of gel bands. Furthermore, the size of DGGE bands is usually less than 500 bp, so the DNA sequence information obtained from gel bands is limited and phylogenetic identification may be poor in the case of novel sequences having less than 85% identity to known sequences (Hugenholtz et al., 1998). Finally, gel-to-gel
variation and the lesser sensitivity (compared to fingerprints obtained by methods using capillary electrophoresis or quantitative PCR) are limiting to ensure reproducibility and detection of minor populations and subtle changes (Talbot et al., 2008). Schematic diagram presenting the process of DGGE is illustrated in Figure 8.

Figure 8. Schematic diagram illustrating the process of DGGE (modified from http://wiki.biomine.skelleftea.se/biomine/molecular/index_11.htm)

1.2.2.2 Phylogenetic analysis

Once the sequences of environmental DGGE bands are generated, sequence alignment is performed to allow more effective organization and visualization of analysis. Sequence alignment is a way of arranging the sequences of DNA to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences (Mount, 2009). Aligned sequences of nucleotide residues are typically represented as rows within a matrix. Gaps are inserted between the residues so that identical or similar characters are aligned in successive columns. The most common method in molecular biology to align sets of nucleotide sequences is to generate a multiple sequence alignment progressively by first aligning the most similar sequences and then adding
successively less related sequences or groups to the alignment until the entire query set has been incorporated into the solution. Progressive alignment results are dependent on the choice of "most related" sequences and thus can be sensitive to inaccuracies in the initial pairwise alignments. Most progressive multiple sequence alignment methods additionally weight the sequences in the query set according to their relatedness, which reduces the likelihood of making a poor choice of initial sequences and thus improves alignment accuracy. Methods used for multiple sequence alignment can be based on T-Coffee (Tree based Consistency Objective Function for alignment Evaluation) software (Notredame et al., 2000) or more commonly ClustalW (Thompson et al., 1994). During the progressive alignment using ClustalW method, the most similar sequences, so those with the best alignment score are aligned first. Then progressively more distant groups of sequences are aligned until a global alignment is obtained. Alignments are also used to aid in establishing evolutionary relationships by constructing phylogenetic trees, which are used to classify the evolutionary relationships between homologous genes represented in the genomes of divergent species. The degree to which sequences in a query set differ is qualitatively related to the sequences' evolutionary distance from one another. A high sequence identity score suggests that the sequences in question have a comparatively young most recent common ancestor, while low identity suggests that the divergence is more ancient. This approximation, which reflects the "molecular clock" hypothesis that a roughly constant rate of evolutionary change can be used to extrapolate the elapsed time since two genes first diverged, assumes that the effects of mutation and selection are constant across sequence lineages. It does not, therefore, account for possible difference among organisms or species in the rates of DNA repair or the possible functional conservation of specific regions in a sequence.

The most common methods for constructing phylogenetic trees from molecular data are classified into Distance methods, Parsimony methods, and Likelihood methods (Nei & Kumar, 2000). Recently, an enormous increase in the size of data sets for orthologous genes from diverse species, and of homologous sequences from multigene families, has caused even a moderate exploration of topological (tree) space to become impractical because of the enormous amount of computational time required. This has led to the extensive use of the neighbor-joining (NJ) method
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(Saitou & Nei, 1987a). The NJ method quickly generates a final tree for large phylogenies under the principle of minimum evolution by clustering neighboring sequences in a stepwise manner it minimizes the sum of branch lengths (Saitou & Nei, 1987a) and thus examines multiple topologies (Tamura et al., 2004). Consequently, this method has been frequently used to present phylogenetic information and there are many examples of successful application of the NJ method to describe AD (Lee et al., 2008; McKeown et al., 2009a; O'Reilly et al., 2009b; Siggins et al., 2011c).

Once the sequences of environmental DGGE bands are identified and aligned they are compared and matched against other DNA sequences present in the GeneBank database using an internet-based algorithm tool such as BLAST. BLAST is designed to look for local alignments, i.e. maximal regions of high similarity between the query sequence and the database sequences, allowing for insertions and deletions of sites (Altschul et al., 1990). For each comparison, BLAST reports a goodness score and an estimate of the expected number of matches with an equal or higher score than would be found by chance, given the characteristics of the sequences. When this expected value is very small, the sequence from the database is considered a "hit" and a likely homologue to the query sequence.

1.2.2.3 Quantitative PCR

In contrast to the conventional end-point detection PCR, quantitative real-time PCR (qPCR) technology is based on the detection of fluorescence during amplification of target DNA (Higuchi et al., 1993). A fluorescent molecule that reports an increase in the amount of DNA is therefore proportional to the increase in fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled sequences and specific primers or probes. Specialized thermal cyclers equipped with fluorescence detection modules are used to monitor the fluorescence as amplification occurs. The measured fluorescence reflects the amount of amplified product in each cycle.

The main advantage of real-time PCR over conventional PCR is that real-time PCR allows determination of the starting template copy number with accuracy and high sensitivity over a wide dynamic range (Heid et al., 1996). Real-time PCR results can
either be qualitative (presence or absence of a sequence) or quantitative (number of
copies of DNA). Real-time PCR that is quantitative is also known as qPCR. In
contrast, conventional PCR is at best semi-quantitative. Additionally, real-time PCR
data can be evaluated without gel electrophoresis, resulting in reduced experiment
time and increased throughput. Finally, because reactions are run and data are
evaluated in a closed-tube system, opportunities for contamination are reduced and
the need for postamplification manipulation is eliminated (Heid et al., 1996).

To understand how real-time PCR works, it is important to examine the amplification
plot (Figure 9). In this plot, the PCR cycle number is shown on the x-axis, and the
fluorescence from the amplification reaction, which is proportional to the amount of
amplified product in the tube, is shown on the y-axis (Heid et al., 1996). The
amplification plot shows two phases, an exponential phase followed by a non-
exponential plateau phase. During the exponential phase, the amount of PCR product
approximately doubles in each cycle. As the reaction proceeds, however, reaction
components are consumed, and ultimately one or more of the components becomes
limiting. At this point, the reaction slows and enters the plateau phase (cycles 28–40
in Figure 9). Initially, fluorescence remains at background levels, and increases in
fluorescence are not detectable (cycles 1–18 in Figure 9) even though product
accumulates exponentially. Eventually, enough amplified product accumulates to
yield a detectable fluorescent signal. The cycle number at which this occurs is called
the threshold cycle, or C_T (Heid et al., 1996). Since the C_T value is measured in the
exponential phase when reagents are not limited, qPCR can be used to reliably and
accurately calculate the initial amount of template present in the reaction (Heid et al.,
1996). The C_T of a reaction is determined mainly by the amount of template present
at the start of the amplification reaction. If a large amount of template is present at
the start of the reaction, relatively few amplification cycles will be required to
accumulate enough product to give a fluorescent signal above background. Thus, the
reaction will have a low, or early, C_T. In contrast, if a small amount of template is
present at the start of the reaction, more amplification cycles will be required for the
fluorescent signal to rise above background. Thus, the reaction will have a high, or
late, C_T. This relationship forms the basis for the quantitative aspect of real-time
PCR (Heid et al., 1996).
Two main qPCR chemistries have been used in the study of anaerobic bioreactors: the TaqMan and the SYBR Green technologies (Figure 10). The principal advantage of the TaqMan technology is that it is more target-specific than SYBR Green technology, which uses only forward and reverse primers (Harms et al., 2003). For quantification, a standard curve must be produced from serial dilutions (usually 10-fold) of either genomic DNA from a pure culture of target bacteria, PCR-amplified DNA segments, or a plasmid containing target DNA insert. Standard DNA has to be accurately quantified by commercial kits containing double stranded DNA binding dyes. Once the concentration of standard DNA is determined, the number of SSU rRNA gene copies can be calculated from the average molecular weight of 660 Da for one DNA base pair, the Avogadro’s number, and the number of base pairs of the standard DNA using Equation 1 (Whelan et al., 2003):

\[
\text{DNA (copy)} = \frac{6.02 \times 10^{23} \text{ (copy mol}^{-1}) \times \text{DNA amount (g)}}{\text{DNA length (bp) \times 660 (g mol}^{-1} \text{bp}^{-1})}
\]

**Figure 9.** Amplification plot. Baseline-subtracted fluorescence is shown. (Source 2006 Bio-Rad Laboratories, Real-Time PCR Applications Guide)
Figure 10. qPCR chemistries (a) SYBR Green and (b) TaqMan technologies. (Source 2006 Bio-Rad Laboratories, Real-Time PCR Applications Guide)

Better sensitivity and reproducibility of qPCR than conventional PCR or conventional hybridization techniques (e.g. dot blotting) are very useful in studies requiring a large number of samples (Talbot et al., 2008). Moreover, in anaerobic reactors methanogenic consortia are hard to obtain by conventional cultivation because of slow growth rate and obligate anaerobiosis (Raskin et al., 1994). The work of Yu et al. (2005b), who designed four TaqMan primers and probe sets that were specific for each of the four orders of methanogens associated with bioreactors represents an important contribution for quantification of methanogens at the order and family taxonomic levels in anaerobic processes or in environmental samples by qPCR. Consequently as the specific primer/probe sets have been designed many studies used quantitative real-time PCR methodology to detect and quantify methanogens at the order and family levels (Yu et al., 2005a; Yu et al., 2005b). This
allowed to monitor dynamics of the methanogenic communities across a variety of environmental and operating conditions during AD (Lee et al., 2009; McKeown et al., 2009a; O'Reilly et al., 2009b; Siggins et al., 2011b; Siggins et al., 2011c).

1.2.3 Statistical Analysis

Multivariate statistical analyses of fingerprints and environmental parameter data can provide new insights into the impact of operating conditions on changes in community composition or metabolic activities (Talbot et al., 2008). Multivariate statistical analyses have been previously employed in soil (Mills et al., 2006; Ritchie et al., 2000) and aquatic microbial communities (Bernhard et al., 2005) and more recently in anaerobic digestion (Lee et al., 2008; Miura et al., 2007; O'Reilly et al., 2010; Siggins et al., 2011b) to evaluate environmental parameters modulating microbial community changes estimated from fingerprints. McCune and Grace (2002) described the main statistical tools that may be used to relate species abundance in a community to environmental conditions. In present study selected methods are described in detail.

1.2.3.1 Hierarchical cluster analysis

Hierarchical clustering methods are applied to obtain a grouping of similar environmental samples and are visualized with a dendrogram (tree diagram) (Talbot et al., 2008). Unweighted pair-group method with arithmetic mean (UPGMA) and Ward’s method are the most appropriate clustering methods to generate dendrograms from fingerprints data (McCune & Grace, 2002). The UPGMA cluster analysis has been commonly employed to monitor microbial community dynamics in anaerobic bioreactors (Connaughton et al., 2006b; Lee et al., 2008; Madden et al., 2010; Siggins et al., 2011c).
1.2.3.2 Nonmetric Multidimensional Scaling (NMS) ordination

Nonmetric Multidimensional Scaling (NMS) is an ordination technique aiming to arrange the items as a way of graphically summarizing complex relationships, extracting one or a few dominant patterns from an infinite number of possible patterns (McCune & Grace, 2002). In other words, NMS ordination plot is generated whereby more similar samples in terms of community composition are located closer to each other and vice versa. The main advantages of NMS are that: (i) the analysis avoids the assumption of linear relationships among variables; (ii) it relieves the “zero-truncation problem”; and (iii) it allows the use of any distance measure or relativization (Clarke & Ainsworth, 1993). It is, therefore, reported to be the most generally effective ordination method for ecological community data and should be the method of choice unless a specific analytical goal demands another method (McCune & Grace, 2002).

1.2.3.3 Moving window analysis

Moving window analysis allows time-based analysis of microbial community data, which is not possible with other statistical methods like NMS and cluster analysis (Possemiers et al., 2004). The community similarity between two consecutive time points is calculated using Sørensen (Bray-Curtis) distance measure in the PC-ORD software (McCune & Grace, 2002) and can be used as the indicator of community variation in response to the environmental perturbations. The values for moving window analysis range from 0 to 1, with 1 being identical and 0 being completely unrelated. Moving window analysis was previously demonstrated to be a valuable tool for monitoring microbial community dynamics in anaerobic bioreactors (Falk et al., 2009; Freeman et al., 2008; Siggins et al., 2011c; Wittebolle et al., 2008).
1.3 Scope of this thesis

The main objective of this study was to evaluate the feasibility of low-temperature anaerobic digestion for the treatment of complex, dairy based wastewaters. Such an application of low-temperature anaerobic digestion presents an attractive option to reduce the treatment costs associated with wastewaters discharged at sub-mesophilic temperatures, especially in temperate climatic regions. Additionally, increasing demand for dairy products will result in large increases in the quantities of dairy waste produced in Ireland in the coming years. This increase in production should be combined with more sustainable, energy-efficient wastewater treatment technologies, such as LTAD. The potential for full-scale implementation of this technology will be maximized through greater understanding of the complex microbial interactions underpinning the process and insights into the impact of reactor configuration on process efficiency. In this study, anaerobic bioprocess performance was, therefore, linked with microbial physiological analysis (specific methanogenic activity assays) and 16S rRNA gene fingerprinting techniques (DGGE, qPCR) to monitor the adaptation of the microbial community structure in response to temperature changes and bioreactor configuration.

Chapter 2 investigated development of the methanogenic community structure in two different reactors, an Expanded Granular Sludge Bed (EGSB) and an Inverted Fluidized Bed (IFB) bioreactor, during 200-day trial at 37°C. Five methanogenic order or family-specific primer and probe sets were employed in q-PCR assays. Quantitative community shifts were visualized using Nonmetric multidimensional scaling (NMS) technique, based on the real-time PCR data. Methanogenic community dynamics associated with operational changes were also monitored using moving window analysis. Additionally, changes in archaeal community structure in both reactors were examined using denaturing gradient gel electrophoresis (DGGE). The obtained microbial information was linked with the variations in process performance and operating conditions (i.e., hydraulic retention time (HRT)), in the IFB and EGSB reactors tested.
Chapter 3 investigated community dynamics of IFB and the EGSB bioreactors during operational temperature transitions from 37°C to 25°C, and from 25°C to 15°C, over a 430-day trial. The methanogenic community structure and population dynamics were examined qualitatively by DGGE, and quantitatively by q-PCR, and the results were statistically analysed and compared. It was hypothesized that the process performance and microbial community structure and population dynamics can be influenced by two different reactor configurations during transition from mesophilic to psychrophilic reactor operation and changes applied in the operating conditions [i.e., loading rate and hydraulic retention time].

Chapter 4 evaluated the feasibility of anaerobic digestion of complex, dairy-based wastewater at 10°C. To determine the limits of stable bioreactor performance, the expanded granular sludge bed (EGSB) was subjected to variable organic loading rates, defined by different HRT and variable influent concentrations. Additionally, changes in bacterial and archaeal community structure were monitored using DGGE and q-PCR to investigate the influence of operating conditions [i.e. increases in organic loading rates] on microbial dynamics during the 335-day trial.

Chapter 5 explored the application of low-temperature (10°C) anaerobic digestion (LTAD) for the treatment of complex, dairy-based wastewater in an inverted fluidized bed (IFB) bioreactor. The effect of variable organic load, defined by different HRT and variable influent concentrations on the bioprocess performance of IFB has been assessed during a 335-day trial at 10°C. Changes in bacterial and archaeal community structure were examined using DGGE and qPCR, respectively, to investigate the influence of operating conditions on the microbial community structure and population dynamics.

Chapter 6 outlines main conclusions of this research and offers future recommendations.
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Chapter 2

Quantitative and qualitative analyses of methanogenic community development in high-rate anaerobic bioreactors.

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Abstract

Methanogenic community structure and population dynamics were investigated in two anaerobic reactors treating a dairy wastewater, an Inverted Fluidized Bed (IFB) and Expanded Granular Sludge Bed (EGSB) during operation at 37°C. A combination of real-time PCR, denaturing gradient gel electrophoresis and statistical techniques was employed to analyse the 16S rRNA genes of methanogenic communities. Distinct methanogenic communities developed in the IFB and EGSB reactors reflecting step-wise reductions in the applied hydraulic retention time from 72 to 12 h during the 200-day trial. The aceticlastic family *Methanosarcinaceae* was only detected in the IFB and the order *Methanomicrobiales* was also much more abundant in this reactor, while the aceticlastic family *Methanosetaeaceae* was more abundant in the EGSB throughout the whole trial. The hydrogenotrophic order, *Methanobacteriales*, predominated in both reactors under all applied operational conditions. Nonmetric multidimensional scaling (NMS) and moving window analyses, based on absolute and relative abundance quantification data, demonstrated that the methanogenic communities developed in a different manner in the IFB, compared to the EGSB reactor. In our study, relative abundance-based quantification by NMS and moving window analysis appeared to be a valuable molecular approach that was more applicable to reflect the changes in the anaerobic digestion process than approaches based either on qualitative analysis, or solely on absolute quantification of the various methanogenic groups. The overall results and findings provided a comparative, quantitative and qualitative insight into anaerobic digestion processes, which could be helpful for better future reactor design and process control.
2.1 Introduction

Dairy wastewater is a complex substrate composed of easily degradable carbohydrates, mainly lactose, and bioavailable proteins and lipids (Angelidaki et al., 1999; Fang et al., 2000; Yu et al., 2000). The chemical oxygen demand (COD) concentration of dairy effluents varies significantly, but since dairy waste streams are usually warm and strong, anaerobic digestion (AD) is often an ideal treatment option (Wheatley 1990). Indeed, AD is an advantageous treatment option for several reasons including: (1) no requirement for aeration, (2) low volumes of excess sludge, and (3) smaller footprints and lower land requirements than aerobic treatment processes (Demirel et al., 2005).

The characteristics of the effluents arising from the production of various dairy products, such as milk, butter, yoghurt, ice-cream, desserts and cheese, are also variable depending on production systems, methods and operations used (Vidal et al., 2000). The diversity and complexity of dairy waste streams implies that different anaerobic treatment applications are required to optimally enhance the process efficiency and economic feasibility of AD treatment. Engineering optimized reactor configurations is among the most widely studied approach to improve AD of high-strength effluents (Alvarado-Lassman et al., 2008). However, although several anaerobic digesters with new configurations exist, there is still little information on how the microbial communities underpinning the different reactors (i.e., an Expanded Granular Sludge Bed (EGSB) and an Inverted Fluidized Bed (IFB) bioreactor) behave and respond. EGSB systems use self-immobilized granular sludge, whereas IFB reactors rely on inert carrier materials supporting attached growth of active biomass. The EGSB is a variant of the up-flow anaerobic sludge blanket (UASB) concept, and in this reactor type, the up-flow liquid velocity (usually recommended to be > 4 m h\(^{-1}\)) is the key operating feature, which expands the sludge bed and maximizes sludge-wastewater contact (Seghezzo et al., 1998). The IFB bioreactor has been identified as a promising design for AD (Garcia-Bernet et al., 1998). The novelty of this configuration arises from the use of floatable particles with a specific density lower than the liquid, such that the particles are fluidized downward (Garcia-Calderon et al., 1998). Due to the large specific area of support particles available for biomass retention, this technology offers advantages in the treatment of high-strength effluents by using reduced spaces and shorter hydraulic
retention times (Alvarado-Lassman et al., 2008). The liquid and the produced biogas are flowing in opposite directions, which help for bed expansion (Arnaiz et al., 2003). Therefore, the down-flow (or inverse) configuration reduces energy requirements, because of the low fluidization velocities (Garcia-Calderon et al., 1998) when compared to up-flow systems.

The links between changes in microbial community and perturbations in anaerobic digesters are not well understood and there may even be changes in community without apparent changes in performance. There is a need for more comprehensive studies on this topic, which can be done by aid of high throughput molecular tools (Talbot et al., 2008). Culture-free molecular techniques, particularly based on 16S rRNA genes, have been successfully applied to numerous microbial ecology studies, and helped us to link microbial community structure and population dynamics to process performance (Fernandez et al., 2008; Lee et al., 2008). Many studies concerning anaerobic reactors have focused only on qualitative techniques, such as DGGE (Muyzer et al., 1993), and thus quantitative population dynamics of anaerobic bioreactors are still in its infancy. In particular, there is no information regarding quantitative comparison of different types of anaerobic reactors during transitional changes. Besides the community diversity and composition, the quantitative changes in microbial communities represent a significant factor affecting process performance (Wittebolle et al., 2005). Quantitative information about community structure can be therefore very useful in diagnosing problems with process performance or comparing anaerobic digesters.

Acidogenic bacteria and the methanogen are the two major groups underpinning AD. However, methanogenesis is usually a rate-limiting step and requires effective control for successful operation of most AD systems (Yang et al., 2003; Yu et al., 2005; Yu et al., 2006). Five methanogenic groups: three hydrogenotrophic orders and two aceticlastic families are considered to cover most methanogens in anaerobic digesters (Yu et al., 2005; Lee et al., 2009). Consequently in the present study, the methanogenic community structure of the EGSB and the IFB bioreactors was quantitatively investigated using five methanogenic order or family specific primer and probe sets employing real-time PCR. Quantitative community shifts were visualized using Nonmetric multidimensional scaling (NMS) technique, based on
real-time PCR data. Methanogenic population dynamics associated with operational changes were monitored using moving window analysis (Wittebolle et al., 2008). Additionally changes in archaeal community structure in both reactors were examined using denaturing gradient gel electrophoresis (DGGE). The obtained microbial information was linked with the variations in process performance and operating conditions (i.e., hydraulic retention time (HRT)), in the IFB and EGSB reactors tested.

