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LOW-TEMPERATURE
ANAEROBIC DIGESTION
AS A CORE TECHNOLOGY FOR THE
SUSTAINABLE TREATMENT OF
MUNICIPAL WASTEWATER

A Thesis Submitted to the National University of Ireland for the
Degree of Doctor of Philosophy
by

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SEPTEMBER 2013

Head of Department: Professor James P. O’Gara
Research Supervisor: Professor Vincent O’Flaherty
Acknowledgements

Abstract

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Abstract

The currently applied paradigm for municipal wastewater treatment in the European Union does not meet basic sustainability criteria. Indeed, it runs counter to the stated goals of recent European Council policies regarding sustainable development. This disposal based linear system uses aerobic microbiology as the core technology. This results – in the case of activated sludge plants, for example – in a requirement for large capital investment, heavy usage of fossil fuels, high technology operational control and the generation of large quantities of sludge requiring treatment before safe reuse/recycle. A direct anaerobic treatment approach is proposed in this study would assist in meeting sustainability criteria and offer significant advantages to the conventional treatment approach. Anaerobic digestion (AD) has long been recognised as a sustainable waste/wastewater treatment strategy. The major application of high-rate AD has been for high-strength industrial wastewaters, e.g. food processing, brewing, etc., where mesophilic high-rate AD has been tremendously successful. There is great potential for expanding the application of anaerobic wastewater treatment. One area of particular interest is AD for the treatment of low-strength, high volume wastestreams which are discharged at sub-mesophilic (<20°C) or low temperatures (e.g. municipal wastewater in countries with temperature climates). The implementation of anaerobic treatment of these low-temperature wastestreams is severely limited by economics, whereby a significant energy input to heat bioreactors would be required for treatment to proceed within a mesophilic temperature range, therefore negating the cost saving associated with direct anaerobic treatment.

The objective of this thesis was to investigate the feasibility and applicability of direct AD of municipal wastewater under Irish conditions; low-temperature anaerobic digestion (LtAD). Process technology trials were supported by microbial physiology and 16S rRNA gene community analyses.

In the first phase of this study (chapter 2), the feasibility of long-term (>1 year), low-temperature (10-15°C) anaerobic bioreactor operation, for the treatment of synthetic sewage, was investigated. The effect of temperature on the bioprocess was investigated through the use of a mesophilically (37°C)
operated control bioreactor. Three hybrid bioreactors (R1-R3) were seeded with a mesophilic inoculum, and used to treat a synthetic sewage wastewater at 37, 15 and 10°C respectively. Organic loading rates (OLRs) of 0.5-6 kg chemical oxygen demand (COD) m⁻³ d⁻¹ and hydraulic retention times (HRTs) of 1.5-12 h were applied during a 1.5-year trial. Despite transient disimprovements, mean total COD removal efficiency and methane biogas concentrations exceeded 70% and 50%, respectively, for all bioreactors. Specific methanogenic activity (SMA) testing indicated that a psychroactive biomass developed in the low-temperature bioreactors. The data obtained suggest that a mesophilic inoculum can physiologically adapt to sub-optimal temperature, and efficiently treat low-strength wastewater at temperatures as low as 10°C.

In the second phase of this study (chapter 3) two hybrid bioreactors (R4 and R5), were each seeded with a mesophilic biomass, and employed for the treatment of synthetic sewage at 12°C and applied OLRs of 0.5-6 kg COD m⁻³ d⁻¹, and HRTs of 2-24 h. Based on the results obtained in chapter 2 the use of an alternative fixed-film matrix material; granulated pumice stone, to enhance process efficiency and stability was evaluated. In addition the reproducibility of LtAD for municipal wastewater treatment was investigated. Stable bioprocess performance was demonstrated with COD removal efficiencies of >70% obtained by both bioreactors. SMA and biodegradability assays demonstrated the development of a psychrotolerant, sewage-degrading consortium within the biomass of both bioreactors. Bacterial communities, as deduced from clone library analysis at the conclusion of the trial, were phylogenetically diverse, consisting of important fermentative and hydrolytic populations. Archaeal methanogenic dynamics indicated that acetoclastic methanogenic activity directly correlated to bioreactor performance.

In the final phase of this study (chapter 4) the feasibility of direct anaerobic treatment of raw and settled sewage under temperate climatic conditions (12°C) was investigated. Two expanded granular sludge bed (EGSB) anaerobic filter (AF) hybrid bioreactors, R6 and R7, were employed to treat raw sewage and settled sewage (primary effluent; the liquid fraction following settlement of primary sludge), respectively, sourced from the city of Galway, Ireland. The bioreactors were operated at HRTs of 3-24 h in a trial
of c.140 days. Successful treatment of both influent types was achieved with consistent effluent quality of <125 mg l⁻¹ COD being obtained at OLRs of 0.2-2.4 kg COD m⁻³ d⁻¹, volumetric loading rates (VLRs) of 1-4 m³ wastewater m⁻³ bioreactor d⁻¹ and HRTs of 3-6 h. Bacterial clone libraries at the conclusion of the trial demonstrated communities that were phylogenetically diverse, similar to those observed in chapter 3, with the identification of key hydrolytic and fermentative populations being achieved. Archaeal clone library analysis indicated that acetoclastic methanogenesis was dominant in both R6 and R7 by the completion of the trial.

In summary, LtAD was demonstrated as an effective and efficient wastewater treatment approach for municipal wastewater. Combining bioprocess monitoring with physiological and molecular analyses, provided valuable insights into the complex microbial communities underpinning the LtAD process.
CHAPTER 1

INTRODUCTION
1.1 General Introduction

“there is little doubt that a cost-effective and efficient anaerobic sewage treatment alternative [to conventional activated sludge] would be one of the most significant advances in waste treatment”

Jewell (1985)

It is widely accepted that sewage is the main point-source pollutant in the world and the disposal of such wastewater without any treatment is a global pollution concern threatening the world’s population (Gijzen, 2001; Tawfik et al., 2006). Municipal wastewater or “sewage” is the term given to the wastewater produced by a community and consists (depending on sewerage system) in the main of three sources – (i) domestic wastewater consisting of household liquid waste from toilets and bathrooms and kitchen activities, such as cooking, washing etc.; (ii) commercial and industrial wastewater, liquid wastes from commercial and industrial activities and processes using the same sewerage system for their effluents. These effluents may or may not be partially treated; (iii) rain-water, surface rainwater from roofs and hard-standing areas and storm-water entering a combined sewerage system (van Haandel and Lettinga, 1994). Municipal wastewater is in quantity the most abundant wastewater type on earth. The treatment of municipal wastewaters in developed countries is currently characterised by a disposal based linear system, incorporating aerobic microbiological treatment as the core technology. While this approach which typically involves primary sedimentation and secondary aerobic biological treatment is effective and achieves high treatment efficiencies, it is an energy intensive process which incurs significant capital and operational cost, while also generating large volumes of sludge requiring further management, which increases the energy footprint, CO₂ emissions and life cycle costs (El-Gohary et al., 1995). In addition while these conventional activated sludge (CAS; Fig. 1.5a) systems are popular in Europe and America, they do not offer a sustainable sewage treatment solution for less wealthy or underdeveloped countries. Sustainability and environmental concerns surrounding the CAS approach
demonstrate the need for a radical shift in the way society and the established sanitary wastewater-engineering world looks at the treatment of municipal wastewater. This realisation has led to intensive research in the field of sustainable environmental protection seeking out low-cost, robust, treatment systems for a wide variety of wastes and wastewaters (Lettinga, 1996; van Lier *et al.*, 2001). The selected alternative treatment technology should satisfy the following sustainability criteria outlined by Lettinga (1995) and Grommen and Verstraete (2002);