2.2 Materials and methods

2.2.1 Reactor operation

Lab-scale EGSB (Figure 11a) and IFB (Figure 11b) reactors (3.6-l working volume each) were continuously operated, at 37°C, for 200 days. Extendosphere™ (Sphere One, Chattanooga, Tennessee, USA) light mineral material composed mostly of silica and traces of aluminum, density of 0.69 g cm⁻³, was used as a carrier material for the IFB reactor. Particle size distribution analyses of virgin carrier material were performed using Mastersizer (Malvern Instruments) to determine particle distribution and percentage of each fraction. The range of particle size distribution was broad, from 73.6 μm to 2000 μm, but the majority of particles were in the range of 194-236 μm.

The seed sludge used to inoculate both reactors was obtained from a full-scale internal circulation (IC) anaerobic digester located at the Carbery Milk Products (Ballineen, Co Cork, Ireland). The volatile solids (VS) concentration used to inoculate bioreactors was 60 g l⁻¹ and granular seed sludge was crushed prior to inoculation. Both reactors were fed with a synthetic dairy wastewater (4 g COD l⁻¹) buffered with NaHCO₃ and fortified with macro- (10 ml l⁻¹) and micro- (1 ml l⁻¹) nutrients (Shelton & Tiedje 1984; Arnaiz et al., 2003). The applied hydraulic retention time (HRT) was decreased in a stepwise manner from 72 to 12 h during the operation of both reactors.
2.2.2 DNA extraction

Total genomic DNA was extracted from seed sludge and biomass obtained from the reactors at various points during the reactor trial (Figure 12). All biomass samples (50 ml) were mechanically disrupted by manual grinding with a pestle and mortar and diluted 10-fold with deionised and distilled water (DDW). Cells from each 1 ml sample were harvested by centrifugation at 13,000 rpm for 5 min, followed by decantation of the supernatant. The residual pellet was washed with 1 ml of DDW, and centrifuged again in the same manner to ensure a maximal removal of residual medium. After two washing cycles, pellet was resuspended in 1 ml of DDW.

Total DNA in the suspension was extracted using an automated nucleic acid extractor (Magtration System 6GC, PSS, Chiba, Japan). Purified DNA was eluted with 100 µl of Tris–HCl buffer (pH 8.0) and stored at -20°C for further analyses. DNA extraction was performed in duplicate.

Figure 11. Laboratory scale anaerobic reactors: a) Expanded Granular Sludge Bed (EGSB) and b) Inverted Fluidized Bed (IFB).
2.2.3 qPCR

Real-time PCR (qPCR) analysis was performed using a LightCycler 480 instrument (Roche, Mannheim, Germany) using three methanogenic order-specific primer and probe sets: *Methanobacteriales* (MBT), *Methanomicrobiales* (MMB), *Methanococcales* (MCC) and two methanogenic family-specific primer and probe sets: *Methanosarcinaceae* (Msc), *Methanosaetaceae* (Mst) as described previously (Yu et al., 2005; Lee et al., 2009). Each reaction mixture of 20 µl was prepared using the LightCycler 480 Probe Master kit (Roche Diagnostics): 2 µl of PCR-grade water, 1 µl of each primers (final concentration 500 nM), 1 µl of the TaqMan probe (final concentration 200 nM), 10 µl of 2× LightCycler® 480 Probes Master, and 5 µl of template DNA. Amplification was performed in a two-step thermal cycling procedure: predenaturation for 10 min at 94°C followed by 40 cycles of 10 s at 94°C and 30 s at 60°C. All DNA templates were analyzed in duplicate.

Quantitative standard curves were constructed as previously described (Yu et al., 2006) using the representative strains corresponding to each primer and probe sets targeting the following methanogenic groups: MBT (*Methanobacterium formicicum* M.o.H. (DSM 863) and *Methanobrevibacter arborophilicus* DH1 (DSM 1536)); MMB (*Methanospirillum hungatei* JF1 (DSM 864) and *Methanomicrobium mobile* BP (DSM 1539)); MCC (*Methanococcus vannielii* SB (DSM 1224) and *Methanococcus voltae* PS (DSM 1537)); Msc (*Methanosarcina acetivorans* C2A (DSM 2834), *Methanosarcina barkeri* MS (DSM 800), *Methanosarcina mazei* Go1 (DSM 3647)); Mst (*Methanosaeta concilli* GP6 (DSM 3671)). For each standard solution, a 10-fold serial dilution series of 10^1 to 10^9 copies/µl was generated and analyzed by real-time PCR in duplicate with its corresponding primer/probe set. Based on copy concentration of each serial dilution and values obtained from qPCR, the standard curves were constructed. Subsequently the values of slope and intercept from each standard curve corresponding to order or family level were used to calculate copies concentration in the template samples. Final results were expressed as copies of 16S rRNA genes/ml.
2.2.4 Statistical analysis

Nonmetric multidimensional scaling (NMS) was performed, based on the real-time PCR results, to visualize the methanogenic community shifts during the operation. Based on 16S rRNA gene concentration data, two different matrices: an absolute quantity-matrix and relative abundance matrix of each target methanogenic group, respectively, were analyzed employing Sørensen distance measure in the PC-ORD software ver. 5.0 (McCune & Grace 2002). The absolute quantity-matrix was based on the amount of rDNA detected by qPCR assay for each order-specific sets (MBT and MMB) and family-specific sets (Msc and Mst). The relative abundance matrix was created using the ratio between the rDNA concentration detected by the qPCR assay quantified for each order/family-specific sets (MBT, MMB, Msc and Mst) and the total rDNA concentration of methanogens detected in the sample.

The NMS plot mirrors the relationships between the community profiles by closely locating the communities with high similarity.

Moving window analysis was also carried out based on the absolute quantity and relative abundance matrices to monitor the variations in methanogenic community composition associated with decreases in the applied HRT in the two bioreactors. Moving window analysis was previously demonstrated to be a valuable tool for monitoring microbial population dynamics (Wittebolle et al., 2008). The community similarity between two consecutive phases with different HRTs was calculated using PC-ORD software ver. 5.0 (McCune & Grace 2002) and used as the indicator of community variation in response to the corresponding HRT change.

2.2.5 Archaeal DGGE

Archaeal 16S rRNA genes were amplified by PCR using the primers ARC 787F and ARC 1059R (Takai and Horikoshi, 2000). To stabilize the melting behavior of the PCR products, a 40-bp GC-clamp was attached at the 5’-end of the forward primer (Muyzer et al., 1993). Touchdown PCR was conducted using thermal cycler G-Storm (Gene Technologies Ltd., Essex UK) and the following protocol was applied: initial denaturation at 94°C for 10 min; 20 cycles of denaturation at 94°C for 1 min, annealing at 65°C to 55°C (reducing 0.5°C per cycle) and elongation at 72°C for 1 min; followed by 20 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and
final elongation at 72°C for 30 min (Janse et al., 2004). DGGE was performed using a D-Code system (BioRad Hercules, CA). Fifteen µl of the PCR product were loaded onto 8% acrylamide gel containing a 40 to 65% denaturant gradient (100% denaturant contained 7 M urea and 40% (v/v) formamide). After electrophoresis, the DGGE gel was stained with ethidium bromide and destained for 20 min, respectively. Gel image was captured using a UV transillumination camera. Bands of interest were cut directly from the gel using a sterile razor blade and eluted in 40 µl of DDW. Two µl of eluted DNA solution were further amplified using the ARC787F and ARC1059R primers, without the GC clamp. The PCR products were gel-purified and cloned into pGEM-T Easy vector (Promega, Madison, WI). The cloned gene fragments were sequenced using T7 primer and compared against the Ribosomal Database Project (RDP) database. Sequencing alignment and phylogenetic analyses were performed using MEGA 4 software (Tamura et al., 2007). All nucleotide sequence data reported in this study were deposited in the GenBank database under accession numbers GQ429188-GQ429206.

### 2.2.6 Analytical methods

Biogas and effluent from both reactors were routinely sampled to analyze methane content and residual chemical oxygen demand (COD) concentration according to Standard Methods (APHA, 1998). All analyses were performed in duplicate. Analysis of effluent volatile fatty acids (VFA), by heated (85°C) and agitated headspace, were performed in a Varian Saturn 2000 GC/MS system, with CombiPAL autosampler (Varian Inc., Walnut Creek, CA). Separation was carried out on a Varian Capillary column, CP-WAX 58 (FFAP) CB (25 m length x 0.32 mm i.d. x 0.2 µm film thickness, Varian). The injector volume was 2 ml and the injector temperature was 250°C. The carrier gas was helium and the flow rate was 1 ml/min. The temperature program was as follows: 50°C (20 sec) to 110°C (20 sec) at a rate of 2°C/min; from 110°C to 200°C (20 sec) at a rate of 20°C/min. The MS-detector was operated in the scan mode in the range of 40-150 m/z at a temperature of 210°C. Identification of VFAs was achieved by matching chromatographic retention times and spectra of standard compounds (acetic-, butyric-, iso-butyric-, propionic-, valeric- and iso-valeric acid). Calibration curves of standard VFAs were conducted
and used for relative concentration of VFAs in effluent headspace samples and then expressed in mg l\(^{-1}\).

2.3 Results and discussion

2.3.1 Process performance

The start-up of the IFB reactor was prolonged and perturbed, with large fluctuations in COD removal efficiency (COD RE) and effluent VFA concentration, whereas the EGSB performance remained relatively constant throughout the initial period of operation (Figure 12 & Figure 13). The high initial effluent VFA concentration corresponded to poor COD removal efficiency by the IFB reactor (Figure 12). After 2 months of operation, the IFB reactor stabilized, with a marked decrease in VFA concentration and significant improvement in COD removal. From day 60-160 of the trial, both the EGSB and IFB reactors performed very similarly in terms of COD removal (>80%; Figure 12), effluent VFA concentration (<80 mg l\(^{-1}\); Figure 13) and >60% biogas methane content (Appendix 1) at an applied HRT of 72 h. With further decreases in the applied HRT from 72 down to 18 h (Figure 12), the two reactors exhibited comparable performance with no significant deterioration recorded (Figure 12). A further decrease in HRT to 12 h, however, caused a significant performance drop (i.e., decrease in COD RE and increase in residual VFA concentration) in the IFB (Figure 12 & Figure 13), indicating that a loading rate threshold may have been reached. On the other hand, the EGSB showed no marked change in performance and maintained a similar level of treatment efficiency, even at the shortest HRT tested (Figure 12 & Figure 13).
Figure 12. Chemical Oxygen Demand (COD) Removal Efficiencies of IFB and EGSB reactors. Arrows indicate biomass sampling points.

Figure 13. Volatile Fatty Acids (VFA presented as sum of: acetic-, butyric-, iso-butyric-, propionic-, valeric- and isovaleric acid); acetic acid and propionic acid concentrations in IFB and EGSB reactors.
2.3.2 qPCR of methanogenic communities

In contrast to the conventional end-point detection PCR, quantitative real-time PCR (qPCR) technology based on the detection of fluorescence during amplification of target DNA (Higuchi et al., 1993) has better sensitivity and reproducibility than conventional PCR and can be easily used in studies requiring a large number of samples (Talbot et al., 2008). TaqMan qPCR primers and probe sets for the methanogenic orders: Methanobacteriales, Methanomicrobiales and the two families: Methanosarcinaceae and Methanosaetaceae; demonstrated satisfactory specificity to allow quantitative comparison between the two bioreactors to be made and for correlation of the molecular data with process performance changes.

In our study, the EGSB and IFB bioreactors displayed a noticeable disparity in terms of the quantitative composition of the methanogenic community by real-time PCR (Figure 14). The order Methanobacteriales (MBT) was commonly the most dominant methanogen group in both reactors (i.e., 63-86% in the IFB and 52-71% in the EGSB), in terms of 16S rRNA gene concentration, during the trial. The 16S rRNA gene concentration of Methanobacteriales, in both reactors, remained stable at approximately 10^8 copies/ml, regardless of changes in operating conditions, which were characterized by decrease in HRT from 72-12 h (Figure 14). The fact that Methanobacteriales were predominant throughout the trial, in both reactors, at all HRT, although it was not dominant group in the seed, was an interesting finding. It is not clear whether high initial 16S rRNA gene concentration in the seed (i.e., 1.4 × 10^9 copies/ml), influent composition or other factors had a stimulating effect on growth of this group – but this should now be investigated.

The hydrogenotrophic order Methanomicrobiales (MMB) was detected in both the EGSB and IFB reactors but its quantitative dynamics differed in each reactor configuration. Although the initial 16S rRNA gene concentration of Methanomicrobiales in the seed sludge was approximately 10^7 copies/ml in both reactors, the MMB were much more abundant in the IFB reactor during the trial (18-90 fold greater than EGSB; Figure 14). The 16S rRNA gene concentration of Methanomicrobiales in the IFB biomass, during the operational period when the applied HRT was reduced from 72-18 h, stabilized between 10^6 - 10^7 copies/ml (Figure 14). Following a further decrease in HRT to 12 h, however, the concentration
of Methanomicrobiales significantly increased to $6 \times 10^7$ copies/ml. The Methanomicrobiales population was much less abundant in the EGSB reactor during the operational period corresponding to the HRT reduction from 72 to 18 h (1.2-2.2×10^5 copies/ml; Figure 14). A slight increase in the 16S rRNA gene concentration of $6.7 \times 10^5$ copies/ml was, however, recorded following the reduction in HRT to 12 h (Figure 14). In both reactors, therefore, the HRT change from 18 to 12 h seemed to evoke an increase in the abundance of this hydrogenotrophic group, which may be due to higher organic loading rate (OLR), or some morphological or biokinetic traits of the group. It is generally accepted that reducing the applied HRT, at a constant influent concentration (as was in case of this study at 4 g COD l⁻¹), will increase the OLR (Mahmoud et al., 2003). The applied HRT change from 18 to 12 h resulted in OLR increase of 50% (5.3 g COD l⁻¹ day at 18h HRT to 8 g COD l⁻¹ day at 12 h HRT), which had a direct and positive influence on the abundance of Methanomicrobiales. Although our results differ from those observed by Rincón et al. 2008, who reported a stable methanogenic community of Archaea, at every OLR tested, the archaeal community in their study was represented only by the genus Methanosaeta. Hypothetically, an increased abundance of Methanomicrobiales might be correlated with more diverse bacterial communities, and Rincón et al. (2008) did observe higher number of bacterial phylotypes at increased OLR; but this was not determined during our study.

The aceticlastic family, Methanosacetaceae (Mst), was the most abundant group in the seed biomass and 16S rRNA gene concentration of this group was detected at $2 \times 10^9$ copies/ml (Figure 14). During reactor operation between 72-18h HRT, the 16S rRNA gene concentration of Methanosacetaceae in the IFB was 3.0-4.3×10^7 copies/ml (Figure 14). On the other hand, Methanosacetaceae was about >2.6 fold more abundant in the EGSB (16S rRNA gene concentration of 1.0-1.5 x10^8 copies/ml; Figure 14). An increase in the 16S rRNA gene levels was observed, following a decrease in HRT to 12 h, to 8.3×10^7 copies/ml and 3.2×10^8 copies/ml in IFB and EGSB reactors, respectively.

Previous studies have revealed the importance of Methanoseta spp. in determining the development of granules in EGSB reactors (Liu et al., 2002; Collins et al., 2003). Methanoseta spp. rods appear to provide a network, within the granule, to which
other bacteria become associated. It is generally accepted that abundant *Methanoseta* spp. improve granulation and result in more stable reactor performance (Liu *et al*., 2002). The predominance of *Methanoseta* in our EGSB reactor (>2.6 fold higher population compared to the IFB), therefore, could be associated with more stable reactor performance and lower in-reactor VFA concentrations, particularly during the first two months of the trial (Figure 12 & Figure 13). It has been reported that *Methanosarcina* spp. have higher maximum growth rates on acetate than *Methanoseta* spp., but that the minimum threshold for acetate utilization by *Methanoseta* spp. is 5-10 times lower than for *Methanosarcina* spp. (Zinder 1990; Jetten *et al*., 1992). These kinetic data indicate that a selection for granules in anaerobic systems dominated by *Methanoseta* spp. should be favored by low steady-state acetate concentration. Additionally, a decrease in HRT from 72-12h seemed to enhance the granulation of initially crushed biomass in the EGSB reactor, which was particularly prominent after transition from 18-12 h HRT (visual examination). It has also been reported previously that increases in upflow liquid velocity and reduction in applied HRT have a stimulating influence on granulation (Alphenaar *et al*., 1993).

The appearance of the family *Methanosarcina* in anaerobic digesters, by contrast, has been associated with high in-reactor acetate concentrations, accompanied by process deterioration (Collins *et al*., 2003; O’Reilly *et al*., 2009). VFA and acetic acid concentration data from the EGSB and IFB reactors apparently confirmed this observation (Figure 13). Throughout the 200 day trial, the VFA concentration in EGSB reactor effluent was relatively constant, not exceeding 80 mg l⁻¹ in the transitional periods associated with HRT decreases. Low VFA and therefore, low acetate concentrations, in the EGSB, especially during start-up, did not favor growth of *Methanosarcina* and this tendency was maintained until the end of trial. On the other hand, the high initial VFA concentration (210-320 mg l⁻¹) where acetate constitutes 36-76% (80-220 mg l⁻¹) apparently stimulated the growth of *Methanosarcina* in the IFB reactor. In addition, it should be noted that elevated concentrations of propionate (60-160 mg l⁻¹; 22-56% of all VFA; Figure 13) in the IFB reactor effluent, during the start-up period, may have played a role in stimulating the growth of propionate-oxidizing syntrophic bacteria (Zheng *et al*., 2006). Hypothetically, the presence of propionate-oxidizing syntrophic consortia could be linked with the more dynamic population of *Methanomicrobiales*, which was
observed in the IFB system (Figure 14). Higher levels of Methanomicrobiales have previously been reported (Zheng et al. 2006) in a reactor fed a mixture of glucose and propionate to enhance the growth of propionate-oxidizing syntrophic consortia.

An interesting observation was that, although Methanosaetaceae was the dominant group in the seed sludge, once the reactors were started up, the Methanobacteriales become the predominant group (Figure 14). The dominance of Methanosaetaceae in the original (seed) sludge is unsurprising and has been previously demonstrated in other studies (Raskin et al., 1994; McHugh et al., 2003; Sawayama et al., 2006), but the predominance of Methanobacteriales, in both reactors, at all HRT’s as recorded in this study, is unusual and has not, to our knowledge been reported previously in high-rate anaerobic sludge reactors.

Quantitative PCR results demonstrated that the aceticlastic family Methanosarcinaceae (Msc) was only detected in the IFB reactor (Figure 14). The initial 16S rRNA gene concentration of Methanosarcinaceae in the seed sludge, 1.8 ×10^5 copies/ml, markedly increased during the IFB reactor trial (to 3.5 ×10^6 copies/ml; Figure 14). In case of the EGSB biomass, the Methanosarcinaceae 16S rRNA gene concentration was under the real-time PCR reaction detection limit (i.e., 1.6×10^4 copies/ml) throughout the trial. It is possible that Methanosarcinaceae were out-competed by Methanosaetaceae, due to the competitive growth relationship between both families for acetate, and that Methanosarcinaceae were washed out from EGSB system (Yu et al., 2006). As mentioned earlier, low acetate concentrations, as observed in the EGSB reactor, create an unfavorable environment for Methanosarcinaceae, which tend to predominate at higher acetate concentrations as identified in the IFB reactor.

The order Methanococcales (MCC) was not detected in either of the reactors, presumably since organisms from this group require high-salt conditions for they growth (0.3-9.4% (w/v) NaCl), which are not normally found in anaerobic reactors (Boone et al., 2001).
Figure 14. Absolute quantification of methanogenic communities in the IFB and the EGSB bioreactors. Methanogenic groups: MBT (*Methanobacteriales*), MMB (*Methanomicrobiales*), MSC (*Methanosarcinaceae*), MST (*Methanosaetaceae*).

2.3.3 Statistical analysis of the quantitative shifts in methanogenic communities

NMS analysis avoids the assumption of linear relationships among variables and it is reported to be the most generally effective ordination method for ecological community data (McCune & Grace, 2002). In both NMS plots (Figure 15 a & b), the cumulative $r^2$ represented by the axes was > 0.9, the final stress value was < 2.5, and instability was < $10^{-4}$, indicating that our results met the criteria for an excellent representation (McCune & Grace, 2002).

Based on output from the absolute quantity-matrix a wide shift was visualized, by NMS, between the methanogenic communities present in biomass samples taken from the EGSB during the 18h HRT and 12h HRT operational periods (Figure 15 a). This change in community abundance corresponded to a 2.3-fold increase in the absolute numbers of rDNA of all methanogens, detected by qPCR, between these two consecutive time points. In particular, an increase in the absolute values of the predominant group, the *Methanobacteriales*, could possibly have some influence on
the NMS output, indicating that highly-weighted effect of dominant population (especially with respect to evenness) might be a drawback in using an absolute matrix. It has been reported previously that all distance measures (including Sørensen distance, which was used in this study) lose sensitivity with increasing environmental distance (McCune & Grace, 2002). That would imply that the higher the absolute number, the greater the difference even a small change will make, although it is well known that Sørensen distance is not greatly influenced by outliers or elevated values and loses sensitivity over a distance about half the length of the environmental gradient (McCune & Grace, 2002). On the other hand, methanogenic communities from the IFB, taken at the same time points, were closely located on the NMS output, based on the absolute quantity-matrix (Figure 15 a), indicating the lesser change in the absolute numbers of rDNA of all methanogens detected by qPCR in the IFB reactor (i.e. a 1.5-fold increase). The absolute numbers of rDNA of all methanogens, detected by qPCR, were almost twice greater in the EGSB between the samples taken during the 18 to 12 h HRT operational periods (3.9×10^8 copies/ml to 9.0 ×10^8 copies/ml), when comparing to IFB (2.6×10^8 copies/ml to 4.0×10^8 copies/ml), possibly meaning that the greater the number is, the bigger the difference that will be visualized on the NMS plot based on absolute numbers.

By contrast, NMS output based on the relative abundance matrix, showed a remarkable shift in community structure between the same biomass samples taken from the IFB reactor during the 18h and 12h HRT periods (Figure 15 b). This was paralleled by the rapid increase in Methanomicrobiales, Methanosarcinaceae and Methanosaetaceae (10-fold increase in the MMB, 4.6-fold increase in the Msc and 2-fold increase in the Mst) during the corresponding period (Figure 14). Interestingly, the relative abundance matrix did not show any great shift in the target microbial populations in the samples simultaneously taken from EGSB reactor – the obverse of the situation when output was based on the absolute quantification matrix. This result was consistent with the much smaller increases in the size of the populations, recorded during this period (2.4- fold increase in MBT, 3- fold increase in MMB and 2- fold increase in Mst).
Figure 15. Nonmetric Multidimensional Scaling (NMS) analysis based on a) absolute quantity and b) relative abundance of target methanogenic groups measured by real-time PCR.

Moving window analysis was also applied to investigate the dynamic shifts in community compositions of the IFB and EGSB reactors. It was previously demonstrated to be a useful tool to visualize large differences in microbial community dynamics between two reactors, which were inoculated with the same sludge (Wittebolle et al., 2008). Two approaches, again based on absolute quantity and relative abundance matrices as in case of NMS analysis, were employed to visualize shifts in quantitative community composition (Figure 16 a & b). Moving window output, based on the absolute quantity matrix, demonstrated that the variation in each reactor was ≤ 20% during operation within the HRT range of 72 to 18 h. A much greater (40%) change in the EGSB community was detected in the reactor biomass, however, following reduction in the applied HRT from 18 to 12 h, far larger than the 20% change, which was recorded for the IFB reactor at the same time (Figure 16 a). On the other hand, and supporting the findings based on NMS, the relative abundance-based analysis exhibited that the EGSB community was relatively stable with < 20% variation throughout the trial (≤ 5% within the HRT range of 48 to 12 h), whereas the IFB community showed a marked change of >20%, after the decrease in HRT to 12 h (Figure 16 b).
These results clearly demonstrated that the community changes derived from the two different matrices were significantly different (Figure 15 a & b, Figure 16 a & b). Given the performance of the IFB reactor between the 18 and 12 h HRT periods, however, with a decrease in COD removal efficiency (Figure 12) and a corresponding increase in effluent VFA concentrations (Figure 13), the moving window analysis based on the relative abundance of target methanogenic groups appeared to better reflect the performance changes in our systems. Additionally, results from moving window analysis of relative abundance correspond well to the relative abundance NMS matrix, where the community profiles of IFB biomass, sampled during the 18h HRT period were most widely dispersed and distantly located from biomass sampled during the 12 h period.

The selection of appropriate tools for analysis of molecular data from anaerobic bioreactor (or other environmental samples) is important so that useful information on the relationship between community structure and process performance can be generated. This study suggests that the outputs derived from a relative abundance matrix could possibly avoid potentially distorted outputs, due to the influence of one dominant variable as presented, for example, by the absolute quantity-matrix relationship between EGSB biomass sampled during the 18 h and 12 h HRT periods. The relative abundance-based method, although based on reliable absolute quantification, possibly has a more meaningful correlation to process performance and might be useful, therefore, in diagnosing and/or assessing anaerobic digesters, especially in systems (such as the retained biomass systems used in this study) operated under reasonably constant conditions with no drastic changes in total microbial mass. When dealing with a system with a high fluctuation of active biomass (e.g. suspended biomass continuously stirred tank reactors), the absolute quantity-matrix may be more applicable – this suggestion should be investigated further.
**Figure 16.** Moving window analysis based on a) absolute quantity and b) relative abundance of target methanogenic groups measured by real-time PCR.

### 2.3.4 Archaeal DGGE and phylogenetic analysis

In order to link the Order and Family data from qPCR with basic information on diversity and phylogeny of the methanogens in the IFB and EGSB biomass, community fingerprinting was carried out using DGGE. This approach has been used widely in environmental microbiology to study diversity and relative abundance shifts of microbial populations in complex systems, including anaerobic bioreactors (Lee *et al.*, 2008, Fernandez *et al.*, 2008, Diaz *et al.*, 2006). Extracted DNA samples were analyzed to investigate changes in methanogenic community structure, with respect to the HRT changes, in the reactors tested. A total of 19 bands, designated AD 1-19, were visually detected (Figure 17) and aseptically excised from the gel for subsequent sequencing analyses. The phylogenetic affiliations of the band sequences were determined by comparing against the GenBank database (Table 4).

AD 6, 11, 13, 15, 16, 17 and 18 were dominant in both EGSB and IFB reactors, and they were all affiliated to the order *Methanobacteriales* (MBT). Four of them (i.e., AD 6, 11, 13 and 18) were closely related (> 97% sequence similarity) to *Methanobacterium* species with 98.9-100% similarities. AD18, affiliated to *M.*
beijingense, was previously isolated from an EGSB bioreactor treating synthetic glucose wastewater (O’Reilly et al., 2009). M. formicicum (AD 6, 11, 13), was previously observed in anaerobic sludge (Collins et al., 2003). The other three bands (i.e., AD 15, 16, 17), were not closely related to any known species with < 90% similarities.

Metanomicrobiales (MMB) related bands (i.e., AD 1, 2, 3, 9 and 14) were detected only in the IFB reactor. Four of them (i.e., AD 1, 2, 9 and 14) were commonly closely related to Methanoculleus species: M. bourgensis, M. palmolei, M. marisnigri with 98.2-98.5% similarities and were previously reported in sludge samples from EGSB (Collins et al., 2003), while AD 3 was closely related to Methanospirillum hungatei (98.2%), also formerly observed in anaerobic sludge (Fernandez et al., 2008; O’Reilly et al., 2009). Interestingly, abundant MMB populations were mainly detected by DGGE in the IFB reactor following the transition to a 12 h HRT (apart from band AD14 which was also visible at 18 h HRT). This corresponded very well with the increased 16S rRNA gene concentration detected by qPCR in 12 h HRT IFB biomass sample. It has been previously reported that some Metanomicrobiales, especially M. bourgensis, M. palmolei and M. marisnigri require acetate as a growth factor, or are greatly stimulated by this organic compound (Ollivier et al., 1986; Maestrojuan et al., 1990; Zellner et al., 1998).

Sequences AD12 and 19 were closely related to Methanosarcina mazei and Methanosarcina lacustris with 99.6%-100% similarity (Table 1). M. mazei has been frequently observed in anaerobic digestion systems (Diaz et al., 2006) and is able to utilize a variety of substrates, including acetate, H₂/CO₂, methanol and methylamines (Boone et al., 2001). All Methanosaetaceae-related bands (i.e., AD 4, 5, 7, 8 and 10) were apparent in both reactors and closely related to Methanosaeta concilii with 99.6% similarity. Although DGGE is a qualitative method, stronger band intensity observed in samples from EGSB suggested predominance of M. concilii-like organisms in this reactor (Figure 17).

As stated above, the majority of detected Metanomicrobiales populations were related to Methanoculleus species. It is suggested, therefore, that the dominance of MMB in IFB reactor was directly related to the higher in-reactor acetate
concentrations, which enhanced the specific growth rate of those organisms. The absence of MMB-related bands in EGSB samples (although detected by qPCR) can be explained by the much greater concentration of MMB in the IFB (18-90-fold higher during the trial) and relatively lower sensitivity of DGGE (Talbot et al., 2008), compared to qPCR.

Figure 17. Denaturing Gradient Gel Electrophoresis (DGGE) profiles of temporal archaeal 16S rRNA genes from the IFB and EGSB reactors indicating the excised bands used for sequencing and phylogenetic analysis. Phylogenetic affiliation to order or family level: MBT (Methanobacteirales), MMB (Methanomicrobiales), MST (Methanosetaeae), MSC (Methanosarcinaceae).
Table 4. Phylogenetic affiliation of the 16S rRNA gene sequences from DGGE bands.

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2.4 Conclusion

Our study showed that quantitative methanogenic community composition data, expressed both as absolute and relative abundances, can provide valuable and comparative information on the condition of anaerobic wastewater treatment systems. NMS and moving window analysis, based on relative abundance matrices, were apparently more relevant for linkage of methanogenic population shifts and reactor performance during steady state conditions in this specific study. Distinctive community development profiles were recorded in the IFB and EGSB reactors, which were influenced by variations in reactor performance and in-reactor VFA concentrations during the trial.
2.5 References


Appendix 1: % Biogas CH$_4$ data of IFB and EGSB (Chapter 2)
Chapter 3

Microbial community structure and dynamics in anaerobic fluidized-bed and granular sludge-bed reactors: influence of operational temperature and reactor configuration.

A version of this chapter was accepted to: Microbial Biotechnology

Abstract

Methanogenic community structure and population dynamics were investigated in two different, replicated anaerobic wastewater treatment reactor configurations (Inverted fluidized bed (IFB) and Expanded granular sludge bed (EGSB)) treating synthetic dairy wastewater, during operating temperature transitions from 37˚C to 25˚C, and from 25˚C to 15˚C, over a 430-day trial. Nonmetric multidimensional scaling (NMS) and moving window analyses, based on quantitative real-time PCR data, along with denaturing gradient gel electrophoresis (DGGE) profiling, demonstrated that the methanogenic communities developed in a different manner in these reactor configurations. A comparable level of performance was recorded for both systems at 37 and 25˚C, but a more dynamic and diverse microbial community in the IFB reactors supported better stability and adaptative capacity towards low-temperature operation. The emergence and maintenance of particular bacterial genotypes (phylum Firmicutes and Bacteroidetes) was associated with efficient protein hydrolysis in the IFB, while protein hydrolysis was inefficient in the EGSB. A significant community shift from Methanobacteriales and Methanosetaeae dominated community towards Methanomicrobiales-predominated community was demonstrated during operation at 15˚C in both reactor configurations.
3.1 Introduction

Bioenergy production from waste streams is a key component in the global development of sustainable energy sources (Demirel et al., 2010). Indeed, anaerobic digestion (AD) is poised to replace aerobic microbiological treatments as the core process of waste-to-energy technologies for enhanced sustainability in the coming decades (Verstraete & Gusseme, 2011). During AD, organic substrates are sequentially degraded by fermentative and acetogenic bacteria to simple precursor compounds, such as acetate, H$_2$/CO$_2$, formate and methanol, from which methanogenic Archaea produce a methane-rich biogas. Temperature can influence the rate and path of carbon flow during methanogenesis by affecting the activity of particular microbial groups and the structure of the microbial consortia (McKeown et al., 2009a; O'Reilly et al., 2009b; Siggins et al., 2011c).

Low-temperature AD (LTAD) has emerged as an economically attractive waste treatment strategy, which confers considerable advantages over conventional mesophilic (~30°C) and thermophilic (~55°C) treatments, primarily due to the capacity to treat the wide variety of cool, dilute wastewaters, previously considered as not suitable for AD (McKeown et al., 2011). LTAD has been successfully applied at laboratory- and pilot-scale, using a variety of reactor types, for the treatment of a broad range of wastewaters (for example, (Bergamo et al., 2009; Collins et al., 2006a; Collins et al., 2003; Lettinga et al., 2001; McHugh et al., 2004; McKeown et al., 2011; Syutsubo et al., 2008). LTAD is an attractive technology because the process is stable, simple to operate and requires very low energy input. Improved reactor designs enable high rates of methanogenic conversion at low temperatures through a combination of: (i) high mixing intensities (i.e., facilitating high rates of mass transfer); and (ii) enhanced retention of psychroactive biomass (Alvarado-Lassman et al., 2008; Lettinga et al., 2001; McKeown et al., 2011). However, failure of the bioreactors to retain granular sludge during LTAD, may lead to severe hydraulic wash-out of psychroactive sludge (Lettinga et al., 1999), therefore non-granule-based systems, using inert nuclei to promote re-granulation could be of advantage during psychrophilic reactor operation (McKeown et al., 2009b).

Knowledge gaps remain, however, regarding the nature and function of the microbial populations involved in LTAD, which are a deterrent to full-scale applications.
This information deficit is mainly due to the complex relationship between wastewater characteristics, process conditions and dynamics in microbial community structure. In an attempt to link microbial functional groups with process performance, we studied community dynamics in two different methanogenic anaerobic reactor configurations [i.e., an inverted fluidized bed (IFB) containing fixed biomass on the fluidized support particles and an expanded granular sludge bed (EGSB) containing crushed granular biomass], during operational temperature transitions from 37°C to 25°C, and from 25°C to 15°C. The present study is a continuation of the experiment described in Chapter 2 (Bialek et al., 2011), therefore a similar experimental approach has been employed. The methanogenic community structure and population dynamics were examined qualitatively by DGGE, and quantitatively by real-time PCR targeting the 16S rRNA genes, and the results were statistically analysed and compared. We hypothesized that the process performance and microbial community structure and population dynamics can be influenced by two different reactor configurations during transition from mesophilic to psychrophilic reactor operation and changes applied in the operating conditions [i.e., loading rate and hydraulic retention time].

### 3.2 Materials and methods

#### 3.2.1 Reactor operation and biomass sampling

Replicate laboratory-scale IFB (IFB1 and IFB2) and EGSB (EGSB1 and EGSB2) reactors treating synthetic skimmed dairy wastewater (Table 7) were operated continuously for 430 days, in three periods (I to III) differentiated by operating temperature, from 37°C (period I, days 1-107), 25°C (period II, days 108-234), and 15°C (period III, days 235-430). This study is a continuation of the 200 day experiment at 37°C described in Chapter 2 (Bialek et al., 2011), therefore similar experimental approach has been employed. All reactors were operated at 24 h hydraulic retention time (HRT), until day 294, when the HRT was changed to 48 h and further to 36 h on day 409. The performance of the reactors was evaluated on the basis of organic loading rate (OLR), chemical oxygen demand (COD), removal efficiency (RE), elimination capacity (EC) and proteins removal efficiency (PRE). During periods I-II, the reactors were subjected to a fixed loading (OLR of 167 mg
COD l⁻¹ h⁻¹ and HRT of 24 h) and during period III (15°C), the reactors were subjected to variable loading rates of: 167, 83 and 111 mg COD l⁻¹ h⁻¹, corresponding to 24 h, 48 h and 36 h HRTs, respectively. The length of each period was determined based on the stability of the reactor performance over time. RE, OLR and EC were calculated as described by Kumar et al. (2011).

For microbial community analysis, biomass samples were directly collected from each reactor at every temperature tested and designated accordingly for the duplicate IFB and EGSB reactors as: T1 (37°C, Day 106); T2 (25°C, day 195); T3a (15°C, Day 298), T3b (15°C, Day 365); T3c (15°C, Day 409), T3d (15°C, Day 430). Biomass was sampled in duplicate (2×50 ml). Biomass for T1 and T2 was sampled during steady state and prior to changing operating conditions. During 15°C operation (T3), biomass was sampled more frequently to reflect the influence of operating and environmental conditions on the microbial community composition. Samples were first mechanically disrupted by manual grinding with a pestle and mortar and diluted 10-fold with deionised and distilled water (DDW) prior to DNA extraction as described previously in Chapter 2 (Bialek et al., 2011). All DNA extractions were performed in duplicate.

Biomass samples collected directly from each reactor were also fixed for scanning electron microscopy analysis (SEM) using the protocol described by Katuri et al. (2010).

### 3.2.2 qPCR

Real-time PCR (qPCR) analysis was performed using a LightCycler 480 instrument (Roche, Mannheim, Germany). In this study qPCR analysis was performed using two methanogenic order-specific primer and probe sets: *Methanobacteriales* (MBT), *Methanomicrobiales* (MMB), and two methanogenic family-specific primer and probe sets: *Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst), which cover most methanogens in anaerobic digesters (Lee et al., 2009; Yu et al., 2005a; Yu et al., 2005b). Methanogens are classified into five orders within the domain *Archaea* which can be grouped into two guilds, aceticlastic and hydrogenotrophic methanogens, determined by methane production pathways. Aceticlastic methanogens include only *Methanosarcinales* which comprises two families,
Methanosaetaceae utilizing only acetate and Methanosarcinaceae utilizing acetate as well as various methyl compounds and hydrogen (Boone et al., 2001). Because those two aceticlastic methanogens play crucial role in overall methanation (i.e., >70% of methane is originated from acetate in most anaerobic systems) and the significant physiological differences between the two aceticlastic families (Hori et al., 2006; Yu et al., 2006), they were monitored at family level, instead of order level. Hydrogenotrophic methanogens comprise of four orders, i.e., Methanobacteriales, Methanococcales, Methanomicrobiales, and Methanopyrales, which utilize only H₂+CO₂, formate or methanol to produce methane (Boone et al., 2001). Because the Methanopyrales members are not likely to be present in anaerobic processes due to their extremely high growth temperature (>80°C) (Boone et al., 2001) and the members of Methanococcales (MCC) are not normally found in anaerobic reactors, presumably since organisms from this group require high-salt conditions for their growth (0.3-9.4% (w/v) NaCl) (Boone et al., 2001), these two orders were left out of consideration in this study. All DNA templates were analysed in duplicate. Quantitative standard curves were constructed using the representative strains corresponding to each primer and probe set, targeting the specific methanogenic groups (MBT, MMB, Msc, Mst), as described previously (Bialek et al., 2011; Yu et al., 2005b).

3.2.3 Statistical analysis

Nonmetric multidimensional scaling (NMS) was performed based on the real-time PCR results to visualize the quantitative shifts of methanogenic populations during reactor operation (McCune & Grace, 2002). The absolute quantity matrix of the target methanogenic groups detected by the qPCR assay (MBT, MMB, Msc and Mst) was created. Moving window analysis was also carried out based on the absolute quantity matrix to monitor the variations in methanogenic community composition associated with decreases in the applied temperature of consecutive samplings in all reactors (Bialek et al., 2011; Wittebolle et al., 2008). The community similarity between two consecutive time points was used as the indicator of community variation in response to the corresponding temperature change. Sørensen distance
measure in the PC-ORD software ver. 5.0 (McCune & Grace, 2002) was employed for both analysis.

### 3.2.4 Archaeal and bacterial DGGE

Archaeal and bacterial 16S rRNA genes were amplified by PCR using the primer sets ARC GC 787F and ARC 1059R (Takai & Horikoshi, 2000), BAC GC 338F and BAC805 R (Yu et al., 2005a; Yu et al., 2005b), respectively. Touchdown PCR, PCR products purification, sequencing, sequencing alignment and phylogenetic analyses were performed as described previously in Chapter 2 (Bialek et al., 2011). DNA from DGGE experiments were sequenced in Germany by Eurofins MWG Operon. Phylogeny was calculated using the Neighbor-Joining method (Saitou & Nei, 1987). Bacterial distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and archaeal distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The bootstrap consensus trees inferred from 1000 replicates are taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985).

The Unweighted Pair Group Method with Arithmetic mean (UPGMA; (McCune & Grace, 2002)) was selected to perform statistical analysis of the DGGE profiles. The scanned DGGE gel image was processed with Phoretix 1D (previously TotalLab TL120 software; TotalLab Ltd, Newcastle upon Tyne, UK) to construct a binary matrix, where the presence or absence of each band was scored with 1 or 0, respectively, without considering band intensity. Construction of the dendogram via hierarchical clustering was performed based on the Sørensons (Bray-Curtis) distance measurement using MEGA 4 software (Tamura et al., 2007).

All nucleotide sequence data reported in this study were deposited in the GenBank database under accession numbers ARC: JF952003-JF952011 and BAC: JF927800-JF927829.


3.2.5 Analytical analysis

Biogas and effluent from all reactors were sampled every second day to analyse respectively, the biogas methane content and residual effluent COD concentration according to Standard Methods (APHA, 2005). Analysis of effluent volatile fatty acids (VFA) were performed in a Varian Saturn 2000 GC/MS system, with CombiPAL autosampler (Varian Inc., Walnut Creek, CA) as described previously in Chapter 2 (Bialek et al., 2011). Total protein quantification in the effluent samples was performed using the RC DC protein assay kit (Bio-Rad). All analyses were performed in duplicate.

3.3 Results

3.3.1 Process performance

The process efficiency depended on the operational temperature (Figure 18): during operation at 37°C (period I) and 25°C (period II), the replicated IFB and EGSB reactors exhibited a similar level of performance exceeding 80% COD removal efficiency (RE) (Elimination capacity (EC) of 139±13 and 148±8 mg COD l⁻¹ h⁻¹ for IFB1 and IFB2; EC of 152±8 and 155±8 mg COD l⁻¹ h⁻¹ for EGSB1 and EGSB2) and >80% Protein Removal Efficiency (PRE; Figure 18); with >60% methane content in the biogas (Appendix 2). The effluent concentrations of volatile fatty acids (VFA) fluctuated, with maximum values of >1500 mg COD l⁻¹ in the IFB and EGSB reactors.

The decrease in operational temperature to 15°C resulted in a gradual reduction in the treatment efficiency, an instantaneous decrease in residual VFA concentrations (<200 mg COD l⁻¹) and a significant drop in PRE, to 60%, in the reactors (Figure 18). The IFB reactors, however, recovered after the temperature decrease and recorded stable >70% COD RE within 30 days (Figure 18). The EGSB reactors displayed a greater initial decline in performance (EGSB1 to 60% COD RE, EC of 105 mg COD l⁻¹ h⁻¹ and EGSB2 to 50% COD RE, EC of 86 mg COD l⁻¹ h⁻¹), although EGSB1 returned to a more stable COD RE afterwards (Figure 18).