- No dilution of high strength wastes with clean water.
- Maximum possible recovery and re-use of treated water and by-products (i.e. biogas energy, fertilization).
- Application of efficient, robust and reliable treatment/conversion technologies, which are low cost (in construction, operation, and maintenance), which have a long life-time and are straightforward with respect to operation and maintenance.
- Applicable at a wide range of scales.
- Acceptable to the general population, leading to a high level of self-sufficiency in all respects.

Anaerobic wastewater treatment appears to satisfy the above criteria. The notion of applying direct anaerobic treatment to municipal wastewater treatment has been postulated in a number of research papers, for example, Mergaert *et al.* (1992) states it is often questioned why aerobic treatment of sewage is not replaced more rapidly by the economically more attractive and conceptually more holistic direct anaerobic treatment. Chunjuan *et al.* (2009) also suggests that the direct treatment of sewage is undoubtedly an attractive and appropriate alternative. The anaerobic approach is advantageous, compared to aerobic technologies, with respect to its environmental and economic impact, e.g. low operating costs, compact construction, and low surplus sludge production and, primarily in many cases, net production of energy as biogas (Lettinga, 1995; McHugh *et al.*, 2003). Biogas is similar to natural gas and can be utilised as a renewable energy source. The implementation of an approach such as high-rate anaerobic wastewater
treatment as a low-technology, cost-efficient alternative to conventional aerobic technologies would offer huge socio-economic benefits representing a significant advancement in the field of industrial, municipal, and domestic wastewater treatment, and has been considered as the core to sustainable waste management (Zeeman and Lettinga, 1999; Hammes et al., 2000; Gijzen, 2002). However significant knowledge gaps remain regarding the nature and function of the microbial populations involved in the process, and indeed the appropriate configuration for such a system, and it is this which has limited the broad application and adoption of this technology. A multidisciplinary research approach is now required in order to realise the potential of anaerobic digestion as a sustainable ‘green’ technology which facilitates the recovery of renewable energy and nutrients from municipal wastewater which is an undesirable and abundant organic waste.

1.2 Anaerobic Digestion of Organic Matter

Anaerobic Digestion (AD) is a natural microbial process and the term is used to describe the biological conversion of organic matter to biogas, consisting primarily of methane (CH₄; 50-80%) and carbon dioxide (CO₂; 20-50%) with trace amounts of other gases (McHugh et al., 2003). This methanogenic transformation process takes place independent of inorganic electron acceptors such as oxygen, nitrate, sulphate, sulphur or oxidised metal ions (Hattori, 2008). The naturally occurring process is responsible for carbon recycling in a variety of anaerobic environments, including wetlands, rice fields, aquatic sediments, manure, and the intestines of animals (Chouari et al., 2005). Historical evidence suggests that anaerobic digestion is one of the oldest biological processes known to man. Anecdotal tales indicate that biogas, the bi-product of the anaerobic digestion process, was used to heat bath water in Assyria in the 10th century BC and in Persia in the 16th century. Awareness of the anaerobic digestion process developed in Europe when Count Alessandro Volta noticed in 1776 that “combustible air” was being formed from decaying vegetation in streams, bogs and lakes. A further realisation of the anaerobic digestion process occurred in France, in 1860, with a report by Louis Mouras that stated: “under anaerobic conditions, some
of the organic matter in sewage was liquefied” (Forster, 1985). The “combustible air” that Volta noticed was methane, produced by the action of microorganisms known as methanogens. The microbiological nature of methanogenesis was elucidated more than a century ago (Koster, 1988).

1.2.1 The Microbiology of Anaerobic Digestion
AD requires the coordinated, cooperative and sequential action of a number of trophic groups of microorganisms, for the complete mineralization of organic matter to methane in the absence of oxygen (Zeikus, 1982). The AD process involves two evolutionarily distinct kingdoms of microorganisms, the bacterial and archaeal trophic groups which work synergistically to mineralize a wide range of organic substrates to methane and carbon dioxide. These microbes, identified on the basis of the substrates used and metabolic end products generated, cooperate sequentially in order to achieve degradation of a variety of polymeric and monomeric substrates. Four distinct trophic groups/stages have been identified in the AD process, namely (i) hydrolysis, (ii) acidogenesis, (iii) acetogenesis and (iv) methanogenesis (Fig. 1.1).

![Figure 1.1 Flow chart of anaerobic digestion: 1 – Hydrolytic/fermentative bacteria; 2 – Obligate hydrogen producing acetogens; 3 – Homoacetogenic bacteria; 4a – Acetoclastic methanogens; 4b – Hydrogenotrophic methanogens; 5 – Fatty acid synthesizing bacteria; 6 – Syntrophic acetate oxidation.](image)
AD of organic matter occurs by the sequential cooperative action of a number of different bacterial trophic groups (Fig. 1.1; Zeikus, 1982; Ferry, 1999; Lokshina and Vavilin, 1999). These microorganisms cooperate sequentially in order to achieve the degradation of a variety of polymeric and monomeric substrates.

The AD process is initiated by the action of facultative and obligate fermentative bacteria, whose enzymes facilitate the hydrolysis of high molecular-weight complex polymeric substances such as proteins and polysaccharides, to low molecular-weight soluble monomeric sugars, amino acids, long-chain fatty acids and alcohols (Zeikus, 1979; Britz et al., 1994; Fig. 1.1). Further fermentation of the monomeric products by these, and other non-hydrolytic fermentative bacteria, results in the generation of a wide variety of fermentation end-products, including acetate, formate, methanol, H2 and CO2 (Fang et al., 2002). This initial fermentative activity is known as ‘acidogenesis’.

The products of acidogenesis are further oxidised to acetate, hydrogen and carbon dioxide, in a process referred to as ‘acetogenesis’ (Schink, 1992; Stams, 1994), and mediated by the obligate hydrogen producing acetogens (OHPA). Under standard conditions, the oxidation of substrates such as butyrate, propionate or ethanol, to acetate, hydrogen and/or formate, or acetate, hydrogen/formate and carbon dioxide, is an endergonic reaction. It is only when the hydrogen partial pressure is lowered, for example, by the presence of hydrogen or formate-utilising methanogens, that the reaction becomes exergonic. Therefore, OHPA bacteria must always grow in syntrophy with hydrogen-utilising methanogens, sulphate-reducing bacteria (SRB), or homoacetogens, in order to facilitate interspecies hydrogen transfer and gain energy from growth on the products of the acidogenesis phase (Schink, 1992; Li et al., 1994). Many syntrophic associations have been described in the literature (Stams, 1994; Schink, 2000, 2002), with optimum temperatures and pH levels for growth of between 25°C and 45°C and 6.3 and 8.5, respectively. The action of the OHPAs is considered the link between the initial fermentation stages and the ultimate methanogenic phase.