The applied hydraulic retention time (HRT) was increased to 48 h on day 294, resulting in an increase in the treatment efficiency of all four reactors (Figure 18). An
unexpected temperature decrease (to 6°C), due to a heating pump failure on day 350, destabilized all systems but a more pronounced deterioration in the PRE (13% PRE), was observed in the EGSB system (Figure 18). After recovery and stabilization, where the COD RE of all four reactors exceeded 80%, the applied HRT to both systems was reduced to 36 h, on day 409. Interestingly, the performance of the IFB reactors remained very stable with >78% COD RE and >77% PRE (Figure 18), while a significant transient drop in the treatment efficiency of the EGSB reactors occurred (to 50% COD RE and 55% PRE; Figure 18).
Figure 18. Process performance of the inverted fluidized bed (IFB) (A) IFB2 (B) IFB1 and expanded granular sludge bed (EGSB) (C) EGSB2 (D) EGSB1 reactors.
3.3.2 Bacterial DGGE and UPGMA cluster analysis

Comparative PCR-DGGE (denaturing gradient gel electrophoresis) analysis of the bacterial populations in the reactors identified configuration-dependent differences. Unweighted Pair Group Method with Arithmetic mean (UPGMA) cluster analysis revealed that the composition, and development during the trial, of the bacterial portion of the microbial communities in the IFB and EGSB reactors was distinctively different (e.g. <58% similarity between IFB2 and EGSB2; Figure 19). Identical DGGE profiles (100% similarity) were recorded from the IFB reactor at 37°C (IFB T1) and 25°C (IFB T2). The profiles changed, however, following the temperature reduction to 15°C (IFB T3abcd; 72% similarity). Likewise, similar communities (>95% similarity) at 37°C (EGSB T1) and 25°C (EGSB T2) within the EGSB reactor changed immediately after temperature reduction to 15°C (EGSB T3ab; 86% similarity). Further, subsequent changes in the EGSB bacterial populations (EGSB T3cd) resulted in < 72% similarity to the former profiles (Figure 19).

![Figure 19](image)

Figure 19. Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of the 16S rRNA gene fragments generated from bacterial denaturing gradient gel electrophoresis (DGGE) profiles of IFB2 and EGSB2 biomass. Similarity calculated by Sørensons (Bray-Curtis) distance measurement. B1-B30 indicate bands used for sequencing and phylogenetic analyses.
3.3.3 Bacterial and archaeal phylogenetic analyses

In total, 30 DGGE bands, designated as B1-B30, were retrieved from the bacterial DGGE gel and used for subsequent sequencing analysis. Ten bands out of the 30 (B1, B2, B7, B10, B11, B16, B18, B19, B20, B24) were unique to the IFB reactor and 15 common bands were noted between the IFB and EGSB reactors (Table 5; Figure 20a). In the archaeal DGGE gel, a total of 9 bands (A1-A9) were retrieved and sequenced (Table 6; Figure 20b).
### Table 5. Phylogenetic affiliation of the 16S rRNA gene sequences from bacterial DGGE bands B1-B30 (accession numbers: JF927800-JF927829).

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<th>Band number</th>
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<th>Nearest species and taxon details</th>
<th>Accession no.</th>
<th>Reactor biomasses containing the respective bands</th>
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<td>Proteobacteria</td>
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<td>Seed; IFB&amp;EGSB T1 T2 T3 abcd (Day106, 195, 298, 365, 409, 430)</td>
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<td></td>
<td>Methanobacterium subterraneum</td>
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<td>DQ649330</td>
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<td>Methanobacterium palustre</td>
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<td>DQ649333</td>
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<tr>
<td>A8 JF952010</td>
<td>Methanospirillum hungatei</td>
<td>Methanomicrobiales</td>
<td>99</td>
<td>AB517987</td>
<td>EGSB T3 abcd (Day 298, 365, 409, 430)</td>
</tr>
<tr>
<td>A9 JF952011</td>
<td>Methanobacterium petrolearium</td>
<td>Methanobacteria</td>
<td>99</td>
<td>AB542742</td>
<td>Seed; IFB&amp;EGSB T1 T2 T3 abcd (Day 106, 195, 298, 365, 409, 430)</td>
</tr>
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<td></td>
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<td>Methanobacterium palustre</td>
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<td>99</td>
<td>DQ649333</td>
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Figure 20. Neighbor joining tree illustrating the phylogenetic affiliations of the 16S rRNA gene sequences obtained from: (A) bacterial DGGE bands B1-B30 (accession numbers: JF927800-JF927829) and (B) archaeal DGGE bands A1-A9 (accession numbers: JF952003-JF952011). Reactor biomasses containing the respective bands are given in parenthesis.
3.3.4 Dynamic changes in archaeal populations observed by real-time PCR

The EGSB and IFB reactors displayed a noticeable disparity in terms of the quantitative composition of the archaeal populations within the methanogenic community during the trial (Figure 21 a & b). In particular, a notable difference between the samples taken during mesophilic (37°C-25°C) and psychrophilic (15°C) reactor operation was an increase in the relative abundance and absolute quantity of the hydrogenotrophic order Methanomicrobiales (MMB), which became by far the most prominent group in all reactors under low-temperature conditions. During mesophilic operation, Methanomicrobiales accounted for only 2.5-3.6% (2.9 - 4.3×10⁶ copies/ml) of the total methanogenic 16S rRNA gene concentration in IFB2 and IFB1 and 0.0-3.3% (1.4×10⁴ - 1.1×10⁶ copies/ml) in EGSB2 and EGSB1, respectively. The emergence to predominance of Methanomicrobiales was evident in all reactors. In IFB1 and IFB2, the relative abundance of this group had reached 52.8% (1.5×10⁷ copies/ml) and 96.7% (4.7×10⁸ copies/ml) of the total methanogenic 16S rRNA gene concentration by the end of the trial. In EGSB2 and EGSB1 Methanomicrobiales accounted for 45.7% (3.1×10⁷ copies/ml) and 87.7% (1.2×10⁸ copies/ml) of the total methanogenic 16S rRNA gene concentration by day 430.

The aceticlastic family Methanosetaeaceae (Mst) was the most abundant group in the seed biomass accounting for 58.4% of the total methanogenic 16S rRNA gene concentration. During 37°C reactor operation, the 16S rRNA gene concentration of Methanosetaeaceae was detected at 2.1 - 3.1×10⁶ copies/ml (18.0-26.2% of the total methanogenic 16S rRNA gene concentration) in the IFB2 and IFB1, respectively. In EGSB2 and EGSB1, the 16S rRNA gene concentration of Methanosetaeaceae was 5.0×10⁶ and 1.5×10⁷ copies/ml (16.1 and 42.9% of the total methanogenic 16S rRNA gene concentration), respectively. At the end of the trial at 15°C, an increase in the 16S rRNA gene concentration of this group was detected at 9.9×10⁶ and 1.1×10⁷ copies/ml (2.0 and 38.9% of the total methanogenic 16S rRNA gene concentration) in IFB2 and IFB1, respectively. A similar trend was observed in EGSB1 and EGSB2 with the 16S rRNA gene concentration of 1.5 - 3.0×10⁷ copies/ml (10.6-45.0% of the total methanogenic 16S rRNA gene concentration).
The aceticlastic family, *Methanosarcinaceae* (Msc), was only detected (i.e., >1.28×10^3 copies/µl) in the IFB reactors and this could have been influenced by presence of this group during previous experiment as described in Chapter 2 (Bialek et al., 2011), since present experiment is a continuation of the former. The 16S rRNA gene concentration of this group showed a marked increase from 1.6×10^4 and 6.5×10^3 (0.1 and 0.1% of the total methanogenic 16S rRNA gene concentration) at 37˚C to 5.3×10^5 and 2.6×10^6 copies/ml (1.8 and 0.5% of the total methanogenic 16S rRNA gene concentration) at 15˚C in IFB1 and IFB2, respectively, at the beginning and at the end of the trial. *Methanosarcina* numbers could have increased and remained above the detection limit (presumably by retention in biofilms during mesophilic operation with high accumulation of VFA) when the IFB reactors were later operated at 15˚C, although no accumulation of acetic acid (reported to be associated with appearance of *Methanosarcina* and process deterioration (O'Reilly et al., 2009b) was observed during that period (period III; Figure 18).

The hydrogenotrophic order *Methanobacteriales* (MBT) was the most dominant group at mesophilic temperatures and the 16S rRNA gene concentration of this group was detected at 8.3-9.3×10^6 copies/ml (70.1-79.4% of the total methanogenic 16S rRNA gene concentration) in IFB1 and IFB2, and 1.8 - 2.6×10^7 copies/ml (53.8-83.9% of the total methanogenic 16S rRNA gene concentration) in EGSB1 and EGSB2, respectively. Following a reduction in the temperature to 15˚C, a decrease in the 16S rRNA gene concentration of this group was observed at 1.9 - 3.4×10^6 copies/ml (6.2-0.7% of the total methanogenic 16S rRNA gene concentration) in IFB1 and IFB2, and 2.4 - 6.4×10^6 copies/ml (1.7-9.4% of the total methanogenic 16S rRNA gene concentration) in EGSB1 and EGSB2, respectively.
Chapter 3

A

16S rRNA gene copy concentration (copies/ml)

- Methanobacteriales
- Methanomicrobiales
- Methanosarcinaceae
- Methanosaetaceae

Seed, T1 (106 d), T2 (195 d), T3a (298 d), T3b (365 d), T3c (409 d), T3d (430 d)
Chapter 3

Figure 21. Absolute quantification of the 16S rRNA gene concentration of the methanogenic/archaeal populations during transition from mesophilic (37 to 25°C) to psychrophilic (15°C) reactor operation in the (A) IFB and (B) EGSB reactors.
3.3.5 Quantitative shifts in archaeal populations

Quantitative shifts in the methanogenic communities were visualized using Nonmetric multidimensional scaling (NMS) technique and moving window analysis, based on real-time PCR data. Unlike presented in Chapter 2 (Bialek et al., 2011), the absolute quantity-matrix based on 16S rRNA gene concentration data appeared to be more applicable in describing the transition from mesophilic to psychrophilic temperature operation with community shift towards hydrogenotrophic methanogens during the 430-day trial.

In the NMS plot, the cumulative $r^2$ represented by the axes was $> 0.9$, the final stress value was $< 5$, and instability was $< 10^{-4}$ (Figure 5). This indicates that our results meet the criteria for an excellent representation (McCune & Grace, 2002). The NMS results revealed that the archaeal populations in the reactors shifted in a different manner and grouped separately in response to decreasing temperature (Figure 22 a), despite the identical operating conditions. This suggests that the reactor configuration may have a significant effect on the shaping of the methanogenic community structure. The absolute quantity matrix clearly demonstrated a remarkable shift in community structure of the IFB reactors when lowering the temperature (I1 and I2 T2 to T3a). Wide shifts, which were especially prominent after transition to 15°C (I1 and I2 T3a to T3d), were paralleled by the rapid increase in *Methanomicrobiales* and *Methanosarcinaceae* populations during the corresponding period. Interestingly, the EGSB matrices showed much smaller shifts in community structure following temperature decrease (E1 and E2 T2 to T3a). Subsequently, at 15°C a similar trend as in the IFB reactors was observed in the community shift of the EGSB reactors, although much slower than in the IFB reactors. This shift can be mostly attributed to increasing *Methanomicrobiales* and *Methanosacetaceae* populations (E1 and E2 T3a to T3d). Consequently, the IFB and EGSB matrices were located distantly based on samples taken during 15°C operation, demonstrating the different community composition of IFB and EGSB reactors, which could be attributed to slower adaptation of the EGSB biomass (Figure 22 a).

Moving window analysis was applied to observe dynamic shifts in the community composition of the IFB and EGSB reactors (Figure 22 b). An approach based on the absolute quantity of target methanogenic groups was employed to visualize shifts in
quantitative community composition. Moving window output of the IFB reactors showed a significant dissimilarity of 87-88% in the IFB1 and IFB2 between T2 (day 195) and T3a (day 298), and between T3a (day 298) and T3b (day 365), respectively. In contrast, the output from the EGSB reactors indicated 56-51% dissimilarity in the EGSB1 and EGSB2 between T3b (day 365) and T3c (day 409), and between T3c (day 409) and T3d (day 430), respectively. The time difference in shifts of the methanogenic communities between the IFB and EGSB reactors could indicate that the IFB reactors responded much quicker to the temperature decrease of 15°C, while in the EGSB reactors this change was slower and methanogenic communities required more time to adapt (Figure 22 b).
3.4 Discussion

3.4.1 Process performance

Dairy wastewater is a complex substrate composed of easily degradable carbohydrates, mainly lactose, and less bioavailable proteins and lipids (Fang, 2000; Tommaso et al., 2003), which are responsible for the typical problems associated with high-rate anaerobic digestion of dairy waste effluents (Perle et al., 1995). Hydrolysis of proteins and lipids is reported to strongly decline with decreasing temperature, especially approaching 15°C (Tommaso et al., 2003). Given these reports, and considering the fact that skimmed dairy wastewater (Table 7) was used in the present study, decreased protein degradation/hydrolysis can be assumed to be mainly responsible for the declining process performance in our digesters.

Casein is the major protein in milk (up to 80% of the total proteins) and in dairy effluents. When fed to acclimated anaerobic reactors, degradation of casein is rapid, due to strong proteolytic activity, and the degradation products are non-inhibitory (Perle et al., 1995). This was likely the case in the systems investigated, with >80%
PRE recorded during acclimated mesophilic conditions of 37-25°C (Figure 18). The fluctuations in effluent VFA concentration (Figure 18) observed at mesophilic temperatures were therefore, most probably due to rapid hydrolysis and fermentation of carbohydrates and proteins into VFA. A gradual decrease in COD RE (Figure 18), however, occurred immediately after the temperature reduction to 15°C, coupled with an instantaneous decrease in residual VFA concentrations and significant drop in PRE of ca. 60% in the IFB and EGSB reactors. Since no VFA build-up was observed at 15°C, it is considered that protein hydrolysis had become the rate-limiting step.

Following the immediate drop in PRE to 60%, the IFB system demonstrated a successful adaptation to low temperature operation and, after a brief temporal instability, >78% COD RE and >77% PRE was recorded at psychrophilic, steady-state operation (Figure 18). On the other hand, the EGSB system displayed a slower adaptation to low temperature operation, with performance at 58% PRE 45 days after the temperature drop, and ongoing unstable performance, with minimum values of 13% PRE and < 60% COD RE (Figure 18).

<table>
<thead>
<tr>
<th>Table 7. Composition of skimmed-milk powder.</th>
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<tr>
<td>Parameter</td>
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<td>Proteins</td>
</tr>
<tr>
<td>Sugars</td>
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<td>Fats</td>
</tr>
<tr>
<td>Others</td>
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<tr>
<td>Total</td>
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We propose that the better flexibility and adaptability of the IFB biomass to low temperature might originate from the spatial arrangement of fixed fluidized biomass that developed in the IFB (Appendix 3) playing an important role in differences in transfer of intermediates and optimal degradation of substrates (Grotenhuis et al., 1991). At low temperatures, the viscosity of effluents increases and, therefore, the diffusion of soluble compounds will drop, particularly in sludge bed reactors that become less easily mixed (Lettinga et al., 2001). There was no evidence of granulation of the biomass in the EGSB reactor, which was seeded with crushed
granular sludge as inoculum and the biomass remained a non-granular flocculant sludge throughout the trial (Appendix 3). Further, the advantage of the IFB configuration over the EGSB in this experiment might arise from the use of floatable particles with a specific density lower than the liquid, thus particles were fluidized downward (Garcia-Calderon et al., 1998) and better substrate–biomass contact might be attained. Due to the large specific area the support particles can retain more biomass (Alvarado-Lassman et al., 2008), which is especially crucial during transitional and permanent changes in operating conditions, like the temperature variation investigated in this study.

3.4.2 Microbial population dynamics

Understanding the impact of disturbances, such as a temperature shocks or permanent temperature decrease, on process stability and performance, will undoubtedly shed more light on the process of LTAD when placed in the context of microbial community dynamics. Together with increased knowledge on the impact of reactor configuration on the functional stability of microbial communities, informed decisions can be enabled regarding the optimal reactor type and process conditions for a given wastewater. A robust LTAD system must possess the ability to maintain process stability in response to disturbances and it has been reported that in general systems with more dynamic communities have greater functional and process stability (Carballa et al., 2011; Hashsham et al., 2000; Werner et al., 2011). We thus considered our data in the context of the model proposed by Allison and Martiny (2008), which divides the population dynamics that maintain community function over time into three basic mechanisms (resistance, resilience or redundancy) to address the process performance of the IFB and EGSB reactors investigated.

A microbial composition is resistant if it is similar across a variety of environmental conditions and, therefore, it is difficult to perturb from an original state (Allison & Martiny, 2008). Initially, identical bacterial populations at mesophilic temperatures, as observed by the UPGMA cluster analysis suggested resistance of the bacterial populations composition (Figure 19): 100% similarity between IFB T1 (37°C) and IFB T2 (25°C) and >95% similarity between EGSB T1 (37°C) and EGSB T2 (25°C).
A similar trend was observed with the archaeal populations' behaviour between the two studied mesophilic temperatures where, for example, the EGSB and the IFB populations showed 80% similarity between T1-T2 based on the NMS and moving window matrix (Figure 22 a & b). The methanogenic community composition was thus resistant to the temperature change from 37-25°C, indicating metabolic flexibility and physiological tolerance to the applied disturbance in this mesophilic temperature range. However, further temperature reduction to 15°C showed that the community was sensitive to the disturbance and resulted in an altered microbial composition (Figure 22 a & b), indicating that the community responded to the applied temperature disturbance using one of the mechanisms discussed below.

The microbial composition of reactor biomass is resilient if it is sensitive to a disturbance and changes, but quickly recovers to its initial composition (Allison & Martiny, 2008). This mechanism was not observed in the systems and timescale investigated because after the disturbance the community did not return to original composition. Werner et al. (2011) reported that resilience was important to maintain function of syntrophic populations over time in brewery wastewater treatment facilities. These authors concluded that syntrophic bacteria had very specialized metabolic functions within the overall trophic structure, which made them more likely to rebound after disturbance, rather than undergo competitive growth with different syntrophs that have a similar function in the microbial consortium.

In our study, the temperature differential from 25°C to 15°C applied to the IFB and EGSB reactors might have been the major reason why communities did not show a resilience mechanism. Indeed, whether the microbial composition rebounds, or not, is possibly determined by the degree of disturbance and importance of the disturbance on the process stability and performance. It may be possible, for example, that the microbial composition of the anaerobic reactor subjected to only slight variation or disturbance with respect to environmental conditions might return to its original composition after such disturbance. The study of Madden et al. (2010) provides evidence to support this hypothesis. These authors investigated the effect of transient (but severe) perturbations on the methanogenic community structure and process performance of replicate EGSB-based reactors. Cluster analyses of DGGE
data suggested that temporal shifts in microbial community structure were predominantly independent of the applied perturbations (Madden et al., 2010).

When the community composition is sensitive, and not resilient, it might produce process rates similar to the original community, if the members of the community are functionally redundant (Allison & Martiny, 2008). The highly dynamic community structure, therefore, during well-functioning periods may be explained by the functional redundancy among diverse phylogenetic groups, allowing oscillations of their populations, due to the presence of a reservoir of species able to perform the same ecological function with no effects on the reactor performance (Briones & Raskin, 2003; Zumstein et al., 2000). The stability of reactor performance, especially that of the IFB reactors, after the applied temperature disturbance and consequent change in methanogenic community composition, could be explained by functional redundancy in one, or more, steps of the methanogenic pathway.

### 3.4.3 Microbial community composition

Methanogenesis can proceed through two parallel pathways, where acetate and/or hydrogen and carbon dioxide are converted into methane, termed as aceticlastic methanogenesis and hydrogenotrophic methanogenesis, respectively. Under certain conditions, homoacetogenic bacteria can compete with hydrogenotrophic methanogenesis for hydrogen (hydrogen is used to reduce carbon dioxide to acetate; (Lovley & Klug, 1983). Homoacetogenesis has been observed under psychrophilic conditions, and some studies have reported that homoacetogens have a better ability to adapt to low temperatures than hydrogenotrophic methanogens (Kotsyurbenko et al., 2001; Nozhevnikova et al., 2003). No acetic acid accumulation (Figure 18 presented as sum of VFA) was, however, observed at 15°C in this study. Competition between these groups did become apparent, when the temperature of the reactors was reduced to 15°C, as indicated by the bacterial DGGE results (Figure 19). Sequence B21 was present in both systems, only after the transition to 15°C, and showed 99% similarity to *Acetobacterium carbinolicum* and *Acetobacterium psammolithicum* (phylum *Firmicutes*; Table 5). Both organisms were formerly described as psychroactive homoacetogenic bacteria, capable of growing at temperatures from 1
to 35°C, with optimal growth between 20 and 30°C (Conrad et al., 1989; Simankova et al., 2000). Enhanced activity of psychrotolerant homoacetogenic bacteria, followed by acetoclastic methanogenesis, has been previously reported to be important in the degradation of organic matter under low temperature conditions (Schulz & Conrad, 1996). There are also reports that homoacetogenic formation of acetate could bypass the formation of fatty acids and H₂, which could explain suppressed VFA production in our systems at 15°C, similar to that described for the decomposition of organic matter in anoxic paddy soil at low temperature (Chin & Conrad, 1995), or for the acidic sediment of Knaack Lake (Conrad et al., 1987). No increase was noted, however, in the maximum specific methanogenic activity with acetate as substrate, following temperature decrease to 15°C, of the biomass sampled from either reactors (data not shown) and the quantitative analysis of methanogenic community structure did not record a large increase in acetoclastic methanogens (Figure 21).