Homoacetogenic bacteria are a diverse group of mesophilic (Balch et al., 1977, Zeikus, 1979, Braun et al., 1981); and thermophilic (Wiegel et al.,
1981; Leigh et al., 1984) species. Their role in anaerobic digesters is still unclear but most species are capable of autotrophic and heterotrophic growth, generating acetate as the sole end-product from either H2/CO2 or multi-carbon compounds (Li et al., 1994). Zeikus (1979) and Li et al. (1994) proposed that homoacetogens contribute to the preservation of a low H2 partial pressure in anaerobic bioreactors, thereby increasing process stability. However, their persistence appears to be based on their heterotrophic ability and metabolic versatility rather than on their H2-utilising ability (Ryan et al., 2008). There is also some evidence to suggest that they may be important under specific ‘stress’ conditions such as high ammonium concentrations (Blomgren et al., 1990) or at high temperatures (van Lier, 1995).

The final step in the anaerobic treatment of wastewater is methanogenesis. Methanogens are strictly anaerobic Archaea that can be subdivided into two groups: (i) hydrogenophilic or hydrogenotrophic species, which form methane by the reduction of H2 with CO2 and (ii) acetoclastic or acetotrophic methanogens, which generate methane by acetate decarboxylation (Fig. 1.1; Ferry, 1999).

Acetoclastic methanogenesis:
\[ \text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2 \]

Hydrogenothrophic methanogenesis:
\[ 4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \]

Acetoclastic methanogens are considered the more important methanogenic species. For example 70% of the total methane generated during AD of domestic sewage is via this pathway (Grotenhuis, 1992; Lettinga, 1995). Consequently, these species are considered to be critical for successful anaerobic digestion (Lettinga, 1995). However hydrogenothrophic methanogens also play a key role in the overall anaerobic process regulating the low partial pressure of hydrogen, essential for the functioning of the intermediate trophic group, OHPA (Stams, 1994; Coates et al., 1996; Schink, 1997).

The use of culture-independent methods has recently highlighted the role of many other, as yet uncultured, groups of microbes in AD, such as the
non-thermophilic Crenarchaeota (Collins et al., 2005). The prevalence of Crenarcheota has been documented in both mesophilic (Chouari et al., 2005; Levén et al., 2007) and low-temperature anaerobic systems, where they can represent a significant portion of total archaeal microbiota (Collins et al., 2005; McHugh et al., 2005). However, the exact role of these groups remains unknown, and much research remains to be carried out in this area to elucidate the role and impact of these groups on the AD process (Collins et al., 2005).

1.2.2 Anaerobic Digestion for Waste/Wastewater Treatment

AD of organic material has long been recognised. However it has taken some time to this point where it is now a well-established technology for the treatment of waste and wastewater streams, in man-made anaerobic environments, referred to as bioreactors or digesters.

The process has historically been applied for the stabilization of animal manures and municipal waste (Iza et al., 1991). The first full-scale application of anaerobic sewage treatment was in a bioreactor resembling a septic tank in the late 18th century called the ‘Mouras Automatic Scavenger’. The early 20th century saw the development of primitive bioreactors such as Cameron’s septic tank which aimed to control and optimise the decay of human/animal waste (McCarty, 2001). Later improvements on this early design came during the 20th century with designs such as the ‘Imhoff’ tank. However widespread acceptance and application of AD for the treatment of waste was held back as a result of the coupling of solids and liquid phases, which necessitated lengthy resistance times and consequently large reactor volumes (Iza et al., 1991).

The rapid escalation in energy costs in the 1970s led to increased attention and intensive research in the field of AD and it was during this period that the major breakthrough in the advancement of anaerobic wastewater treatment came with the development of high-rate bioreactors (van Lier et al., 2001). The success of these systems is attributed to bioreactor designs in which solid retention time (biomass) was uncoupled from hydraulic retention time (wastewater) (Lettinga et al., 2001). This uncoupling has enabled the application of relatively high loading rates, while maintaining long solid retention time at short hydraulic time (of the order of hours) and is
achieved by retaining the active biomass within the system by sedimentation and/or immobilisation. This is achieved through the use of a fixed/moving support matrix or by self-immobilisation as flocs or granules. In these systems, wastewater flows through the anaerobic biomass where purification takes place through a combination of biological, physical and chemical interaction. The use of such bioreactors has led to more stable and efficient process operation and reduced bioreactor volumes, making the economics and applicability of anaerobic bioreactor technology more favourable (McHugh et al., 2003). The introduction of high-rate bioreactors also expanded the scope and application of AD to a wide range of substrates such as food processing and other industrial wastewaters (Colleran, 1992). Indeed high-rate AD is now an established and proven technology for the treatment of a wide variety of industrial wastewaters (Bouallagui et al., 2005; Elmitwalli et al., 2001; Macarie, 2000; Rincon et al., 2006). This has been achieved through the fusion of two disciplines, namely science and engineering; leading to greater scientific knowledge and understanding of the microbial processes involved and engineering developments resulting in ever-improving anaerobic bioreactor designs (Iza et al., 1991). Many different types of high-rate systems have been developed over the last three decades including; anaerobic filter (AF) (Young and McCarty, 1969), the up-flow anaerobic sludge blanket (UASB) (Lettinga et al., 1980), fluidised and expanded bed bioreactors (Schwitzbaum and Jewell, 1980), down flow stationary fixed film bioreactor (Murray and van den Berg, 1981) and the baffled bioreactors (Barber and Stuckey, 1999). Variations of the UASB design include; the expanded granular sludge bed (EGSB) system (de Man et al., 1988), the UASB - septic tank reactor (Bogte et al., 1993), the hydrolytic up-flow sludge bed (HUSB) (Wang, 1994), the staged multi-phase anaerobic (SMPA) bioreactor (van Lier, 1995) and the two stage AF-anaerobic hybrid (AH) system (Elmitwalli, 2000). These designs can be broadly divided into three classifications based on the means by which they retain microbial biomass: (i) suspended growth systems; (ii) attached growth systems; (iii) granular sludge-based systems (e.g. UASB, EGSB; Fig. 1.2).
Figure 1.2 Schematic of major anaerobic bioreactor configurations, and relative organic loading capacities, based on various forms of biomass retention (adapted from van Lier, 2008).