Phylogenetic analyses of the archaean DGGE bands A1-A3 (Figure 20 b, Table 6) indicated that hydrogen-utilizing Methanocorpusculum-like organisms (order Methanomicrobiales) were prominent after transition to low temperature. The Methanocorpusculum-like organisms deduced from A3 of the IFB system were likely to be the major hydrogenotrophic population in the low temperature reactor from day 365 (T3b, 15°C) onwards. In case of EGSB reactor some part of the Methanomicrobiales population shifted towards Methanospirillum-like organisms deduced from A8 (T3a, 15°C) from day 298 onwards. Many studies have indeed documented that methanogenesis predominantly proceeded through the hydrogenotrophic route in low-temperature anaerobic reactors (McKeown et al., 2009a; O'Reilly et al., 2009; Syutsubo et al., 2008). In these situations, conditions with low hydrogen availability and high biomass concentration seemed to favor hydrogenotrophic methanogens due to their higher affinity for H₂ and thus they out-competed homoacetogens for hydrogen (Kotsyurbenko, 2005). It has been further proposed that hydrogen metabolism is thermodynamically and metabolically more favorable than acetate-utilisation; and that a higher level of hydrogen can be retained in the system (i.e., increased gas solubility) at low temperature (Kotsyurbenko, 2005; Lettinga et al., 2001). Supporting this, other authors (McKeown et al., 2009a; O'Reilly et al., 2009; Siggins et al., 2011) clearly demonstrated the temporal
methanogenic community shifts towards the dominance of hydrogenotrophs, especially the order *Methanomicrobiales*, in low-temperature reactors compared to mesophilic systems. Hydrogenotrophic *Methanomicrobiales* showed a 1600- to 2200- fold increase in the 16S rRNA gene concentration from it’s minimum to maximum, corresponding to mesophilic and psychrophilic reactor operation in the IFB and EGSB reactors, respectively (Figure 21). Indeed, given this remarkable rise low temperature appeared to be the major factor facilitating the emergence to dominance of this group in our reactors.
3.5 References


Chapter 3


Appendix 2: % Biogas CH₄ data of IFB1, IFB2 and EGSB1, EGSB2 (Chapter 3)
Appendix 3: Reactor configuration and zoom in showing Scanning Electron Microscopy (SEM) images of the biofilm biomass from (A) the inverted fluidized bed (IFB) and (B) expanded granular sludge bed (EGSB) reactors.
Chapter 4

Quantitative and qualitative analysis of microbial community development during high-rate, low-temperature anaerobic digestion of dairy wastewater in an EGSB reactor.

A version of this chapter was submitted to: Bioresource Technology

Abstract

The feasibility of low-temperature (10°C) anaerobic biotreatment of dairy wastewater was investigated. Stable and efficient treatment performance was observed at an applied organic loading rate (OLR) of 0.5-2 kg COD m\(^{-3}\) d\(^{-1}\) with mean COD RE >85%. The process was dependent on the OLR applied and values >2 kg COD m\(^{-3}\) d\(^{-1}\) resulted in process deterioration. OLR also influenced microbial community dynamics. An increased abundance of hydrogenotrophic methanogenic groups (Methanomicrobiales and Methanobacteriales) was recorded, using quantitative polymerase chain reaction (qPCR) analysis, by the end of the 335 day trial. Despite the perturbations in OLR applied during the 10°C bioreactor trial, the levels of aceticlastic Methanoseta spp. were maintained at stable levels.
4.1 Introduction

The application of low-temperature anaerobic digestion (LTAD) with temperatures ≥10°C has been successfully demonstrated at laboratory- and pilot-scale granular sludge-based bioreactors (Bergamo et al., 2009; Collins et al., 2006a; Collins et al., 2003; Lettinga et al., 2001; McHugh et al., 2004; McKeown et al., 2011; Syutsubo et al., 2008). Although LTAD with temperatures ≤10°C has also been studied, to date most studies have examined low-strength, simple VFA-based wastewaters (McKeown et al., 2009b; Siggins et al., 2011a; Syutsubo et al., 2008). Up to date there are only few reports describing LTAD (≤10°C) of more difficult, low-strength recalcitrant industrial wastestreams (i.e. solvent-containing wastewaters; (Enright et al., 2009; Takahashi et al., 2011). Therefore, the potential for high-rate anaerobic digestion of complex industrial wastewaters at temperatures ≤10°C remains largely unexplored.

Dairy wastewater is defined as a complex substrate (Angelidaki et al., 1999; Fang, 2000) and high rate anaerobic treatment of dairy wastes (or any industrial wastewater) can often be problematic, even at mesophilic temperatures (Demirel et al., 2005). To our knowledge, there are no reports exploring the feasibility of LTAD to digest dairy effluents at temperatures ≤10°C. Most studies reporting treatment of dairy wastewater concentrate on: (i) diversity of dairy effluents; (ii) reactor configuration; (iii) physico-chemical treatment methods; and (iv) thermodynamics to enhance the treatment efficiency (Demirel et al., 2005; Kushwaha et al., 2011). There is, therefore, a lack of information regarding microbial community structure and dynamics in such systems. It is generally accepted that the composition of influent wastewater (Akarsubasi et al., 2005; Leclerc et al., 2004; Lee et al., 2009), environmental parameters such as temperature (Bergamo et al., 2009) as well as operating conditions (McHugh et al., 2003; Wilderer et al., 2002) will all influence the microbial composition and dynamics within high-rate anaerobic digesters. Additionally, in general a complex substrate composition allows development of a microbial community with a higher metabolic diversity compared to a substrate composed of only a few components (McHugh et al., 2004; McHugh et al., 2003). More studies should thus be undertaken to understand the nature and function of the microbial populations involved in LTAD of dairy wastewater.
In light of this, the aim of this study was to investigate the high-rate anaerobic digestion of dairy wastewater at 10°C. The process performance and microbial community structure and population dynamics were investigated during the course of this study. To determine the limits of stable bioreactor performance, the expanded granular sludge bed (EGSB) was subjected to variable organic loading rates, defined by different hydraulic retention times (HRT) and variable influent concentrations. Additionally changes in bacterial and archaeal community structure were monitored using denaturing gradient gel electrophoresis (DGGE) and real-time PCR to investigate the influence of operating conditions [i.e. increases in organic loading rates] on microbial population dynamics during the 335-day trial.

4.2 Materials and methods

4.2.1 Reactor operation and biomass sampling

A laboratory-scale EGSB bioreactor (7.2 l working volume Appendix 4, configuration as described in Chapter 2 (Bialek et al., 2011)) treating synthetic skimmed dairy wastewater was continuously operated, at 10°C, for 335 days. The seed sludge used to inoculate the bioreactor was sourced from the previous experiment described in Chapter 3 (Bialek et al., 2012) and the VSS concentration inoculated into the bioreactor was 17.5 g l⁻¹. Effluent was recirculated at an applied upflow velocity of 2.5 m h⁻¹.

The trial period was divided into six operational phases (PI-PVI; Table 8). PI (48 h HRT, day 0-146), PII (24 h HRT, day 147-230), PIII (18 h HRT, day 231-265), PIV (12 h HRT, day 266-294), PV (day 295-322) characterized by fixed HRT (12 h HRT) and 20% increments in the OLR from 2-5 kg of COD m⁻³d⁻¹), PVI (day 323-335) characterized by fixed HRT (12 h HRT) and return to fixed organic loading rate of 2 kg of COD m⁻³d⁻¹. The performance of the reactor was evaluated on the basis of chemical oxygen demand (COD) removal efficiency (RE).

For microbial community analysis, biomass samples were directly collected from the EGSB bioreactor on Days: 0, 140, 226, 255, 294 and 335. All biomass were sampled twice (2×50 ml) before changing operating conditions and were first mechanically
disrupted by manual grinding with a pestle and mortar and diluted 4-fold with deionised and distilled water (DDW) before DNA was extracted as described previously in Chapter 2 (Bialek et al., 2011). All DNA extractions were performed in duplicate.

Biomass samples collected directly from the bioreactor were also fixed for scanning electron microscopy analysis (SEM). Fixation was undertaken following the protocol described by Katuri et al. (2010).

### 4.2.2 Specific methanogenic activity testing

Biomass sampled from the bioreactor on day 0 and day 335 (trial conclusion) was screened for metabolic capability using specific methanogenic activity (SMA) values, performed using the pressure transducer technique (Coates et al., 1996). Acetate (30 mM) and H₂/CO₂ (80:20, v/v) were employed to determine activity of acetoclastic and hydrogenotrophic methanogens, while propionate (30mM), butyrate (15mM) and ethanol (30mM) were used as indirect methanogenic substrates to determine activity of syntrophic populations. Vials without any substrate or with the addition of N₂/CO₂ (80:20, v/v) in case of hydrogenotrophic assays served as controls. All activity assays contained 2-5 g VSS l⁻¹ and were performed in triplicate. Assays on day 0 were performed at 15 and 37°C, assays on day 335 were performed at 10 and 37°C, and are expressed as ml CH₄ gVSS⁻¹ day⁻¹.

### 4.2.3 qPCR

Real-time PCR (qPCR) analysis was performed using a LightCycler 480 instrument (Roche, Mannheim, Germany) using two methanogenic order-specific primer and probe sets: Methanobacteriales (MBT), Methanomicrobiales (MMB), and two methanogenic family-specific primer and probe sets: Methanosarcinaceae (Msc) and Methanosaetaceae (Mst), as described previously (Bialek et al., 2011; Lee et al., 2009; Yu et al., 2005a; Yu et al., 2005b). All DNA templates were analysed in duplicate. Quantitative standard curves were constructed using the representative strains corresponding to each primer and probe set, targeting the specific methanogenic groups (MBT, MMB, Msc, Mst), as described previously (Yu et al., 2006).
4.2.4 Archaeal and bacterial DGGE

Archaeal and bacterial 16S rRNA genes were amplified by PCR using the primer sets ARC 787F and ARC 1059R (Takai & Horikoshi, 2000), BAC 338F and BAC805 R (Yu et al., 2005a; Yu et al., 2005b), respectively. To stabilize the melting behaviour of the PCR products, a 40-bp GC-clamp was attached at the 5’-end of each forward primer (Muyzer et al., 1993). Touchdown PCR was conducted using a G-Storm thermal cycler (Gene Technologies Ltd., Essex UK) and the applied protocol was as described previously in Chapter 2 (Bialek et al., 2011). Further, two µl of eluted DNA solution were amplified using the archaeal (ARC787F and ARC1059R) and bacterial (BAC 338F and BAC805 R) primers without the GC clamp. The PCR products were gel-purified and cloned into pGEM-T Easy vector (Promega, Madison, WI). The cloned gene fragments were sequenced using T7 primer and compared against the GeneBank and Ribosomal Database Project (RDP) databases. Sequencing alignment and phylogenetic analyses were performed using MEGA 4 software (Tamura et al., 2007). Phylogeny was calculated using the Neighbor-Joining method (Saitou & Nei, 1987b). Bacterial and archaeal distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The bootstrap consensus trees inferred from 1000 replicates are taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

Unweighted Pair Group Method with Arithmetic mean (UPGMA; (McCune & Grace, 2002)) was selected to perform statistical analysis of the DGGE profiles. The scanned DGGE gel image was processed with Phoretix 1D (previously TotalLab TL120 software; TotalLab Ltd, Newcastle upon Tyne, UK) to construct a binary matrix, where the presence or absence of each band was scored with 1 or 0, respectively, without considering band intensity. Construction of the dendogram via hierarchical clustering was performed based on the Sorensons (Bray-Curtis) distance measurement using MEGA 4 software (Tamura et al., 2007). All nucleotide sequence data reported in this study were deposited in the GenBank database under accession numbers ARC (A3-A5): JQ730820- JQ730821 and BAC (B1-B25): JQ730824- JQ730848.
4.2.5 Analytical analysis

Effluent from the reactor was routinely sampled to analyse residual COD concentration according to Standard Methods (APHA, 2005). Analysis of effluent volatile fatty acids (VFA) were performed in a Varian Saturn 2000 GC/MS system, with CombiPAL autosampler (Varian Inc., Walnut Creek, CA) as described previously (Bialek et al., 2011). The total genomic DNA was quantified using the Quant-iT™ dsDNA BR Assay Kit with the Qubit™ fluorometer (Invitrogen, Molecular Probes Inc., Eugene, Oregon, USA). All analyses were performed in duplicate.

4.3 Results and discussion

4.3.1 Bioreactor performance

Figure 23 illustrates the COD removal efficiency (RE) profiles and effluent VFA concentrations associated with the EGSB bioreactor during the trial. The operating parameters and performance are summarized in Table 8. The EGSB reactor demonstrated >85% COD RE and effluent concentrations of <140 mg COD l\(^{-1}\) VFA during steady-state operation of phases PI-IV. During PV, the COD RE suddenly dropped to 48% and in-reactor VFA concentrations peaked at >770 mg COD l\(^{-1}\) on day 300. Most probably the OLR was increased too quickly (up to 4.1 kg COD m\(^{-3}\) d\(^{-1}\)), causing a shock load and resulting in poor bioprocess performance. An applied reduction in OLR to 2.9 kg COD m\(^{-3}\) d\(^{-1}\) resulted in return of the COD RE to >80% and drop in the VFA effluent concentrations to <140 mg COD l\(^{-1}\). Subsequent gradual increases in the OLR up to 5 kg COD m\(^{-3}\) d\(^{-1}\) were followed by fluctuations in the effluent concentrations of VFA with maximum values of >760 mg COD l\(^{-1}\) and gradual drop in the COD RE reaching a minimum value of 60% on Day 322 (maximum OLR of 5 kg COD m\(^{-3}\) d\(^{-1}\)). Interestingly, during PVI (characterized by return to fixed HRT of 12 h and return to fixed OLR of 2 kg COD m\(^{-3}\) d\(^{-1}\)), a rapid improvement (3 days after the maximum OLR was reduced 2.5 times from 5 to 2 kg COD m\(^{-3}\) d\(^{-1}\)) was observed in the bioprocess performance, with mean COD RE exceeding 84% and a decrease in effluent VFA concentrations to <60 mg COD l\(^{-1}\) by the end of trial.
Figure 23. Chemical Oxygen Demand (COD) Removal Efficiency (RE), Volatile Fatty Acids (VFA concentrations; presented as sum of: acetic-, butyric-, iso-butyric-, propionic-, valeric- and iso-valeric) and Organic Loading Rate (OLR) applied to the Expanded Granular Sludge Bed (EGSB).

Table 8. Operational and performance characteristics during phases I to VI of the bioreactor operation.

<table>
<thead>
<tr>
<th>Phases</th>
<th>PI (0-146)</th>
<th>PII (147-230)</th>
<th>PIII (231-265)</th>
<th>PIV (266-294)</th>
<th>PV (295-322)</th>
<th>PVI (323-335)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT (a)</td>
<td>48</td>
<td>24</td>
<td>18</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>OLR (b)</td>
<td>0.5</td>
<td>1.0</td>
<td>1.3</td>
<td>2.0</td>
<td>2.0/5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>CODRE (c)</td>
<td>88±6</td>
<td>89±4</td>
<td>90±2</td>
<td>86±6</td>
<td>74±11</td>
<td>84±4</td>
</tr>
<tr>
<td>SLR (d)</td>
<td>0.03</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.14</td>
</tr>
<tr>
<td>Q (e)</td>
<td>0.0036</td>
<td>0.0072</td>
<td>0.0096</td>
<td>0.0144</td>
<td>0.0144</td>
<td>0.0144</td>
</tr>
<tr>
<td>(C_s) (f)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0/2.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(a\)Hydraulic retention time (h)

\(b\)Organic loading rate (kg COD m\(^{-3}\) d\(^{-1}\))

\(c\)Chemical oxygen demand removal efficiency (%), where values are the phases mean (± s.d.)

\(d\)Sludge loading rate (kg COD kg [VSS]\(^{-1}\) d\(^{-1}\)); n.d.- not determined

\(e\)Flow rate (m\(^3\) d\(^{-1}\))

\(C_s\)Substrate concentration (kg COD m\(^{-3}\))

The retention of psychoactive biomass within low-temperature bioreactors, although crucial for successful high-rate psychrophilic anaerobic digestion (Lettinga et al., 2001), can be challenging and significant loss of granular sludge (up to 43%) due to
biomass wash-out was observed at ≤10°C biotreatment of acidified wastewaters (McKeown et al., 2009b). In our study, c. 14.5 g VSS l⁻¹ Reactor was observed in our bioreactor by the end of the trial (Day 335; PVI), corresponding to a 17% decrease in the VSS during the trial.

The temperature drop strongly affects the physical and chemical properties of the wastewater (Lettinga et al., 2001). This may lead to changes in physicochemical conditions, such as substrate availability, shear stress, and chemical gradients (e.g., pH or oxygen) and may influence biofilm structure (Horn & Hempel, 1998; Stoodley et al., 1998) and thus extracellular polymeric substances composition (Sheng et al., 2010). When we consider that EPS can constitute 50–80% of the organic matter within biofilms composition (Beer & Stoodley, 2006) it becomes apparent that changes in the EPS concentration and amounts may have an impact on the concentration of active biomass within the total organic matter (VSS). In our study, although slight reduction in the VSS concentration was observed by the end of trial, the estimated DNA amount at the beginning (Day 0) and at the end of trial (Day 335) was 0.18±0.01 mg DNA/g VSS and 0.25±0.01 mg DNA/g VSS, respectively. Increase in the DNA concentration per gram VSS, as well as increase in the 16S rRNA gene copy concentration of Archaea and Bacteria (Table 9) by the end of the trial, implies that the EPS or other organic matter was washed out from the biomass (broken or loose granules; Figure 24). This, in turn, raised the concentration of active biomass and thus the DNA concentration. The EPS are the major components in microbial aggregates bonding them together in a three-dimensional matrix (Sheng et al., 2010). That ensures mechanical stability of a biofilm, which is important for stable process performance. It has been observed that the anaerobic aggregates with low amounts of EPS exhibit a strong tendency to float by attachment of gas bubbles to the hydrophobic aggregate surface, which leads to severe biomass losses from the reactors (Beer & Stoodley, 2006). Therefore, more studies are needed to understand the influence of low operational temperature on the biofilm development and parameters influencing the EPS formation and composition.

The decrease in the biomass concentration in our study, concurrently with increase in the applied OLR throughout the trial effectively increased the SLR from 0.03 to 0.14 kg COD kg [VSS]⁻¹ d⁻¹ applied at the beginning (PI; Day 0) and at the end of trial (PVI; Day 335; Table 8). The initial SLR value reported in this study is significantly lower than the SLR values of 0.07-0.49 kg COD kg [VSS]⁻¹ d⁻¹ (Singh et al., 1998)
recommended for the successful start-up for UASB reactors, and much lower than SLR values reported by McKeown et al. (2009b) during low-temperature treatment of acidified wastewaters in EGSB-AF hybrid bioreactor. Singh et al. (1998) also concluded that the suitable SLR value will strongly depend on (i) the waste type (ii) active seed concentration (iii) proper operating and hydraulic conditions (iv) suitable environmental conditions imposed on the system, although the suggested SLR values are based on mesophilic and thermophilic reactor operating temperatures. Although the LTAD is recognized as an attractive technology, few studies reported the effect of operating temperature on the applied SLR.

Figure 24. Scanning Electron Microscopy (SEM) images of the loose biomass granules from the EGSB bioreactor.
4.3.2 Real-time PCR of archaeal populations

At the beginning of trial (PI; Day 0), hydrogenotrophic *Methanomicrobiales* (MMB) accounted for 9.9% (4.9×10^5 copies/ml) of the total methanogenic 16S rRNA gene concentration (Figure 25). With decreasing HRT from 48 to 12h (PI-PIV) a 975-fold reduction in the concentrations of *Methanomicrobiales* (16S rRNA gene concentration of 5.0×10^2 copies/ml) was noted. Interestingly, however, the *Methanomicrobiales* numbers subsequently recovered, with a marked 456-fold increase by the end of the trial (16S rRNA gene concentration of 2.3×10^5 copies/ml; PVI; day 335).

The hydrogenotrophic order *Methanobacteriales* (MBT) was detected at 2.2×10^5 at the beginning of trial (PI; day 0). Following HRT reduction from 48 to 12 h (PI to PIV) *Methanobacteriales* levels decreased, with an 80-fold reduction noted (16S rRNA gene concentration of 2.7×10^3 copies/ml; PIV; day 294). Interestingly, by the end of the trial the level of *Methanobacteriales* also recovered, demonstrating a 47-fold increase (16S rRNA gene concentration of 1.3×10^5 copies/ml; PVI; day 335) (Figure 25).

A reduction in the applied HRT from 48 to 12h (PI to PIV), at a constant influent concentration (1 kg COD m^-3) resulted in increase in the applied OLR (Mahmoud et al., 2003) of 300% (0.5 to 2 kg COD m^-3 d^-1) which had an initial negative influence on the abundance of *Methanomicrobiales* and *Methanobacteriales* at 10°C reactor operation. Although a significant decrease in abundance of those two hydrogenotrophic groups occurred, there was no deterioration in the bioreactor performance during the corresponding periods (>89 % COD RE; PI to PIV; Figure 23). Indeed, a subsequent increase in the abundance of *Methanobacteriales* and *Methanomicrobiales*, as observed by the end of trial (PVI; day 335) seemed to be a stress response induced by increased OLR during PV (increase in OLR from 2 to 5 kg COD m^-3 d^-1), followed by reduction in OLR during PVI (OLR back to 2 kg COD m^-3 d^-1). McHugh et al. (2004) suggested that increased abundance/ proliferation of *Methanomicrobiales* sp. and a parallel decrease in *Methanosarcina* sp./*Methanosaeta* sp. is a stress response due to the combination of high loading rate and low operating temperature encouraging the propagation of hydrogenotrophic methanogens. The numbers of *Methanosacetaceae* did not decrease in response to OLR in our study, but
this was based on DNA concentration, rather than more functionally relevant RNA levels, and thus the activity of the *Methanosetaeaceae* may have been affected during periods of poor reactor performance while the corresponding DNA template concentrations remained stable. Additionally, higher organic loading rates favor the preponderance of acid-forming bacteria, resulting in increased production of volatile acids during anaerobic digestion of a complex type of substrate like dairy wastewater (Demirel & Yenigun, 2004) and, indeed, increased VFA effluent concentrations were observed during PV in our bioreactor. The VFA concentration has been reported to be closely related to the dissolved hydrogen concentration (Cord-Ruwisch et al., 1997; Schink, 1997) and it was suggested that the dissolved hydrogen concentration plays a direct role in the predominance of the hydrogenotrophic methanogens and their affinity to hydrogen concentration (Hori et al., 2006). Increased OLR and VFA in-reactor concentrations were observed to have a positive influence on the abundance of *Methanomicrobiales* during mesophilic and thermophilic reactor operation (Bialek et al., 2011; Hori et al., 2006) but it has not to our knowledge been reported previously during psychrophilic (10°C) bioreactor operation. It is also not clear, why subsequent reduction in OLR during PVI and drop in VFA in-reactor concentrations (Figure 23) had a stimulating effect on the increased abundance of *Methanobacteriales* and *Methanomicrobiales*. However, this observed behavior could have been due to decreased mass transfer resistance, as the diffusion rate increases after a hydraulic shock load (Brito & Melo, 1999). Another possible explanation could be the hysteretic effect, which was attributed by Bhatia et al. (1985a); Bhatia et al. (1985b) to the structure of the cultures inside the flocculated biomass, which can change depending upon the operational conditions. It is likely that the $K_S$ (Monod half saturation constant) increased, therefore increasing the substrate ($H_2$) affinity, when the OLR was raised, and did not return to its previous value when the OLR was decreased (Leitao et al., 2006). Additionally several researchers have suggested that the diffusion rate of substrate through a biofilm is a function of liquid velocity and substrate concentration. Under steady-state conditions, the diffusion rate increases with a higher substrate concentration (Fick's law) and decreases with a higher flow velocity (Beyenal & Lewandowski, 2000). According to these authors, the substrate concentration has a stronger effect on the diffusion rate than the flow velocity, as possibly occurred in our study after transition from PV (constant flow rate of 0.0144 m$^3$ d$^{-1}$ and substrate concentration increasing
from 1 to 2.5 kg COD m\(^{-3}\); Table 8) to PVI (constant flow rate of 0.0144 m\(^3\) d\(^{-1}\) and fixed substrate concentration of 1 kg COD m\(^{-3}\); Table 8).

The aceticlastic family *Methanosaetaceae* (Mst) was the most abundant and stable group during the whole trial, indicating that OLR changes applied during the trial did not influence/perturb this community (Figure 25). The 16S rRNA gene concentration of *Methanosaetaceae* accounted for 85.6\% (4.2×10\(^6\) copies/ml) and 93.7\% (5.3×10\(^6\) copies/ml) at the beginning (PI; Day 0) and at the end (PVI; Day 335) of the trial, respectively. Such results indicate that this aceticlastic family was an important member of the methanogenic community and may be retained within anaerobic biofilms during cold (10°C) bioreactors operation (McKeown et al., 2009a). High abundance of *Methanosaetaceae* has been also associated with stable bioprocess (Díaz et al., 2006) as observed in this study.

The aceticlastic family, *Methanosarcinaceae* (Msc), was not detected (i.e., <5.63×10\(^1\) copies/µl) in the bioreactor throughout the trial. It is likely that under steady operational conditions, as observed in the EGSB bioreactor during PI-PIV, the low prevailing residual acetate concentrations (65±31 mg COD l\(^{-1}\) acetate presented as sum of VFA; Figure 23) coupled with low temperature contributed to the suppression of *Methanosarcina* by *Methanosaeta* (Chin et al., 1999; Griffin et al., 1998). Although fluctuations and increased residual acetate concentrations within the system during PV peaked at c. 670 mg COD l\(^{-1}\) (acetate presented as sum of VFA; Figure 23), which is above the threshold value required to support the growth of *Methanosarcina* (Jetten et al., 1990), this group remained below the detection limit.
4.3.3 Physiological characterization of biomass, specific methanogenic activity (SMA) assay

SMA results indicated the mesophilic nature of the inoculum (day 0) and biomass at the end of trial (day 335), exhibiting higher activity at 37°C than at 10 and 15°C, for all substrates tested (Table 9). Assays carried out at the beginning of the trial (day 0) biomass displayed a preference for hydrogenotrophic methanogenesis at 37 and 15°C. By the end of trial (day 335) SMA values against H2/CO2 at 37°C were almost 2-fold higher than those determined at the beginning (day 0) revealing continued preference towards hydrogenotrophic methanogenesis. However, metabolic activity determined by the end of trial (day 335) at 10°C (the same as bioreactor operational temperature throughout the trial) indicated equal capacity for acetate-mediated and H2/CO2-mediated methanogenesis (Table 9).

The use of an SMA assay, with acetate as sole substrate, determines the metabolic activity of acetoclastic methanogens and it may be possible to correlate it with the absolute quantification of the 16S rRNA gene copy concentration of the aceticlastic methanogens, utilizing acetate as a carbon source. It is well established that the aceticlastic methanogens belong to the order Methanosarcinales comprising two
families, *Methanosarcinaceae* and *Methanosaetaceae* (Boone et al., 2001) and since in our system the family *Methanosarcinaceae* (Msc) was below the detection limit of qPCR the whole acetate conversion could be attributed to the members of *Methanosaetaceae* (Mst) family. Similar value of the 16S rRNA gene concentration of *Methanosaetaceae* at the end and at the beginning of the trial (5.3×10^6 copies/ml; day 335 versus 4.2×10^6 copies/ml; day 0; Table 9) suggest that there was no significant change in the abundance of this group. However the SMA indicates that the metabolic activity against acetate at the end of trial at 37°C was lower (257±6; day 335; Table 9) than at the beginning (366±26; day 0; Table 9). SMA determined at 15°C (day 0) and 10°C (day 335) was always lower than the activity determined at 37°C, although the 16S rRNA gene concentration of *Methanosaetaceae* had the same value at day 0 (37 and 15°C) and at day 335 (37 and 10°C). It has been reported that the temperature reduction may have strong effect on the activity of the aceticlastic methanogens, reducing maximum substrate utilization rates of microorganisms (Kettunen & Rintala, 1997; Lettinga et al., 1999; Rebac et al., 1995; Wu et al., 1993). However, in our study the SMA was determined only at 10°C at the end of experiment; hence it is possible that the value was lower at the beginning of the trial (the same temperature). It was observed previously that due to the biomass adaptation to low temperature, improved methanogenic activity against acetate was observed (McKeown et al., 2009a).

Hydrogenotrophic methanogens comprise of four orders, i.e., *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, and *Methanopyrales*, which utilize only H_2+CO_2, formate or methanol to produce methane (Boone et al., 2001). Because the *Methanopyrales* members are not likely to be present in anaerobic processes due to their extremely high growth temperature (>80°C) (Boone et al., 2001) and the members of *Methanococcales* (MCC) are not normally found in anaerobic reactors, presumably since organisms from this group require high-salt conditions for their growth (0.3-9.4% (w/v) NaCl) (Boone et al., 2001), these two orders were left out of consideration in this study. Despite almost 2-fold decrease in the sum of 16S rRNA gene concentration of the hydrogenotrophic *Methanomicrobiales* (MMB) and *Methanobacteriales* (MBT) at the beginning and at the end of the trial (7.1×10^5 copies/ml at day 0 versus 3.6×10^5 copies/ml at day 335) the SMA activity determined against H_2/CO_2 at 37°C at day 335 was almost 2-fold higher than SMA value determined at day 0 (Table 9). Such results might indicate that although
absolute quantity values of hydrogenotrophic methanogens decreased, their metabolic activity increased during the trial. It is likely that the $K_S$ (Monod half saturation constant) increased, therefore increasing the substrate ($H_2$) affinity, when the OLR was raised during PV, and did not return to its previous value when the OLR was decreased during PVI (Leitao et al., 2006). Nevertheless, the discrepancy between the molecular and physiological data, points out the limitations of studies based only on the DNA and indicates need for more future studies focusing on functional investigations to unravel metabolic activity of the microbial communities underpinning the AD processes.

Although the SMA assays suggested that the biomass retained an essentially mesophilic nature (higher metabolic activity against direct and indirect methanogenic substrates tested at 37°C versus 10°C; Table 9) as was previously observed during cold cultivation (Lettinga et al., 1999; Siggins et al., 2011a; Siggins et al., 2011c) our results indicate that mesophiles may metabolise and grow under sub-optimal temperatures (Rebac et al., 1999). It has been suggested that although psychrophiles are likely to be present in psychrophilically cultivated anaerobic sludges, they are not able to manifest in SMA profiles because of the presence of many more mesophiles (Rebac et al., 1995), although longer trials (>600 days) at low-temperatures could act as selective enrichment for psychophilic methanogenic populations from mesophilic inocula (McKeown et al., 2009b).

**Table 9.** Specific methanogenic activity (SMA) profiles against direct and indirect methanogenic substrates expressed as ml CH$_4$ gVSS$^{-1}$ day$^{-1}$ and the absolute quantification of 16S rRNA gene copy concentration expressed in copies/ml, of day 0 and day 335 biomass.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>T (°C)</th>
<th>Trial day</th>
<th>Specific methanogenic activity (ml CH$_4$ g VSS$^{-1}$ day$^{-1}$)</th>
<th>Absolute quantification by qPCR 16S rRNA gene copy concentration (copies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SMA Substrate</td>
<td>Mst</td>
</tr>
<tr>
<td>EGSB</td>
<td>37</td>
<td>0</td>
<td>Acetate</td>
<td>366±26</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td></td>
<td>$H_2$/CO$_2$</td>
<td>87±10</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>335</td>
<td>Ethanol</td>
<td>257±6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>Propionate</td>
<td>38±4</td>
</tr>
</tbody>
</table>

All SMA values are the mean of triplicates (std. error; n = 3), except * where values are the mean of duplicates (std. error; n=2). Absolute quantification of 16S rRNA gene copy concentration of the groups: Mst (*Methanosaetaceae*), MBT (*Methanobacteriales*) + MMB (*Methanomicrobiales*), ARC (*Archaea*), BAC (*Bacteria*).
4.3.4 Bacterial DGGE and UPGMA cluster analysis

The UPGMA cluster analysis of bacterial DGGE profiles revealed >96% similarity of all DGGE profiles of the bacterial populations within the whole trial (PI-PVI; EGSB: 0, 140, 226, 255, 294, 335; Figure 26) suggesting resistance of the bacterial community composition (Allison & Martiny, 2008) despite changes in the HRT and OLR applied during the trial.

Figure 26. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of the 16S rRNA gene fragments generated from bacterial Denaturing Gradient Gel Electrophoresis (DGGE) profiles of EGSB biomass. Similarity calculated by Sørensons (Bray-Curtis) distance measurement.

4.3.5 Bacterial and archaeal phylogenetic analysis

Partial 16S gene sequences from 20 out of total 21 excised bacterial DGGE bands grouped within four phyla: Firmicutes, Proteobacteria, Spirochaetes and Bacteroidetes (Figure 27).

The phylum Firmicutes was represented by five bands (B10, B11, B12, B14, B21), which were mostly present in all biomass samples (Figure 27 a). Information on the identity and potential role of some of these bacteria could be tentatively inferred from the phylogenetic analysis. For example, B10 showed 98% similarity to Clostridium aminobutyricum and was present in bioreactor during the whole 10°C trial, which is perhaps surprising since the optimum temperature growth of this organism was reported to be in the mesophilic range (Hardman & Stadtman, 1960). B14 was apparent during the whole trial (except Day 0) and shared 99% similarity with Trichococcus flocculiformis, Trichococcus collinsii and Trichococcus pasteurii,
aerotolerant, fermentative organisms growing with glucose, sucrose and lactose to produce lactate, acetate, formate and other acids (Liu et al., 2002). The phylum Proteobacteria was represented by four bands (B1, B16, B18, B22). Although, B16 and B18 were not closely related they shared 94-95% similarity to Syntrophobacter sulfatireducens, propionate-oxidizing bacterium (Ariesyady et al., 2007). Growth of this organism was observed between 20-48°C (Chen et al., 2005) but it was previously reported during psychrophilic (15°C) anaerobic bioreactor operation (Bialek et al., 2012). Propionate is fermented syntrophically to acetate and CO$_4$ in the presence of hydrogen-/or formate-utilizing methanogens like Methanospirillum hungatei (Chen et al., 2005), which has been identified as band A4 retrieved from archaeal DGGE (Figure 27 b). The presence of Syntrophobacter sulfatireducens like clones during the whole trial in the EGSB reactor putatively suggests that it was important during propionate oxidation, which has been identified as the rate-limiting step during LTAD (Rebac et al., 1999). The emergence of psychrophilic propionate-utilizing populations was correlated with propionotrophic activity during long-term operation under low-temperature conditions (McKeown et al., 2009a).

B22 showed 99% similarity to Thiothrix thioparus, thiosulphate oxidizing bacterium commonly used for Total Reduced Sulphur biofiltration technology (Valdebenito-Rolack et al., 2011) and was apparent at day 140 and 255.

The phylum Spirochaetes was represented by two bands (B15 and B17), which shared 99% similarity to Spirochaetaceae bacterium and were present in all 10°C biomass samples. Although Spirochaetes were reported to be numerically important in psychoactive consortia (McKeown et al., 2009a) their function is not clear.

The phylum Bacteroidetes was represented by nine bands (B2, B3, B4, B5, B7, B9, B19, B24, B25), which were present in all 10°C biomass samples, but that were mostly affiliated to uncultured organisms and their function remains unclear.

In the archaeal DGGE gel, three bands (A3, A4, A5) were retrieved and sequenced (Figure 27b). Band A3 was closely related to hydrogen-utilizing Methanocorpusculum like clones (M. bavaricum, M. parvum, M. labreanum, M. sinense) with 99% sequence similarity. Similar clones were previously observed during the cold adaptation of anaerobic granular and floculant sludge (Collins et al., 2003; McKeown et al., 2009a; O'Reilly et al., 2009b), Bialek et al., 2012). Band A4
showed 99% similarity to hydrogen-utilizing *Methanospirillum hungatei* and was detected in all 10°C biomass samples. This organism has been previously observed during psychrophilic (15°C) anaerobic digestion in EGSB bioreactors (Bialek et al., 2012). Band A5 was closely related to acetoclastic *Methanosaeta concilii* (100% similarity) and was detected throughout the whole trial.

**Figure 27.** Neighbor joining tree illustrating the phylogenetic affiliations of the 16S rRNA gene sequences obtained from: (A) bacterial DGGE bands and (B) archaeal DGGE. Bioreactor biomass samples taken on various days, and containing the respective bands, are given in parenthesis.
4.4 Conclusions

This study demonstrates the feasibility of low-temperature anaerobic biotreatment of complex wastewater. Variations in operating conditions were correlated with process performance and temporal changes in microbial community structure. In particular, this investigation revealed that:

- Stable and efficient biotreatment of complex dairy-based wastewaters, with no accumulation of solids during 10°C reactor operation, is feasible at applied OLR of 0.5-2 kg COD m\(^{-3}\) d\(^{-1}\) with mean COD RE >85%
- OLR influenced the efficiency of bioprocess and values >2 kg COD m\(^{-3}\) d\(^{-1}\) had direct influence on COD RE decrease. The bioreactor, however recovered very quickly (3 days after the maximum OLR was reduced 2.5 times from 5 to 2 kg COD m\(^{-3}\) d\(^{-1}\)) reaching >84% COD RE
- Mesophilic inocula can adapt and grow at sub-optimal temperatures. A decrease in the biomass concentration observed by the end of trial was concurrent with increase in the applied OLR, effectively increasing the SLR to 0.14 kg COD kg [VSS]\(^{-1}\) d\(^{-1}\)
- *Methanosaeta* spp. can be retained within anaerobic biomass and were the most stable methanogenic group with respect to perturbations in OLR applied during bioreactor operation at 10°C
- DNA-based methods may yield false interpretation as indicated by decreased numbers of hydrogenotrophic methanogens despite increased metabolic activity during the trial. This emphasizes the need for functional studies unraveling the activity of microbial communities in response to changes in the operating conditions
4.5 References


Bialek, K., Kumar, A., Mahony, T., Lens, P., O'Flaherty, V. 2012. Microbial community structure and dynamics in anaerobic fluidized-bed and granular sludge-bed reactors: influence of operational temperature and reactor configuration. Microbial Biotechnology, accepted.


Chapter 5

10°C anaerobic digestion of dairy wastewater in the IFB

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Abstract

The application of low-temperature (10°C) anaerobic digestion (LTAD) for the treatment of complex dairy-based wastewater in an inverted fluidized bed (IFB) reactor was investigated. Bioreactor performance was poor throughout the whole trial with mean COD removal efficiency (RE) of 54±17% at applied organic loading rates (OLRs) of 0.5-5 kg COD m$^{-3}$ d$^{-1}$. The applied OLR above 2 kg COD m$^{-3}$ d$^{-1}$ influenced the microbial composition and dynamics. Hydrogenotrophic methanogens: Methanomicrobiales and Methanobacterales were monitored via quantitative polymerase chain reaction (qPCR) and demonstrated 16478-fold and 85-fold decrease in their, abundance, respectively. This suggests that those organisms were inhibited or washed out from the system after the OLR stress was applied and did not regrow even when the conditions were changed and stress removed. The bacterial community in the bioreactor was monitored via denaturing gradient gel electrophoresis (DGGE), and the results of this analysis also suggested an influence of OLR stress on bacterial community structure and population dynamics. Possible shortcomings in the bioreactor operation are indicated, which could be helpful in future design and optimization of fluidized reactors intended for digestion of complex industrial wastewaters during LTAD.
Chapter 5

5.1 Introduction

Although low-temperature AD (LTAD) has been successfully demonstrated at laboratory- and pilot-scale, the majority of recent studies were based on granular sludge systems (Bergamo et al., 2009; Bialek et al., 2011; Collins et al., 2006a; Collins et al., 2003; Lettinga et al., 2001; McHugh et al., 2004; McKeown et al., 2011; Syutsubo et al., 2008). It is well known that temperature (Bergamo et al., 2009) might influence the process performance and microbial community composition and population dynamics during AD and, therefore, bioreactors intended for low-temperature applications should compensate negative effects of low temperature during AD, including increased liquid viscosity.

One aspect of bioreactor design, which would facilitate increased effectiveness of high-rate anaerobic wastewater treatment systems for cold wastewater, is increased contact between retained sludge and wastewater to utilize all the available capacity within the bioreactor. Progress on the optimization of the contact between sludge and wastewater led to the development of an advanced reactor design, known as the inverted fluidized bioreactor (IFB). The IFB bioreactor has been proposed as a promising design for AD (García-Bernet et al., 1998). The novelty of this configuration arises from the use of floatable particles with a specific density lower than the liquid, such that the particles are fluidized downward (García-Calderon et al., 1998). Due to the large specific area of support particles available for biomass retention, this technology offers advantages in the treatment of high-strength effluents by using reduced spaces and shorter hydraulic retention times (Alvarado-Lassman et al., 2008). The liquid and the produced biogas are flowing in opposite directions, which help for bed expansion (Arnaiz et al., 2003). Therefore, the down-flow (or inverse) configuration reduces energy requirements, because of the low fluidization velocities (García-Calderon et al., 1998), when compared to up-flow systems.

Another important factor to be considered during LTAD is the reported failure of the bioreactors to retain granular sludge, which may lead to severe hydraulic wash-out of psychroactive biomass (Lettinga et al., 1999). In this light, non-granule-based systems, using inert nuclei to promote re-granulation might be of advantage during psychrophilic reactor operation. Although many potential advantages are apparent, there are some crucial parameters that restrict a widespread application of IFB technology such as: support material selection, establishment of
reactor operating conditions, and start-up duration (Punal et al., 2000). Successful biofilm formation and stabilization is required and this can range from 90-160 days (Castilla et al., 2000; Michaud et al., 2002). Recent studies, however, using pre-colonized support media reported significantly shortened start-up times (Alvarado-Lassman et al., 2010; Arnaiz et al., 2005).

LTAD at temperatures ≤10°C has been predominantly applied to relatively low-strength, simple VFA-based wastewaters (McKeown et al., 2011; McKeown et al., 2009b; Siggins et al., 2011a; Syutsubo et al., 2008). To date, there are only few reports describing LTAD (≤10°C) of more difficult low-strength industrial wastestreams (i.e. solvent-containing wastewaters (Enright et al., 2009; Takahashi et al., 2011). Therefore, the potential for high-rate anaerobic digestion of complex industrial wastewaters at temperatures ≤10°C with suspended, fluidized biomass remains largely unexplored.

In this context, the objective of this study was to assess the effect of variable organic load, defined by different hydraulic retention times (HRT) and variable influent concentrations, on the bioprocess performance of the inverted fluidized bed (IFB) treating complex dairy-based wastewater at 10°C, during a 335-day trial. Additionally, changes in bacterial and archaeal community structure were examined using denaturing gradient gel electrophoresis (DGGE) and real-time PCR, respectively, to investigate the influence of operating conditions [i.e. increases in organic loading rates] on microbial community dynamics.