By far the most widely applied full-scale AD bioreactors are the granular sludge-based systems. Sludge granules are dense, spherical, particulate biofilms formed by the self-immobilisation of anaerobic organisms under suitable conditions, most notably when grown in an up-flow stream (Guiot et al., 1992; Thaveesri et al., 1995; O’Flaherty and Lens, 2003). Granulation of sludge is anomalous, and is characteristic of up-flow anaerobic sludge bioreactors. Many theories exist that attempt to explain the phenomenon of granulation. These can be categorised into physical, microbial and thermodynamic theories. Physical explanations propose that the phenomenon occurs due to influent and gas up-flow velocities, suspended solids in the effluent or seed sludge, and the wash out and removal of excess sludge from the reactor (Hulshoff Pol et al., 1983). Microbial approaches suggest that the characteristics of the microorganisms present in the reactor sludge are responsible for granulation as a result of the microbes directly agglomerating. The formation of granules through the clustering of species can be as a result of the physiology of the microorganisms, the growth of microbial nuclei, or the ecology of the microbes attaching to certain species etc. (Hulshoff Pol et al., 2004). Thermodynamic theories attempt to explain granulation in terms of the energy involved in the adhesion itself, due to the physio-chemical interactions between cell walls or between cell walls and inert surfaces (Hulshoff Pol et al., 2004). Anaerobic sludge granules permit
the close association of the trophic groups involved in AD (MacLeod et al., 1990). In an anaerobic bioreactor, one pellet of granular sludge forms an operational unit which comprises of the various groups of microbes which are necessary for the anaerobic degradation of organic substances (Lettinga, 1995).

The phenomenon of granulation has many advantages to the AD process; it dispenses with the need for costly support materials and allows the amalgamation of bacteria from different trophic groups, thereby facilitating interspecies transfer of substrates and products by the creation of a unique anaerobic environment (O’Reilly, 2003). The development of stable, well-settling microbial aggregates is a key factor contributing to the successful operation of granular-based bioreactors.

Of the full-scale, granular sludge-based bioreactors in operation, the UASB configuration dominates the industrial market-place, however there is a growing trend towards the EGSB and Internal Circulation® (IC) systems (Fig. 1.3). This is due to their increased organic loading capacity, increased loading capacity lends itself to a decrease in bioreactor size, and as a result it is likely that these systems will eventually replace the UASB configuration in the future (Franklin, 2001).

(a)  
(b)

Figure 1.3 Full-scale implementation of anaerobic bioreactor technology for period (a) 1981-2007 \( (n = 2266) \) and (b) 2002-2007 \( (n = 610) \); UASB: upflow anaerobic sludge bed; IC: Internal Circulation®; EGSB: expanded granular sludge bed; AF: anaerobic filter; CSTR: continuously stirred tank.
reactor; AL: anaerobic lagoon; HYB: hybrid sludge bed-anaerobic filter; FB: fluidised bed (adapted from van Lier, 2008).

Anaerobic treatment technologies are predominantly applied to relatively simple wastestreams, such as those generated by the agro-food, beverage and alcohol distillery industries (Lettinga, 2005). Recently the technology has been applied to treat more challenging wastewaters, such as the pulp and paper, chemical, pharmaceutical and domestic wastestreams (van Lier, 2007). Conventional anaerobic treatments are applied at mesophilic (>20°C) and thermophilic (>45°C) temperature ranges to ensure optimal microbial activity. For this reason, the major application of high-rate AD has been to high-strength industrial wastewaters, e.g. from food processing, brewing, etc., where high-rate AD has been tremendously successful (McHugh et al., 2003).

There is great potential for expanding the application of anaerobic wastewater treatment. One area of particular interest is AD for the treatment of low-strength, high volume wastestreams which are discharged at sub-mesophilic (<20°C) or low-temperatures (e.g. municipal and domestic sewage). The implementation of anaerobic treatment of these low-temperature wastestreams is severely limited by economics, whereby a significant energy input to heat bioreactors would be required for treatment to proceed within a mesophilic temperature range, therefore negating the cost saving associated with direct anaerobic treatment. As a result of the low-strength of such wastewaters, the bioenergy harvest, plus additional energy must be expended to heat the system to within the optimal high-temperature range (Collins et al., 2005). The application of AD technology to direct treatment of municipal and low-strength industrial wastewaters has therefore been delayed. In order for low-strength anaerobic treatment to be economically favourable it would need to proceed under ambient or low-temperatures. The successful implementation of high-rate AD applied at low-temperatures (low-temperature anaerobic digestion [LtAD]), could represent a significant technological breakthrough. Lettinga et al. (1987) fully agreed, commenting that ‘a satisfactory application to raw domestic sewage would represent the maximum possible accomplishment for high-rate anaerobic treatment systems’. The development of LtAD systems offering efficient treatment
comparable to mesophilic AD would greatly extend the scope of AD as a remediation strategy for cold, dilute wastewaters.

1.3 Low-Temperature Anaerobic Digestion (LtAD)

1.3.1 LtAD of Organic Matter

A large portion of the Earth’s biosphere (ca. 85%) experiences temperatures of less than 5°C during some part of the year and over 75% is permanently cold. Yet these environments are rarely sterile and microorganisms can be found wherever liquid particles still exist. Indeed these ecosystems are important in respect of the global biogeochemical cycling of nutrients. Psychrophilic microorganisms have successfully colonized all permanently cold environments from the deep sea to mountain and Polar Regions (D’Amico et al., 2006). Some of these organisms, depending on their optimal growth temperature, are also known by the terms psychrotolerant or psychrotrophic (Morita, 1975). Methanogenesis has been recorded in a diverse range of cold habitats including Arctic and sub-Arctic wetlands, freshwater sediments, high altitude rice paddy soils and animal manures, as well engineered environments such as anaerobic bioreactors and lagoons (Conrad et al., 1989; Nozhevnikora et al., 1997; Kashyap et al., 2003; Kotsyurbenko, 2005; Metje and Frenzel, 2007; Høj et al., 2008; Syutsubo et al., 2008; Zhang et al., 2008) at temperatures as low as 2°C (Nozhevnikora et al., 1997). While mesophilic and thermophilic methanogenesis are comparatively well investigated, the microbial interactions and biochemical pathways involved in low-temperature biomethanation remain poorly understood (Kotsyurbenko et al., 2004; Metje and Frenzel, 2007). A greater recognition of the significance of methane as a greenhouse gas, and consequently in global warming and climate change has led to increased research in the area of biological mineralization and anaerobic methane formation (Galand et al., 2002). In addition, these poorly understood low-temperature environments may potentially harbour a wealth of phylogenetic and microbial diversity.

Under low-temperature conditions, chemical and biological reactions proceed more slowly than under mesophilic conditions, in particular the
initial stage of AD; hydrolysis. Hydrolysis has been identified as the rate-limiting step under mesophilic conditions, a feature which is exacerbated under low-temperatures (Pavlostathis and Giraldo-Gomez, 1991; Fey and Conrad, 2003). Once hydrolysis is achieved the fermentation of monomeric compounds is not rate-limiting as these compounds are readily degradable under low-temperatures (Kotsyurbenko, 2005). Many researchers consider homoacetogenesis as one of the most important processes in low-temperature ecosystems, such as certain lake sediments (Conrad et al., 1989; Nozhevnikova et al., 1997), anoxic pond sediments (Kotsyurbenko et al., 1993) and tundra soil (Kotsyurbenko et al., 1996), with the role of acetogens increasing significantly at low-temperatures ecosystems, as the energy gain from syntrophic volatile fatty acid (VFA) degradation can be limited (Nozhevnikova et al., 2000; Kotsyurbenko, 2005). The development of and stable functioning of syntrophic communities are critically important to successful AD under low temperature (Kotsyurbenko, 2005). Many psychroactive acetogenic and methanogenic microorganisms have been isolated from low-temperature environments (Simankova et al., 2003; Cavicchioli, 2006).

In low-temperature environments it is believed that acetate is the main precursor for methanogenesis in both natural (Küsel and Drake, 1995; Schulz et al., 1997; Fey and Conrad, 2000; Glissman et al., 2004; Kotsyurbenko et al., 2004; Fey et al., 2004) and engineered systems (McHugh et al., 2003; Akila and Chandra, 2007). The action of homacetogenic bacteria consuming hydrogen is thought to account for this phenomenon (Conrad and Wetter, 1990; Kotsyurbenko et al., 1995; Schulz and Conrad, 1996). Homooacetogens have been shown to out-compete hydrogenotrophic methanogens for hydrogen under low-temperature conditions (Conrad et al., 1989; Kotsyurbenko et al., 1996; Schulz and Conrad, 1996). Despite these findings, hydrogenotrophic methanogenesis has in many instances been shown to play an important role in cold terrestrial habitats (Lansdown et al., 1992; Horn et al., 2003; Kotsyurbenko et al., 2007) and LtAD bioreactors (McHugh et al., 2004; Syutsubo et al., 2008; McKeown et al., 2009). Kotsyurbenko (2005) demonstrated that in a balanced system with low-prevailing H2-partail pressures, hydrogenotrophic methanogens may out-compete homacetogens
due to their greater affinity for hydrogen. The implications of hydrogenotrophic methanogenesis as the primary route of carbon flow in low-temperature environments is of particular importance for LtAD of wastewaters as acetoclastic *Methanoseta* species are regarded as key to the formation and maintenance of healthy granular biomass. Recent studies have demonstrated the emergence of hydrogenotrophic methanogenesis as the main route of methane formation, despite the maintenance of a predominantly acetoclastic methanogenic archaeal community (Kelly *et al.*, 2011). The reasons for conflicting reports on the main pathway of methanogenesis under low-temperature conditions are still not fully understood.

### 1.3.2 LtAD of Wastewaters

The effects of low-temperatures on the biological and chemical reaction of microorganisms have been observed by several researchers (Lin *et al.*, 1987; Wu *et al.*, 1993; Collins *et al.*, 2003, McHugh *et al.*, 2004). Anaerobic processes are commonly known to be sensitive to sudden environmental changes (Lau and Fang, 1997). Various factors can affect the anaerobic treatment of wastewaters such as; pH, macro and micro nutrient concentrations, toxic compounds and arguably most importantly temperature; parameters other than temperature are not as detrimental to process operation/stability as operating temperature. In the case of the anaerobic process, with decreasing temperature – (i) the rate of substrate utilisation may be reduced (ii) a decrease in the maximum microbial specific growth may be observed and (iii) methane biogas may become increasingly soluble in the bioreactor liquor (Lettinga *et al.*, 2001). Consequently, full-scale application of LtAD has been for a long time been considered unfeasible and impractical (Rebac, 1998). Significant physical, chemical and biological challenges need to be overcome in order to apply high-rate AD under low-temperature conditions (Lettinga *et al.*, 2001; Collins *et al.*, 2006). The ability of anaerobic bioreactor processes to treat low-temperature wastewater is reliant on five main factors – (i) the quality of the seed inoculum and its development under these conditions (ii) extremely high biomass retention under high hydraulic loading conditions (iii) high biomass and wastewater contact within the bioreactor (iv) the nature of the organic pollutants in the wastewater being
treated, and (v) the bioreactor design being employed and its capacity to retain viable biomass (Lettinga et al., 1999). Recent research has shown that with appropriate bioreactor design and operation, successful low-temperature bioreactor operation is feasible (Lettinga et al., 1999, 2001; Rebac et al., 1999; Collins et al., 2004). The development of the granular sludge-based EGSB-style configuration was an important milestone in the development of LtAD (Kato et al., 1999). Namely the treatment of low-strength wastewaters at low-temperature has benefited from the use of the EGSB bioreactor (Rebac et al., 1999, Kato et al., 1999, Chu et al., 2005), the design of which offers the benefit of greater mixing intensities than alternative bioreactor designs, resulting in greater contact between the wastewater and biomass through increased height to diameter ratio and effluent recirculation (Puyol et al., 2009). It has also been reported that the high recycle rate may play a role in physically stripping dissolved metabolic gases from the bioreactor liquor (Connaughton et al., 2006). EGSB-style systems can achieve high treatment efficiencies, even with very dilute wastestreams (<1 kg chemical oxygen demand [COD] m$^{-3}$ d$^{-1}$; Lettinga et al., 2001), under conditions of low-temperature (2-20°C; Rebac et al., 1999). The EGSB approach has led to the successful LtAD of a wide variety of wastewaters including domestic (Sanz and Fernandez-Polanco, 1990; Luostarinen et al., 2007; Cui et al., 2007), acidified (Kato et al., 1994; Rebac et al., 1995, 1999; van Lier et al., 1997; Lettinga et al., 1999), brewery (Kato et al., 1999; Connaughton et al., 2006), food-processing (Koster and Lettinga, 1985; Lin, 1986; Viraraghavan and Kikkeri, 1990; McHugh et al., 2006), phenolic (Collins et al., 2005; Scully et al., 2006, 2007) and pharmaceutical (Enright et al., 2005, 2007) wastewaters.

The effect of low-temperature conditions on granular sludge-based EGSB systems can occasionally result in a loss in granule integrity as a result of the high up-flow velocities applied. Granule shearing resulting in the loss of active biomass can greatly affect system performance (Rebac et al., 1995; van Lier et al., 1997; Scully, 2007; McKeown et al., 2009). A variation of the EGSB design, the EGSB-Anaerobic Filter (EGSB-AF) hybrid bioreactor, incorporates an AF section at the top of the bioreactor which enables active biomass lost from the granular sludge bed to become entrapped and retained as fixed-film growth on an inert support matrix (McHugh et al., 2004; Collins
et al., 2005). The EGSB-AF bioreactor has to an extent helped in overcoming a lot of the issues associated with LtAD. The AF section supports the maintaince of active biomass, compensating for the slow microbial growth rate at reduced temperatures, while the EGSB itself provides increased biomass and wastewater contact and mass transference through greater bioreactor mixing to that of alterntive bioreactor designs such as the UASB. This is achieved through increased height to diameter ratio and effluent recycle. For those reasons outlined above, the EGSB-AF bioreactor configuration was selected for the current study (Fig. 1.4).