5.2 Materials and methods

5.2.1 Reactor operation and biomass sampling

A laboratory-scale IFB bioreactor (7.2 l working volume; Appendix 4 (Bialek et al., 2011) treated a synthetic, skimmed milk-based dairy wastewater, at 10°C, for 335 days. Extendosphere™ (Sphere One, Chattanooga, Tennessee, USA) light mineral material composed mostly of silica and traces of aluminum, density of 0.69 g cm⁻³ was used as a carrier material as described in Chapter 2 (Bialek et al., 2011). The seed sludge used to inoculate the reactor was sourced from the previous experiment.
described in Chapter 3 (Bialek et al., 2012) and the VSS concentration inoculated into the bioreactor was 17.9 g l\(^{-1}\). Bioreactor operation was divided into six operational phases (PI-PVI; Table 10). The performance of the reactor was evaluated on the basis of chemical oxygen demand (COD) removal efficiency (RE). For microbial community analysis, biomass was directly collected from the IFB bioreactor on Days: 0, 140, 226, 255, 294 and 335. Two samples of biomass (2×50 ml) were taken from the reactor on each sampling day and were first mechanically disrupted by manual grinding with a pestle and mortar and diluted 4-fold with deionised and distilled water (DDW) before DNA was extracted as described previously in Chapter 2 (Bialek et al., 2011). All DNA extractions were performed in duplicate. Biomass samples collected directly from the bioreactor were also fixed for scanning electron microscopy analysis (SEM). Fixation was undertaken following the protocol described by Katuri et al. (2010).

### 5.2.2 qPCR

Real-time PCR (qPCR) analysis was performed using a LightCycler 480 instrument (Roche, Mannheim, Germany) using two methanogenic order-specific primer and probe sets: *Methanobacteriales* (MBT), *Methanomicrobiales* (MMB), and two methanogenic family-specific primer and probe sets: *Methanosarcinaceae* (Msc) and *Methanosaetaceae* (Mst), as described previously (Bialek et al., 2011; Lee et al., 2009; Yu et al., 2005a; Yu et al., 2005b). All DNA templates were analysed in duplicate. Quantitative standard curves were constructed using the representative strains corresponding to each primer and probe set, targeting the specific methanogenic groups (MBT, MMB, Msc, Mst), as described previously (Yu et al., 2006).

### 5.2.3 Archaeal and bacterial DGGE

Archaeal and bacterial 16S rRNA genes were amplified by PCR using the primer sets ARC 787F and ARC 1059R (Takai & Horikoshi, 2000), BAC 338F and BAC805 R (Yu et al., 2005a; Yu et al., 2005b), respectively. To stabilize the melting behaviour of the PCR products, a 40-bp GC-clamp was attached at the 5’-end of each forward
primer (Muyzer et al., 1993). Touchdown PCR was conducted using a G-Storm thermal cycler (Gene Technologies Ltd., Essex UK) and the protocol applied was as described previously in Chapter 2 (Bialek et al., 2011). Further, two microlitres of eluted DNA solution were amplified using the archaeal (ARC787F and ARC1059R) and bacterial (BAC 338F and BAC805 R) primers without the GC clamp. The PCR products were gel-purified and cloned into pGEM-T Easy vector (Promega, Madison, WI). The cloned gene fragments were sequenced using T7 primer and compared against the GeneBank and Ribosomal Database Project (RDP) databases. Sequencing alignment and phylogenetic analyses were performed using MEGA 4 software (Tamura et al., 2007). Phylogeny was calculated using the Neighbor-Joining method (Saitou & Nei, 1987b). Bacterial and archaeal distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The bootstrap consensus trees inferred from 1000 replicates are taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

Unweighted Pair Group Method with Arithmetic mean (UPGMA; (McCune & Grace, 2002)) was selected to perform statistical analysis of the DGGE profiles. The scanned DGGE gel image was processed with Phoretix 1D (previously TotalLab TL120 software; TotalLab Ltd, Newcastle upon Tyne, UK) to construct a binary matrix, where the presence or absence of each band was scored with 1 or 0, respectively, without considering band intensity. Construction of the dendogram via hierarchical clustering was performed based on the Sorensons (Bray-Curtis) distance measurement using MEGA 4 software (Tamura et al., 2007).

All nucleotide sequence data reported in this study were deposited in the GenBank database under accession numbers ARC (A1-A6): JQ730818- JQ730823 and BAC (B2-B27): JQ730825-JQ730850.

### 5.2.4 Specific methanogenic activity (SMA) testing

Biomass, sampled from the bioreactor on day 0 and 335 (trial conclusion), was screened for metabolic capability using specific methanogenic activity (SMA) values, performed using the pressure transducer technique (Coates et al., 1996). Acetate (30 mM) and H₂/CO₂ (80:20, v/v) were employed to determine activity of
acetoclastic and hydrogenotrophic methanogens, respectively, while propionate (30mM), butyrate (15mM) and ethanol (30mM) were used as indirect methanogenic substrates to determine activity of syntrophic populations. Vials without any substrate or with the addition of N₂/CO₂ (80:20, v/v), in case of hydrogenotrophic assays, served as controls. All activity assays contained 2-5 g VSS l⁻¹ and were performed in triplicate. Assays on day 0 were performed at 15 and 37°C, assays on day 335 were performed at 10 and 37°C, and the results were expressed as ml CH₄ gVSS⁻¹ day⁻¹.

5.2.5 Analytical analysis

Effluent from the reactor was sampled every second day to analyse residual COD concentration according to Standard Methods (APHA, 2005). Analysis of effluent volatile fatty acids (VFA) were performed in a Varian Saturn 2000 GC/MS system, with CombiPAL autosampler (Varian Inc., Walnut Creek, CA) as described previously in Chapter 2 (Bialek et al., 2011). The total genomic DNA was quantified using the Quant-iT™ dsDNA BR Assay Kit with the Qubit™ fluorometer (Invitrogen, Molecular Probes Inc., Eugene, Oregon, USA). All analyses were performed in duplicate.

5.3 Results and discussion

5.3.1 Bioreactor performance

Figure 28 illustrates the COD removal efficiency (RE) profiles and effluent VFA concentrations associated with the IFB bioreactor during the trial. The operating parameters and performance are summarized in Table 10. The IFB bioreactor showed low COD RE throughout the whole trial (Figure 28). PI was characterized by unstable COD RE, fluctuating between 48-91%, although in-reactor effluent VFA concentrations were <200 mg COD l⁻¹. During PII, the COD RE dropped to an average of 55±4 % and effluent VFA concentrations peaked at c. 446 mg COD l⁻¹. Erratic bioreactor performance was observed during PIII and PIV with COD RE
fluctuating between 9-54% and effluent VFA concentrations peaked at c. 750 mg COD l⁻¹ (PIV, day 293). During PV (characterized by increase in OLR from 2 to 5 kg COD m⁻³ d⁻¹) there was initial drop in the COD RE down to 19% when the OLR was increased up to 2.9 kg COD m⁻³ d⁻¹, followed by a slight improvement in the COD RE when the OLR was further quickly increased up to 4.1 kg COD m⁻³ d⁻¹ and then decreased back to 2.9 kg COD m⁻³ d⁻¹. Subsequent gradual increases in the applied OLR, up to 5 kg COD m⁻³ d⁻¹, resulted in less fluctuations in the COD RE (44±6%), although effluent VFA concentrations fluctuated between 328-2050 mg COD l⁻¹. The return to a fixed OLR of 2 kg COD m⁻³ d⁻¹ during PVI resulted in initial decrease in the COD RE down to 21%, followed by slight improvement in the COD RE (40±7%) and a decrease in the VFA effluent concentrations to <140 mg COD l⁻¹ by the end of trial (Figure 28).

The slight improvement in the COD RE in the bioreactor during PV, when the OLR was gradually increasing up to 5 kg COD m⁻³ d⁻¹ is surprising, since it is accepted that high influent concentration will cause poor mixing if the flow rate (down flow velocity) is not adequate (Mahmoud et al., 2003). In general, poor bioprocess efficiency throughout the whole trial could be a reflection of not adequate mixing intensity due to the characteristics of down-flow configuration and low down flow velocity (1.5 m h⁻¹), when compared to similar systems (Arnaiz et al., 2003). Additionally, Extendosphere particles covered with biomass tended to adhere to each other forming conglomerates (Figure 29) which easily settled at the bottom of the bioreactor and supported loss by sedimentation (Alvarado-Lassman et al., 2008). It has been previously observed that the overgrowth of attached biomass to the support particles can provoke agglutination and modifies the bioparticle density, inducing bed stratification (Buffière et al., 1998). Buffière et al. (1998) noticed that this has an influence on the hydrodynamics of the fluidized layer and on the kinetics of organic carbon elimination by methanogenic biofilms. Furthermore, it has been demonstrated that the excessive accumulation of fixed biomass leads to a progressive decrease of biofilm specific activity associated with mass transfer limitations (Alves et al., 1999) and this could have been one of the reasons of inefficient performance observed in our bioreactor.

Although Alvarado-Lassman et al. (2008) reported successful application of the IFB technology, with the capability to apply OLR of 70 kg m⁻³ d⁻¹ of COD with an average COD RE higher than 90%, the reactor was operated by the authors in the
mesophilic temperature range. It is generally accepted that low temperatures (10°C as investigated in this study) show a very strong negative effect on metabolic activity of the anaerobic microorganisms and bioprocess performance (Lettinga et al., 2001) because, like other biological processes, anaerobic digestion depends strongly on temperature. Moreover, a drop in temperature results in changes in physical and chemical properties of the wastewater, which may considerably affect operation of the treatment systems (Bergamo et al., 2009). At low temperatures, the viscosity of liquids is increased that in consequence changes the hydraulic shearing force on the particles (Mahmoud et al., 2003) and the biomass in the reactor is less easily mixed, especially at low biogas production rates. Furthermore, at temperatures below 20°C the solubility of fats, particles and organic polymers is slow and may constitute the limiting stage of the process causing instability of the reactor (Speece, 1996).

Figure 28. Chemical Oxygen Demand (COD) Removal Efficiency (RE), Volatile Fatty Acids (VFA concentrations; presented as sum of: acetic-, butyric-, iso-butyric-, propionic-, valeric- and iso-valeric) and Organic Loading Rate (OLR; red line) applied to the Inverted Fluidized Bioreactor (IFB).
Table 10. Operational and performance characteristics during phases I to VI of the bioreactor operation.

<table>
<thead>
<tr>
<th>Phases</th>
<th>PI</th>
<th>PII</th>
<th>PIII</th>
<th>PIV</th>
<th>PV</th>
<th>PVI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days</td>
<td>0-146</td>
<td>147-230</td>
<td>231-265</td>
<td>266-294</td>
<td>295-322</td>
<td>323-335</td>
</tr>
<tr>
<td>HRT(^a)</td>
<td>48</td>
<td>24</td>
<td>18</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>OLR(^b)</td>
<td>0.5</td>
<td>1.0</td>
<td>1.3</td>
<td>2.0</td>
<td>2.0/5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>CODRE(^c)</td>
<td>69±10</td>
<td>55±4</td>
<td>37±13</td>
<td>38±14</td>
<td>44±8</td>
<td>33±9</td>
</tr>
<tr>
<td>Q(^d)</td>
<td>0.0036</td>
<td>0.0072</td>
<td>0.0096</td>
<td>0.0144</td>
<td>0.0144</td>
<td>0.0144</td>
</tr>
<tr>
<td>Cs(^e)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0/2.5</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \)Hydraulic retention time (h)
\( ^b \)Organic loading rate (kg COD m\(^{-3}\) d\(^{-1}\))
\( ^c \)Chemical oxygen demand removal efficiency (%), where values are the phases mean (±s.d.)
\( ^d \)Flow rate (m\(^{3}\) d\(^{-1}\))
\( ^e \)Substrate concentration (kg COD m\(^{-3}\))

Figure 29. Scanning Electron Microscopy (SEM) images showing conglomerates of many extendosphere particles covered with biomass. (A) three particles grouped (B) many particles trapped in biomass incrusted with depositions of calcium.

5.3.2 Real-time PCR of archaeal populations

The 16S rRNA gene concentration of hydrogenotrophic order *Methanomicrobiales* initially increased from 2.7×10\(^5\) copies/ml (5.1%) on day 0 up to 1.2×10\(^6\) copies/ml (69.3%) on day 140 (PI). Subsequent HRT reductions from 48h to 12h (PI to PIV) resulted in fluctuations of the 16S rRNA gene concentration of *Methanomicrobiales* and this group was detected at 4.2×10\(^5\) copies/ml (43%; PIV; day 294). However, by the end of the trial (PVI; day 335), the 16S rRNA gene concentration of *Methanomicrobiales* significantly decreased to 2.5×10\(^1\) copies/ml corresponding to 16478-fold decrease (Figure 30).
The hydrogenotrophic order *Methanobacteriales* (MBT) was detected at $1.4 \times 10^5$ copies/ml at the beginning of trial (PI; day 0). Following the HRT reduction from 48 to 12 h (PI to PIV), a decrease in the 16S rRNA gene concentration of this group was recorded and by day 294 it was detected at $3.6 \times 10^4$ copies/ml. Subsequently, the 16S rRNA gene concentration of *Methanobacteriales* significantly decreased by the end of trial (day 335) to $4.2 \times 10^2$ copies/ml corresponding to 85-fold reduction (Figure 30).

The significant decrease in the 16S rRNA gene concentrations of *Methanobacteriales* and of *Methanomicrobiales* indicate that stress induced by increases in the OLR during PV (increase in OLR from 2 to 5 kg COD m$^{-3}$ d$^{-1}$) had a negative effect on those methanogenic groups during 10°C bioreactor operation. Although operational conditions applied during PVI (day 335) were the same as applied during PIV (OLR of 2 kg COD m$^{-3}$ d$^{-1}$), before the OLR shock loads, both hydrogenotrophic groups could have been washed out of the IFB bioreactor, outcompeted by different microorganisms or were suppressed due to unfavorable conditions. Wang et al. (2009) concluded that the concentration of propionic acid in the system below 300 mg l$^{-1}$ did not have negative effects on the methanogenic bacteria. Demirel and Yenigun (2002) and Wang et al. (2009) reported that when the concentration of propionic acid reached 900 mg l$^{-1}$, the activity of acidogenic bacteria became repressed, resulting in greatly decreased degradation rates of VFAs and consequent VFA accumulation. Once the VFA accumulation went beyond the endurance of acidogenic bacteria, the pH of the reactor decreased rapidly and the activity of methanogenic bacteria became repressed and the methane yield decreased, leading to further accumulation of acetic acid and finally to complete cessation of methane production (Wang et al., 2009). Although, in our study the max. residual propionic acid concentration within the system did not exceeded 260 mg COD l$^{-1}$, our bioreactor was operated at 10°C, as opposed to 37°C described by (Wang et al., 2009), therefore the effect of propionic acid and VFAs accumulation on methanogenic bacteria during low operating temperature is not known.

Another possible hypothesis explaining inhibition of hydrogenotrophic methanogens could be the toxic effect of ammonium ion ($\text{NH}_4^+$) and free ammonia (FA) ($\text{NH}_3$), being the main products of the biodegradation of proteins in anaerobic conditions.
(Vidal et al., 2000), on methanogenic bacteria (Koster & Lettinga, 1988), although, this was not monitored in our study. There is, however conflicting information in the literature about the sensitivity of aceticlastic and hydrogenotrophic methanogens. Some research based on the comparison of methane production and growth rate indicated that the inhibitory effect was in general stronger for the aceticlastic than for the hydrogenotrophic methanogens (Angelidaki & Ahring, 1992; Borja et al., 1996), while others observed the relatively high resistance of acetate consuming methanogens to high total ammonia nitrogen (TAN) levels as compared to hydrogen utilizing methanogens (Wiegant & Zeeman, 1986; Zeeman et al., 1985). Also, significant difference in inhibiting ammonia concentrations reported in the literature can be attributed to the differences in substrates and inocula, environmental conditions (temperature, pH), and acclimation periods (Angelidaki & Ahring, 1994; Chen et al., 2008; Hashimoto, 1986).

The aceticlastic family *Methanosaetaceae* (Mst) accounted for 92.1% (4.8×10⁶ copies/ml) at the beginning of the trial (day 0). By the end of the trial (day 335), the 16S rRNA gene concentration of *Methanosaetaceae* had decreased to 5.8×10⁵ copies/ml, although the relative abundance increased to 99.9% (Figure 30). Despite, 8-fold reduction in the absolute quantity values, *Methanosaetaceae* seemed to be the least disturbed group, suggesting that OLR changes applied during the trial did not perturb members of this community to such a great extent. Such results also indicate that this aceticlastic family was an important member of the methanogenic community and may be retained within anaerobic biofilms during cold (10°C) bioreactors operation (McKeown et al., 2009a).

The aceticlastic family, *Methanosarcinaceae* (Msc), was detected (i.e., >5.63×10¹ copies/ml) during PI-PIV and accounted for only 0.4% (7.4×10³ copies/ml). At the end of trial (PVI; day 335) *Methanosarcinaceae* were not detected in the bioreactor (Figure 30). Emergence of the fast growing *Methanosarcina sp.* has been described as a result of high acetate levels and has previously been suggested to be associated with process deterioration (Collins et al., 2003; McMahon et al., 2004; O'Reilly et al., 2009b) as was observed in the IFB bioreactor. Fluctuations of the effluent VFA concentrations within the IFB bioreactor during PI-PIV (16-750 mg COD l⁻¹; Figure 28), where acetate constitutes 63-100% (16-660 mg COD l⁻¹) supported the growth
of *Methanosarcina* (Jetten et al., 1990). Additionally, the biomass used to inoculate the IFB bioreactor was sourced from previous run at 15°C where *Methanosarcina* have been detected (Chapter 3; Bialek et al., 2012) and so this group could have remained above the detection limit (presumably by retention in biofilms) when the IFB bioreactor was later operated at 10°C. It is not clear why *Methanosarcina* disappeared from the system at the end of trial (PVI; day 335), since significant accumulation of in-reactor VFA concentrations, where the residual acetate concentration within the system peaked at 1680 mg COD l\(^{-1}\) (Figure 28) would have favored an increased level of this group. Wang et al. (2009) showed that the acetic acid concentration of 2400 mg l\(^{-1}\) had no significant inhibition effect on the activity of methanogenic bacteria, although this was based on mesophilic AD and the influence on particular methanogenic groups was not determined. On the other hand, Vidal et al. (2000) showed that the fast hydrolysis of soluble substrates in carbohydrate-rich wastewaters (skimmed-milk powder) produced inhibition of the acetoclastic methanogenic bacteria, because of the presence of high concentrations of VFA. In our system, although the operational conditions applied during PVI (day 335) returned to those applied during PIV (OLR of 2 kg COD m\(^{-3}\) d\(^{-1}\)) *Methanosarcina* did not regrow/rebound in the bioreactor.

![Figure 30](image)

**Figure 30.** Absolute quantification of the 16S rRNA gene concentration of the methanogenic/archaeal populations during psychrophilic (10°C) IFB operation.
5.3.3 Physiological characterization of biomass

SMA results indicated the mesophilic nature of the inoculum (day 0) and biomass at the end of trial (day 335), exhibiting higher activity at 37°C than at 10 and 15°C, for all substrates tested (Table 11). Assays carried out at the beginning of the trial (day 0) biomass displayed a preference for aceticlastic methanogenesis at 37°C and for hydrogenotrophic methanogenesis at 15°C. However, metabolic activity determined by the end of trial (day 335) at 10°C (the same as bioreactor operational temperature throughout the trial) and at 37°C indicated preference for H₂/CO₂-mediated methanogenesis (Table 11).

A similar approach to that presented in Chapter 4 was used to correlate the metabolic activity (SMA) of aceticlastic methanogens utilizing acetate as sole substrate with the absolute quantification of the 16S rRNA gene copy concentration of the aceticlastic methanogens, utilizing acetate as a carbon source. In this experiment the whole acetate conversion was attributed to the members of two aceticlastic families Methanosetaeaceae (Mst) and Methanosarcinaceae (Msc) (Boone et al., 2001). The sum of 16S rRNA gene concentration of Methanosetaeaceae (Mst) and Methanosarcinaceae (Msc) indicated 8-fold decrease at the end of the trial (4.8×10⁶ copies/ml at day 0 versus 5.8×10⁵ copies/ml at day 335; Table 11). At the same time the SMA activity against acetate displayed almost 3-fold reduction at 37°C at the end of the trial (455±11 at day 0 versus 158±13 at day 335; Table 11). The SMA determined at 15°C (day 0) and 10°C (day 335) was always lower than the activity determined at 37°C, as temperature reduction has been reported to have strong effect on the activity of the aceticlastic methanogens, reducing maximum substrate utilization rates of microorganisms (Kettunen & Rintala, 1997; Lettinga et al., 1999; Rebac et al., 1995; Wu et al., 1993). However, in our study the SMA was determined only at 10°C at the end of experiment; hence it is possible that the value was lower at the beginning of the trial (at the same temperature). It was observed previously that due to the biomass adaptation to low temperature, improved methanogenic activity against acetate was observed (McKeown et al., 2009a).

In case of hydrogenotrophic methanogens the members of two orders Methanobacteriales, and Methanomicrobiales (Boone et al., 2001) were correlated with SMA activity against H₂/CO₂ (as explained in Chapter 4). Despite almost 910-
fold decrease in the sum of 16S rRNA gene concentration of the hydrogenotrophic *Methanomicrobiales* (MMB) and *Methanobacteria* (MBT) by the end of the trial (4.1×10^5 copies/ml at day 0 versus 4.5×10^5 copies/ml at day 335; Table 11) the SMA activity determined against H2/CO2 at 37°C at day 335 was only 1.6-fold lower than SMA value determined at day 0 (244±1 at day 335 versus 391±11 at day 0; Table 11). The SMA determined at 10°C by the end of trial (day 335) also did not show big decrease, when compared to SMA value at the beginning of trail (day 0), although determined at 15°C. Such results might indicate that although absolute quantity values of hydrogenotrophic methanogens significantly decreased, their metabolic activity was not affected to such a high degree.