Figure 1.4. Configuration of lab-scale hybrid expanded granular sludge bed-anaerobic filter bioreactor.

Much research is needed in the disciplines of microbiology and engineering to fully understand the complex interactions and biological processes which occur in LtAD systems and how to best optimise the performance of these systems. This would open up the possibility of incorporating AD as the core technology for municipal wastewater treatment in countries with a temperate climate, thereby realising a major commercial and technological opportunity.
The existing and widely applied CAS treatment and the direct LtAD approach proposed in this study are illustrated below in Figure 1.5.

**Figure 1.5** Schematic of (a), typical process flow of conventional activated sludge plants; and (b) the process flow of the direct LtAD treatment approach proposed in this study; FOG – Fats, oil and grease.
1.4 Application of High-Rate AD for Municipal Wastewater Treatment

1.4.1 Characteristics of Municipal Wastewater

Municipal wastewater or ‘sewage’ is classified as a ‘complex’ wastewater. It is a relatively low-strength wastewater, the strength of which can vary greatly, typically between 200-800 mg l\(^{-1}\) COD (Table 1.2), but can be relatively more concentrated in countries where water consumption is limited e.g. Jordan (Kerstens, 2001). Domestic sewage which is in general the main component of municipal wastewater is characterised by a high fraction of particulate COD, both suspended and colloidal. The biodegradability of the typical COD fractions is moderate as a result of fatty compounds, proteins and detergents (Lettinga et al., 2001). Strong fluctuations are also reported in municipal wastewater temperature, which adds to the complex nature of the wastewater (Zeeman et al., 1997; Elmitwalli, 2000; Lens et al., 2001).

The rate of flow and composition of municipal wastewater can vary significantly from one area to another and over time. The cyclical nature of human activity leads to variable sewage production and concentration throughout the day (Metcalf and Eddy, 1991). Another factor which greatly affects the flow and concentration of municipal wastewater is climate. This is a particular feature of older sewerage designs which handle the combined flows of domestic, commercial, and industrial wastewater, and rainwater. In addition to increased flow, storm water and the resulting surface water runoff can result in an increase in suspended solids and COD concentrations (Deletic, 1998). Many countries have clearly defined wet and dry seasons, where the COD concentration of municipal wastewater would correspond to the seasonal difference in rainfall. Short term fluctuations can also occur as a consequence of commercial/industrial contributions, e.g. tourism high season, festivals, a period of increased industrial production etc. (Castillo et al., 1997; Puñal and Lema, 1999).
Table 1.2. Characteristics of municipal wastewater in different countries.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Egypt a</th>
<th>Turkey b</th>
<th>The Netherlands c</th>
<th>Brazil d</th>
<th>Columbia e</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD (Total)</td>
<td>824.9</td>
<td>410</td>
<td>520</td>
<td>727</td>
<td>267</td>
</tr>
<tr>
<td>COD (Soluble)</td>
<td>270.2</td>
<td>140</td>
<td>147</td>
<td>–</td>
<td>112</td>
</tr>
<tr>
<td>Suspended solids (Total)</td>
<td>310</td>
<td>210</td>
<td>–</td>
<td>492</td>
<td>215</td>
</tr>
<tr>
<td>Suspended solids (Volatile)</td>
<td>277</td>
<td>145</td>
<td>–</td>
<td>252</td>
<td>108</td>
</tr>
<tr>
<td>Nitrogen (as N)</td>
<td>33.8</td>
<td>43</td>
<td>70</td>
<td>44</td>
<td>24</td>
</tr>
<tr>
<td>Ammonia</td>
<td>26</td>
<td>30</td>
<td>48</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td>Phosphorus (Total)</td>
<td>8.9</td>
<td>7.2</td>
<td>18</td>
<td>11</td>
<td>1.3</td>
</tr>
<tr>
<td>Orthophosphate</td>
<td>3.87</td>
<td>4.5</td>
<td>14</td>
<td>8</td>
<td>–</td>
</tr>
</tbody>
</table>

Adapted from: a Orhon et al., (1997); b Tawfik (1988); c,d and e van Haandel and Lettinga, (1994). All values are in mg l⁻¹.

Combined sewerage systems are usually not desirable as the seasonal and periodic fluctuations in flow, concentration and composition may reduce the efficiency of treatment plants, by variations in subjected hydraulic and organic loading rates. This in turn has an impact on operational control, and design of treatment systems. Separate sewerage systems are the preferred situation, uncoupling climatic influence from daily flow per capita. Flow rates and wastewater concentrations in separate sewer systems display characteristic patterns on annual, seasonal, daily and hourly time scales which enable wastewater engineers to more accurately determine the required treatment plant design etc. The implementation of separate sewer systems and/or alterations to existing combined systems could play a significant role in the advancement of alternative treatment approaches such as anaerobic treatment.

1.4.2 Advantages of Anaerobic Treatment of Municipal Wastewater

- No energy input is required for aeration, indeed energy is produced in the form of a valuable by-product, methane gas (Schink, 2002).
- Modern bioreactor designs allow for high hydraulic and organic loading rates to be applied reducing the footprint of the technology (Lettinga et al., 1993).
- The technologies are simple in construction and operation, resulting in low capital and operational costs (Zeeman and Lettinga, 1999).
- Due to the low energy requirements anaerobic technologies can be applied in both centralised and decentralised applications (Kalogo and Verstreate, 2001; Lettinga et al., 2001).

- Anaerobic treatment processes generate considerably less biomass than high rate aerobic processes, 0.5 kg VSS/kg COD removed and 0.1 kg VSS/kg COD removed, respectively (Eckenfelder et al., 1988). In addition sludge is well stabilised and easily dewatered as a result of high SRT (Aiyuk et al., 2006; Martin et al., 2010; Haandel and Lettinga, 1994). Furthermore, viable sludge can be preserved in a dormant or un-fed condition (Lettinga et al., 2001).

- Valuable nutrients (N and P) are conserved with high reuse and recycle potential (Zeeman and Lettinga, 1999).

1.4.3 Drawbacks of Anaerobic Treatment of Municipal Wastewater

- No full-scale experience at moderate/low-temperature.

- Considerable amounts of CH₄, CO₂, H₂ and H₂S may remain in the effluent of anaerobic bioreactors operating at low-temperature because of increased gas solubility, particularly for low-strength wastewaters such as sewage (Lens et al., 2001).

- Post treatment may be necessary depending on effluent discharge standards, with particular attention to nutrient and pathogens, as well as difficult to treat particulate and soluble organic matter (Foresti, 2002).

1.4.4 Applications in Tropical Countries

The UASB bioreactor design has long been considered as the most appropriate anaerobic system to treat sewage because of its simplicity, low investment and operational costs and previous positive experience in the treatment of a wide range of wastewater types (van Haandel and Lettinga, 1994; Mahmoud et al., 2004; Hulshoff Pol et al., 2004; Ghangrekar et al., 2005). In tropical regions, the UASB bioreactor configuration is reported to be the most commonly employed process for anaerobic sewage treatment (Verstraete and Vandevivere, 1999; Foresti, 2002). One stage high-rate anaerobic sewage treatment in tropical regions began in the early 1980s with
the construction of a pilot-plant UASB bioreactor treating sewage in Cali, Colombia. The results obtained from the operation of the Cali plant demonstrated the feasibility of the system, under the environmental conditions and sewage characteristics experienced, with COD and biological oxygen demand (BOD) removal efficiencies higher than 75% being achieved at an average temperature of 25°C (Schellinkhout et al., 1985; Kooijmans et al., 1986). This led to the construction of the first full-scale UASB system for sewage being constructed in Kanpur, India in 1989, which is still in operation. Subsequently, hundreds of UASB systems for the treatment of sewage have been commissioned in several tropical countries such as India, Colombia, Brazil, and Mexico (van Haandel and Lettinga, 1994; von Sperling and Chernicharo, 2005; Aiyuk et al., 2006). The ambient temperature in these countries is relatively high throughout the year (20-35°C), enabling AD of sewage to proceed in the mesophilic temperature range without process heating. The challenge now is to produce innovative technological solutions to bring the benefits of AD for sewage treatment to regions with a colder climate, such as Ireland and many other EU member states (Lettinga et al., 2001).

1.4.5 Applications in Countries with Moderate to Low-Temperature
As outlined above, one stage high-rate anaerobic sewage treatment is now well established in tropical regions, where treatment efficiencies enable the use of low-technology secondary treatment such as trickling filters or polishing ponds for effluent polishing. The implementation of direct anaerobic treatment of municipal wastewater in countries with sub optimal temperatures is an ongoing challenge for researchers in the field of environmental engineering and technology (Lettinga, 2001; Lettinga et al., 2001). Experience with one stage anaerobic treatment systems for sewage has provided limited success and while results of several studies on lab and pilot scale systems operated at low-temperature have opened new perspectives a full-scale application has yet to be realised. The application of one stage anaerobic treatment such as a UASB system for the treatment of complex wastewater containing high levels of suspended solids at low-temperature is generally limited by accumulation of these solids in the sludge bed (Zeeman
and Lettinga, 1999), this will be discussed further below. Some examples of pilot and laboratory scale high-rate anaerobic sewage treatment applications at low-temperature (<20°C) are presented in Table 1.2.
Table 1.2. Some examples of pilot and laboratory scale high-rate anaerobic sewage treatment applications at low-temperature (<20°C).

<table>
<thead>
<tr>
<th>Bioreactor Design</th>
<th>Vol. (m³)</th>
<th>Operating Temp. (°C)</th>
<th>Influent CODtot (mg L⁻¹)</th>
<th>Inoculum</th>
<th>HRT (h)</th>
<th>CODREtot (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGSB</td>
<td>205</td>
<td>16–19</td>
<td>391 b</td>
<td>Self-cultivated on sand</td>
<td>1.5–5.8</td>
<td>30</td>
<td>van der Last and Lettinga (1992)</td>
</tr>
<tr>
<td>UASB</td>
<td>120</td>
<td>&gt;13</td>
<td>391 b</td>
<td>Granular sludge</td>
<td>2-7</td>
<td>16-34</td>
<td>van der Last and Lettinga (1992)</td>
</tr>
<tr>
<td>UASB</td>
<td>20</td>
<td>11–19</td>
<td>150–550</td>
<td>Granular sludge</td>
<td>6.2–18</td>
<td>31–49</td>
<td>de Man et al. (1986)</td>
</tr>
<tr>
<td>UASB</td>
<td>6</td>
<td>10–18</td>
<td>100–900</td>
<td>Granular sludge</td>
<td>9–16</td>
<td>46–60</td>
<td>de Man et al. (1986)</td>
</tr>
<tr>
<td>UASB-ST</td>
<td>1.2</td>
<td>13.8</td>
<td>976</td>
<td>Digested sewage sludge</td>
<td>44.3</td>
<td>33</td>
<td>Bogte et al. (1993)</td>
</tr>
<tr>
<td>UASB-ST</td>
<td>1.2</td>
<td>12.9</td>
<td>821</td>
<td>Digested sewage sludge</td>
<td>57.2</td>
<td>3.8</td>
<td>Bogte et al. (1993)</td>
</tr>
<tr>
<td>UASB-ST</td>
<td>1.2</td>
<td>11.7</td>
<td>1716</td>
<td>Granular sludge</td>
<td>202.5</td>
<td>60</td>
<td>Bogte et al. (1993)</td>
</tr>
<tr>
<td>HUSB</td>
<td>0.2</td>
<td>15.8</td>
<td>650</td>
<td>Digested sludge</td>
<td>3</td>
<td>37-38</td>
<td>Wang (1994)</td>
</tr>
<tr>
<td>UASB+EGSB</td>
<td>0.2+0.12</td>
<td>17</td>
<td>697</td>
<td>Flocculent + granular sludge</td>
<td>3+2</td>
<td>69</td>
<td>Wang (1994)</td>
</tr>
<tr>
<td>UASB+EGSB</td>
<td>0.2+0.12</td>
<td>12</td>
<td>507</td>
<td>Flocculent + granular sludge</td>
<td>3+2</td>
<td>51</td>
<td>Wang (1994)</td>
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<tr>
<td>UASB</td>
<td>0.14</td>
<td>15</td>
<td>721</td>
<td>Flocculent sludge</td>
<td>6</td>
<td>44</td>
<td>Mahmoud et al. (2004)</td>
</tr>
<tr>
<td>UASB</td>
<td>0.14</td>
<td>Warm period</td>
<td>1159–1701</td>
<td>None</td>
<td>10</td>
<td>43–69</td>
<td>Mahmoud (2008)</td>
</tr>
<tr>
<td>UASB</td>
<td>0.14</td>
<td>Cold period</td>
<td>770–1525</td>
<td>None</td>
<td>10</td>
<td>5–57</td>
<td>Mahmoud (2008)</td>
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<tr>
<td>UASB</td>
<td>0.12</td>
<td>8-20</td>
<td>400</td>
<td>Granular sludge cultivated on sugar beet waste</td>
<td>8</td>
<td>30-50</td>
<td>Grin et al. (1983)</td>
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<tr>
<td>UASB</td>
<td>0.12</td>
<td>12-18</td>
<td>420-920</td>
<td>Digested sewage sludge</td>
<td>32-40</td>
<td>48-70</td>
<td>Lettinga et al. (1983)</td>
</tr>
</tbody>
</table>

Table 1.2. (cont.) Some examples of pilot and laboratory scale high-rate anaerobic sewage treatment applications at low-temperature (<20°C).