Additionally, the estimated DNA amount at the beginning (day 0) and at the end of trial (day 335) was 0.25±0.01 mg DNA/g VSS and 0.7±0.01 mg DNA/g VSS, respectively. Despite almost 3-fold increase in the DNA concentration per gram VSS, a marked reduction in the 16S rRNA gene copy concentration of *Archaea* and *Bacteria* (5-fold and 119-fold decrease, respectively; Table 11) was observed by the end of the trial. Although the reason for such disparity is not known, it is tentatively suggested that extracellular debris DNA was trapped in the fluidized biomass on the support particles and persisted in the system. Further studies should, therefore investigate the correlation between the DNA concentration, the active biomass concentration and the 16S rRNA gene copy concentration in relation to mRNA expression levels indicating metabolic activity of the microbial communities underpinning the AD processes (Raes & Bork, 2008).

**Table 11.** Specific methanogenic activity (SMA) profiles against direct and indirect methanogenic substrates expressed as ml CH4 gVSS^-1 day^-1 and the absolute quantification of 16S rRNA gene copy concentration expressed in copies/ml, of day 0 and day 335 biomass.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>T (°C)</th>
<th>Trial day</th>
<th>Specific methanogenic activity (ml CH4 g VSS^-1 day^-1)</th>
<th>Absolute quantification by qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SMA Substrate</td>
<td>16S rRNA gene copy concentration (copies/ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetate</td>
<td>H2/CO2</td>
</tr>
<tr>
<td>IFB</td>
<td>37</td>
<td>0</td>
<td>455±11</td>
<td>391±11</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td></td>
<td>87±6</td>
<td>101±1</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>335</td>
<td>158±13</td>
<td>244±1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>6±1</td>
<td>86±16</td>
</tr>
</tbody>
</table>

All SMA values are the mean of triplicates (std. error; n = 3), except * where values are the mean of duplicates (std. error; n=2). Absolute quantification of 16S rRNA gene copy concentration of the groups: Mst (*Methanosetaeae*) + Msc (*Methanosarcinae*) (Msc), MBT (*Methanobacteria*) + MMB (*Methanomicrobiae*) (MBT), ARC (*Archaea*), BAC (*Bacteria*).
5.3.4 Bacterial DGGE and UPGMA cluster analysis

Comparative PCR-DGGE analysis of the bacterial populations in the bioreactor identified 10% difference between day 0 (seed biomass) and all other DGGE profiles of the bacterial populations (day: 140, 226, 255, 294, 335; Figure 31). Seed biomass was sourced from 15°C laboratory-scale IFB bioreactors described in Chapter 3 (Bialek et al., 2012) and was acclimated to 10°C for 30 days prior to start-up, so this could explain the 10% difference visualized by cluster analysis of bacterial DGGE profiles. The UPGMA cluster analysis of bacterial DGGE profiles revealed >98% similarity of samples during PI-PIV (day: 140, 226, 255, 294; Figure 31). Sample at the end of trial (day 335; PVI) showed >95% similarity to the former profiles suggesting that OLR increments applied during PV had a considerable influence on the bacterial composition, and this was detectable even at the low-resolution as offered by the DGGE (Talbot et al., 2008).

Figure 31. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of the 16S rRNA gene fragments generated from bacterial Denaturing Gradient Gel Electrophoresis (DGGE) profiles of IFB biomass. Similarity calculated by Sørensons (Bray-Curtis) distance measurement.

5.3.5 Bacterial and archaeal phylogenetic analysis

Partial 16S gene sequences from 18 out of total 19 excised bacterial DGGE bands grouped within four phyla: *Firmicutes*, *Proteobacteria*, *Spirochaetes* and *Bacteroidetes* (Figure 32 a).
The phylum *Firmicutes* was represented by five bands (B10, B11, B12, B14, B21), which were mostly present in all biomass samples. Information on the identity and potential role of some of these bacteria could be tentatively inferred from the phylogenetic analysis. For example, B10 showed 98% similarity to *Clostridium aminobutyricum* and was present in bioreactor during the whole 10°C trial, what is somehow surprising since the optimum temperature growth of this organism was reported to be in the mesophilic range (Hardman & Stadtman, 1960). B14 was apparent during the whole trial and shared 99% similarity with *Trichococcus flocculiformis*, *Trichococcus collinsii* and *Trichococcus pasteurii*, aerotolerant, fermentative organisms growing with glucose, sucrose and lactose to produce lactate, acetate, formate and other acids (Liu et al., 2002).

The phylum *Spirochaetes* was represented by two bands (B15 and B17), which shared 99% similarity to *Spirochaetaceae bacterium* and were present in all 10°C biomass samples. Although *Spirochaetes* were reported to be numerically important in psychroactive consortia (McKeown et al., 2009a) their function is not clear.

The phylum *Bacteroidetes* was represented by ten bands (B2, B3, B4, B5, B7, B8, B9, B19, B26, B27), which were mostly present in all 10°C biomass samples, but were affiliated to uncultured organisms and their function remains unclear.

The phylum *Proteobacteria* was represented by one band (B23), sharing 99% similarity with *Dechloromonas denitrificans* and *Dechloromonas hortensis*. The latter is a (per)chlorate-reducing bacteria, utilizing acetate and propionate as electron donors (Wolterink et al., 2005). The former is producing N₂O as an intermediate during the reduction of NO₃⁻ to N₂ and utilizes acetate, propionate, butyrate, iso-butyrate, iso-valerate, lactate, pyruvate, succinate, malate, glutamate and Casamino acids as electron donors (Horn et al., 2005).

In the archaeal DGGE gel, a total of 6 bands (A1-A6) were retrieved and sequenced (Figure 32b). Bands A1-3 were closely related to hydrogen-utilizing *Methanocorpusculum* like clones (*M. bavaricum, M. parvum, M. labreanum, M. sinense*) with 99% sequence similarities. Similar clones were previously observed during the cold adaptation of anaerobic granular and floculant sludge (Bialek et al.,...
Band A4 showed 99% similarity to *Methanospirillum hungatei* and was detected in all 10°C biomass samples, although become very faint by the end of trial. This organism has been previously observed during psychrophilic (15°C) anaerobic digestion in EGSB bioreactors (Chapter 3; Bialek et al., 2012). Bands A5 was closely related to acetoclastic *Methanosaeta concilii* (100% similarity) and was detected throughout the whole trial. Band A6 was only detected in the bioreactor at the beginning of trial (day 0 and 140) and was closely related to acetoclastic *Methanosarcina mazei* and *Methanosarcina lacustris* (99% similarity).
Figure 32. Neighbor joining tree illustrating the phylogenetic affiliations of the 16S rRNA gene sequences obtained from: (A) bacterial DGGE bands and (B) archaeal DGGE. Bioreactor biomasses containing the respective bands are given in parenthesis.
5.4 Conclusions

The low-temperature (10°C) anaerobic biotreatment of complex dairy-based wastewater in the IFB bioreactor displayed low efficiency and unstable performance throughout the trial. Present study shows that organic loading rate (OLR) with values >2 kg COD m\(^{-3}\) d\(^{-1}\) influenced the abundance of hydrogenotrophic methanogens, although the SMA did not record such extreme reduction in the metabolic activity. This indicates need for future studies to elucidate the correlation between DNA-based and function-oriented analyses during LTAD.

The use of inert nuclei (Extendospheres) in the fluidized bed bioreactors to promote re-granulation during psychrophilic reactor operation presents an interesting possibility, but an optimization of this technology is required. Future studies should focus on selecting better support materials for biofilm formation. Additionally, better understanding of physico-chemical properties of biomass and liquids during LTAD will help to optimize reactor configuration and select the optimal operating conditions for the treatment of complex industrial wastewaters.
5.5 References


Appendix 4: Set-up of the EGSB and IFB during 10°C experiment (Chapter 4 and 5).
Chapter 6

Final conclusions and future recommendations

6.1 Final conclusions

This thesis provides new information on the feasibility of anaerobic digestion of complex dairy wastewater, under variable operational conditions. Two different reactor configurations, an Expanded Granular Sludge Bed (EGSB) and an Inverted Fluidized Bed (IFB) bioreactor were employed to assess the effect of those configurations on the development of methanogenic community structure and population dynamics. Additionally, the influence of operating temperature as well as other operating conditions (i.e. organic loading rate and hydraulic retention time) on the process performance and population dynamics was investigated in the course of this study.

This study demonstrated that the two different reactor configurations investigated had a significant influence on the development of microbial community structure and composition (Chapter 2, 3, 4 and 5). The transition from mesophilic to psychrophilic temperatures provoked a shift in the methanogenic community composition, which appeared to be affected in a different manner in the two different reactor configurations, as investigated in this study.

Statistical analyses, based on the quantitative polymerase chain reaction (qPCR) data employed in this study, were demonstrated to be useful techniques to monitor and visualize the methanogenic community shifts associated with operational changes in the bioreactors (Chapter 2 and 3; Bialek et al., 2011; Bialek et al., 2012). Quantitative shifts in the methanogenic communities were visualized using Nonmetric multidimensional scaling (NMS) technique and moving window analysis, based on qPCR data. Based on 16S rRNA gene concentration data, matrices were created based on absolute quantity and relative abundance. The results presented in Chapter 2 (Bialek et al., 2011) suggest that the outputs derived from a relative abundance matrix could possibly avoid potentially distorted outputs, due to the influence of one dominant variable. The relative abundance-based method, although
based on reliable absolute quantification, had a more meaningful correlation of methanogenic population shifts to process performance under reasonably constant mesophilic conditions with no drastic changes in total microbial mass (such as the retained biomass systems used during the 200-day trial; Chapter 2). When dealing with a system with a high fluctuation of active biomass (e.g. suspended biomass continuously stirred tank reactors) or under variable operating conditions with big fluctuations in the methanogenic community composition, however, the absolute quantity-matrix appeared to be more applicable (e.g. in describing the transition from mesophilic to psychrophilic temperature operation with community shift towards hydrogenotrophic methanogens during the 430-day trial; Chapter 3). Those two contradictory findings imply that caution needs to be exercised when selecting appropriate statistical techniques. It is recommended, therefore to apply several different statistical techniques, when searching for the best fit. Overall, those findings indicate no predictive power of the two applied statistical techniques and, although such studies can provide some insight into the functional potential of anaerobic digesters, they cannot predict the actual functions taking place under specific process conditions (Raes & Bork, 2008).

Despite many successful applications of AD, there is a lack of fundamental knowledge of the mechanisms underpinning AD. In particular, the relationship between the dynamic behavior of microbial communities and environmental parameters in AD has hardly been studied (Appels et al., 2011; Tomei et al., 2009). Therefore, there is a pressing need for more and better information on the biology, rates and limitations of bacterially-mediated processes. Changes in feed composition, temperature, pH, etc., strongly influence the activity of microbial populations and the fact that, the activity of a microorganism is dependent on both itself and its environment, is difficult to accommodate (Appels et al., 2011). Mathematical models are widely accepted as a pre-requisite for improvement of AD performance, but a full and completely deterministic model of the process must be able to accommodate the inherent biological complexity of the process (Appels et al., 2011).

Additionally, discrepancies between the molecular and physiological data were observed in the EGSB bioreactor (e.g. a 2-fold decrease in the 16S rRNA gene copy concentration of the hydrogenotrophic methanogens versus almost 2-fold higher metabolic activity against H₂/CO₂; Chapter 4) and IFB bioreactor (e.g. a 910-fold
decrease in the 16S rRNA gene copy concentration of the hydrogenotrophic methanogens although only 1.6-fold lower metabolic activity against H₂/CO₂; Chapter 5). Those results point out the limitations of studies based only on the DNA and indicate the need for more future studies focusing on functional investigations to unravel metabolic activity of the microbial communities underpinning the AD processes. Wendeberg et al. (2012) found out that the abundance and distribution of sulfur- and methane-oxidizing symbionts in eight mussel individuals from hydrothermal vents was not noticeably different, although mRNA expression levels varied markedly. Those authors concluded it would be energetically favorable for the symbionts to respond to these short-term variations in substrate availability with changes in transcriptional activity that can provide adaptations within a short time, whereas changes in symbiont abundance involve interactions between the symbiont and the host that can take days to weeks, and would therefore only be energetically favorable after long-term changes in vent geochemistry (Wendeberg et al., 2012). Similar studies of AD are still in its infancy and since prediction of function based on phylogeny is not complete (Nelson et al., 2011) a need for models, such as ecosystems biology models integrating function-oriented approaches with community structure analysis, is highlighted (Raes & Bork, 2008).

Despite the shortcomings of the two statistical techniques investigated in this study, the results showed that the IFB and EGSB methanogenic biofilm communities developed differently after 200 days operation under mesophilic (37°C) operation (Chapter 2) suggesting that bioreactor configuration is likely to directly influence methanogenic community structure and dynamics. Additionally, temperature reduction from mesophilic (37 and 25°C) to psychrophilic temperature (15°C) appeared to have significant influence on the shaping of methanogenic community structure and consequently the communities of the IFB and EGSB bioreactors were relatively different by the end of 430-day operation (Chapter 3).

Temperature reduction appeared to have a significant impact on the process performance and microbial community structure and dynamics. During mesophilic operation at 37°C and 25°C, the IFB and EGSB reactors exhibited stable performance (>80% COD RE and >80% PRE). However, during 15°C operation the IFB reactors using fluidized biomass attached to support particles (Extendospheres) demonstrated...
quicker adaptation to low temperature operation and more stable performance at psychrophilic, steady-state, operation (>78% COD RE and >77% PRE; Chapter 3). On the other hand, the EGSB reactor displayed slower adaptation to low temperature and unstable performance (minimum values of 13% PRE and < 60% COD RE). The microbial communities in IFB appeared to be more dynamic than in the EGSB reactors and showed greater phylogenetic variability, which may have facilitated maintenance of more efficient reactor performance.

The quantitative real-time PCR analysis highlighted increased abundance of hydrogenotrophic methanogens of the order *Methanomicrobiales*, and especially *Methanocorpusculum*-like organisms, during 15°C bioreactor operation. This is possibly due to the metabolically and thermodynamically more favorable metabolism of hydrogen than acetate at 15°C (Kotsyurbenko, 2005; Lettinga et al., 2001) (Chapter 3). The emergence and maintenance of some bacterial strains (phylum *Firmicutes* and *Bacteroidetes*) possibly enhanced the protein hydrolysis in the low-temperature IFB reactor, further suggesting that reactor configuration has a significant and direct influence on the development of the microbial community structure. Additionally, monitoring of protein concentrations in dairy wastewater effluents might be linked to reactor perturbations, indicating problems with protein hydrolysis, which is often problematic during LTAD (Chapter 3).

The application of a further temperature reduction to 10°C provoked unexpected outcome for the two configurations. The EGSB bioreactor demonstrated stable and efficient biotreatment of complex dairy-based wastewaters with no accumulation of solids under 10°C operation at applied OLR of 0.5-2 kg COD m⁻³ d⁻¹ with mean COD RE >85% (Chapter 4). Although the OLR influenced the efficiency of bioprocess, and values >2 kg COD m⁻³ d⁻¹ had direct influence on COD RE decrease, the bioreactor recovered very quickly (3 days after the maximum OLR was reduced 2.5 times from 5 to 2 kg COD m⁻³ d⁻¹) reaching >84% COD RE (Chapter 4). An increased abundance of hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*), recorded via qPCR analysis by the end of the 335-day trial, could be a stress response induced by overload OLR. Aceticlastic *Methanosetae* spp. appeared to be the most stable methanogenic group, independently of perturbations in OLR applied during the 10°C bioreactor operation (Chapter 4).
On the other hand, the IFB was not stable during 10°C operation and showed poor performance throughout the whole trial with mean COD removal efficiency (RE) of 54±17% at applied organic loading rates (OLRs) of 0.5-5 kg COD m⁻³ d⁻¹ (Chapter 5). Increments in the applied OLR above 2 kg COD m⁻³ d⁻¹ additionally perturbed the system and influenced the microbial composition and dynamics. Hydrogenotrophic methanogens: *Methanomicrobiales* and *Methanobacteriales* monitored via qPCR demonstrated 16478-fold and 85-fold decrease in their abundance, respectively. This suggests that those organisms were inhibited or washed out from the system after the OLR stress was applied and did not regrow even when the conditions were changed and stress removed. The dynamics of bacterial community in the bioreactor monitored via DGGE also suggested an influence of OLR stress on bacterial community structure and population dynamics. Possible problem, which occurred, could have been the conglomeration of particles covered with biomass and in consequence they were washed out from the system or settled down, negating the idea of fluidization.

As the temperature drop strongly affects the physical and chemical properties of the wastewater (Lettinga et al., 2001) this may in turn influence the biofilm structure (Horn & Hempel, 1998; Stoodley et al., 1998) and thus extracellular polymeric substances (EPS) composition (Sheng et al., 2010). Since EPS constitute a significant fraction of the organic matter within biofilms composition (Beer & Stoodley, 2006), changes in the EPS concentration and amounts may have an impact on the concentration of active biomass within the total organic matter expressed in VSS (volatile suspended solids) as observed during 10°C EGSB operation (Chapter 4). In our study, an increase in the DNA amount was observed by the end of 335 day trial, although reduction in the VSS concentration occurred at the same time. Increase in the DNA concentration per gram VSS, as well as increase in the 16S rRNA gene copy concentration of *Archaea* and *Bacteria* by the end of the trial, implies that the EPS or other organic matter was washed out from the biomass (loose granules were observed; Chapter 4). This, in turn, raised the concentration of active biomass and thus the DNA concentration. Since the EPS supports stability of microbial aggregates within biofilm, they are crucial to maintain stable bioprocess (Sheng et al., 2010). It has been observed previously that the anaerobic aggregates with low amounts of EPS exhibit a strong tendency to float by attachment of gas bubbles to the hydrophobic
aggregate surface, which leads to severe biomass losses from the reactors (Beer & Stoodley, 2006). Therefore, more studies are needed to understand the influence of low operational temperature on the biofilm development and parameters influencing the EPS formation and composition as well as granule stability and strength.

The overall findings of this study could be helpful in future design and optimization of reactors intended for LTAD. For example, a conclusion of this study is that bioreactor design can influence methanogenic community composition during LTAD of a synthetic dairy wastewater, and thus more attention should be given to optimize the configuration of reactors intended for low-temperature applications. The EGSB bioreactors, although inoculated with crushed biomass granules, which did not fully re-granulate during the course of this study, demonstrated successful operation, even at the lower temperatures (Chapters 2, 3 and 4). The use of inert nuclei (Extendospheres) in the IFB bioreactors to promote re-granulation during mesophilic and psychrophilic reactor operation presents an interesting possibility, as presented in this study (Chapters 2, 3 and 5). The feasibility of fluidized bioreactor operation was demonstrated during 37, 25 and 15°C biotreatments, but operation at 10°C was challenging and optimization of this technology is needed especially at the lower temperatures (Chapter 5).
6.2 Future recommendations

Results reported in this thesis provide information about the influence of two different reactor configurations on the process performance and microbial community structure and population dynamics during LTAD. However, significant knowledge gaps were identified and the following recommendations are proposed:

- More studies should focus on selecting better support materials for biofilm formation. Materials more susceptible for biofilm formation could help to retain more biomass in the system, which is crucial during LTAD (Lettinga et al., 2001). Colonization experiments using different materials to select the best support media for low-temperature colonization are therefore recommended. Such experiments should also determine optimal operating conditions for different types of wastewaters, as it is known that variations in one or more parameters i.e. the influent type and concentration, flow rate, sludge retention time (SRT), nutrient availability, temperature, pH, presence of xenobiotics, as well as others can affect or define the reactor performance (Chen et al., 2008; Leitao et al., 2006).

- Systematic evaluation of many different reactor configurations is necessary to determine the best bioreactor configuration for the treatment of dairy wastewater under LTAD. The design of bioreactors is generally based on rule of thumb, and bioreactor over-dimensioning, process instability and failures are still common (Appels et al., 2011). Therefore, methodical investigation of the influence of different reactor configurations on the performance and microbial community structure and population dynamics using the same inoculum seed sludge is recommended.

- Currently AD is operated based on relationships between bioreactor performance and empirical operating parameters, but the differences between successful and unsuccessful bioreactors are poorly understood (Chen et al., 2008; Leitao et al., 2006). Therefore better understanding of physico-chemical properties of biomass and liquids during LTAD will help to optimize reactor configuration and choose the best operating conditions.

- An investigation into the influence of low operational temperature on the biofilm development and parameters influencing the extracellular polymeric
substances (EPS) formation and composition as well as granule strength are necessary. It has been observed previously that the anaerobic aggregates with low amounts of EPS show a strong tendency to float by attachment of gas bubbles to the hydrophobic aggregate surface, which may lead to severe biomass losses from the reactors (Beer & Stoodley, 2006). Given that EPS supports stability of microbial aggregates within biofilm (Sheng et al., 2010), information about their content could thus be crucial during LTAD.

- DNA-based methods may yield false interpretation of the community dynamics, therefore function-oriented studies are required to understand influence of temperature transitions and operation at sub-optimal temperatures on the metabolic activity of anaerobic consortia underpinning the LTAD. The application of metaproteomics could provide significant insights into microbial activity, linking microbial community structure to functions and informing on which microorganism is carrying out what function (Siggins et al., 2012). Additionally the application of metagenomics, metatranscriptomics and metaproteomics (Gosalbes et al., 2011; Hettich et al., 2012; Kolmeder et al., 2012; Qin et al., 2010) in conjunction with metabolomics (Halter et al., 2012), which is the study of the intermediate and end-product of cellular processes, could improve our understanding of anaerobic microbial ecosystems.

- Optimization, further development and wider application of AD requires better knowledge of the mechanisms occurring at microscales, which should, in turn, be linked to the macroscale system performance and behavior (Rittmann et al., 2008). Prediction of ecosystem attributes will help with process optimization and development of full-scale LTAD. Therefore incorporating microbial information into integrated models (ecosystem biology modeling) will be useful for diagnosing and/or assessing anaerobic digesters and predicting future reactor performance (Klitgord & Segre, 2011).
6.3 References