<table>
<thead>
<tr>
<th>Bioreactor Design</th>
<th>Vol. (m³)</th>
<th>Operating Temp. (°C)</th>
<th>Influent CODtot (mg L⁻¹)</th>
<th>Inoculum</th>
<th>HRT (h)</th>
<th>CODREtot (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UASB</td>
<td>0.12</td>
<td>18-20</td>
<td>248-581</td>
<td>Granular sludge</td>
<td>12</td>
<td>72</td>
<td>Lettinga et al. (1983)</td>
</tr>
<tr>
<td>UASB</td>
<td>0.12</td>
<td>7-8</td>
<td>464-700</td>
<td>Granular sludge</td>
<td>9-14</td>
<td>57</td>
<td>de Man et al. (1986)</td>
</tr>
<tr>
<td>UASB</td>
<td>0.12</td>
<td>12-20</td>
<td>190-1180</td>
<td>Granular sludge</td>
<td>7-8</td>
<td>30-75</td>
<td>de Man et al. (1988)</td>
</tr>
<tr>
<td>EGSB</td>
<td>0.12</td>
<td>15.8</td>
<td>397</td>
<td>Granular sludge</td>
<td>2</td>
<td>27-48</td>
<td>Wang (1994)</td>
</tr>
<tr>
<td>UASB</td>
<td>0.11</td>
<td>12-18</td>
<td>465</td>
<td>Anaerobically adapted</td>
<td>12-18</td>
<td>65</td>
<td>Monroy et al. (1988)</td>
</tr>
<tr>
<td>ABR</td>
<td>0.1</td>
<td>19</td>
<td>30-700</td>
<td>Flocculent sludge</td>
<td>12.8</td>
<td>67</td>
<td>Gomec et al. (2009)</td>
</tr>
<tr>
<td>ABR</td>
<td>0.1</td>
<td>18</td>
<td>30-700</td>
<td>Granular sludge</td>
<td>9.5</td>
<td>63</td>
<td>Gomec et al. (2009)</td>
</tr>
<tr>
<td>AF+AH</td>
<td>0.06</td>
<td>13</td>
<td>461</td>
<td>Flocculent + flocculent, granular sludge mix</td>
<td>4+8</td>
<td>71</td>
<td>Elmitwalli (2000)</td>
</tr>
<tr>
<td></td>
<td>+0.065</td>
<td></td>
<td></td>
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<td>UASB+UASB</td>
<td>0.042+0.0046</td>
<td>18-20</td>
<td>200-700</td>
<td>Flocculent + granular sludge</td>
<td>8-4+2</td>
<td>74-82</td>
<td>Sayad and Fergalla (1995)</td>
</tr>
<tr>
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<td>13</td>
<td>369</td>
<td>Lab-scale EGSB treating beer wastewater</td>
<td>0.75</td>
<td>66</td>
<td>Chunjuan et al. (2009)</td>
</tr>
<tr>
<td>UASB</td>
<td>0.00645</td>
<td>13 ± 2</td>
<td>165–270</td>
<td>Granular sludge</td>
<td>7.5</td>
<td>24–54</td>
<td>Gomec (2005)</td>
</tr>
<tr>
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<td>456</td>
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<td>67</td>
<td>Elmitwalli (2000)</td>
</tr>
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<td>15</td>
<td>310</td>
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<td>48</td>
<td>Bodik et al. (2000)</td>
</tr>
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<td>Municipal anaerobic digestate</td>
<td>12</td>
<td>37</td>
<td>Bodik et al. (2000)</td>
</tr>
</tbody>
</table>

1.4.6 Issues Associated with LtAD of Municipal Wastewater

The implementation of LtAD for municipal wastewater predominantly depends on three main factors; the characteristics and biodegradability of entrapped solids, the operational temperature of the anaerobic bioreactor, and the solids retention time (SRT). These interlinked parameters are the principal determinants in the design and performance of such systems. These factors are discussed in greater detail below.

One of the more challenging constituents of municipal wastewater are suspended solids and the hydrolysis of these solids. The removal of suspended solids during anaerobic treatment of municipal wastewater occurs initially by physical processes, such as settling, adsorption and entrapment followed by hydrolysis (Seghezzo, 2004). Hydrolysis of these solids proceeds first by conversion mechanisms, such as cell lysis, non-enzymatic decay, phase separation and physical breakdown, followed by chemical hydrolysis (Seghezzo, 2004; Batstone et al., 2004). As mentioned previously, hydrolysis, which is the rate limiting step in the AD process and is greatly influenced by process temperature and SRT (Pavlostathis and Giraldo-Gomez, 1991; Miron et al., 2000; Sanders, 2001). The lower the operational temperature the longer the SRT required to degrade these solids (Zeeman, 1991). Reduced operational temperature not only decreases hydrolysis rates but also results in decreases in biomass growth and substrate utilisation rates (Lettinga et al., 2001). It has been reported by many authors that the content of suspended solids is a primary factor which may affect the performance of an anaerobic bioreactor (Lettinga et al., 1993; Zeeman and Lettinga, 1999; Kalogo and Verstraete, 1999; van Lier et al., 2001). The characteristics and concentration of suspended solids present in wastewater in addition to the hydrolysis potential of the biomass can affect the anaerobic process in a variety of ways:

- A reduction in specific methanogenic activity of anaerobic biomass due to entrapment and adsorption of poorly and non-biodegradable suspended solids (Lettinga and Hulshoff Pol, 1991). The adsorption of dispersed solids onto sludge granules may hinder specific sludge activities, through the phenomenon of sludge wrapping. This is where active biomass becomes colonised by a film of non-biomass matter, which consequently hampers
substrate transport through the active biofilm (Lettinga et al., 1983; Grin et al., 1985).
- A detrimental effect on the formation of granular sludge and the loss of existing granular sludge integrity (Lettinga and Hulshoff Pol, 1991; Grotenhuis 1992).
- Sudden sludge washout in cases of prolonged continuous entrapment in a granular sludge bed (Lettinga and Hulshoff Pol, 1991).
- Dilution of active biomass during anaerobic treatment of municipal wastewater in a granular sludge UASB bioreactor as a result of entrapment of suspended solids (de Man et al., 1986).

Thus many researchers have investigated the feasibility of anaerobic treatment of pre-settled domestic sewage under ambient/low-temperature conditions (van der Last and Lettinga, 1992; Elmitwalli, 2000; Seghezzo, 2004). The belief being that the pre-removal of suspended solids would allow the operation of high-rate systems operating at low HRTs while avoiding problems such as deteriorating sludge quality and poor treatment efficiencies.

Several treatment approaches have been investigated in order to overcome the obstacles encountered during LtAD of municipal/domestic wastewater. Those approaches are outlined below.

- using granular seed sludge can enhance the methanogenic capacity of the bioreactor (Lettinga et al., 1993). However over time as a result of low hydrolysis rate at low-temperature the accumulation of suspended solids from the influent leads to a deterioration in the methanogenic capacity of the biomass (Zeeman and Lettinga, 1999).
- removing suspended solids prior to anaerobic treatment and treating the pre-settled sewage in bioreactors with high up-flow velocity, such as EGSB or anaerobic fluidised bed (AFB) designs (Schwitzenbaum and Jewel 1980; Jewel et al., 1981; Yoda et al., 1985; van de Last and Lettinga, 1992; Kalogo and Verstraete, 2000). The settling out and exclusion of solids from the influent prevents accumulation in the sludge bed and can ensure the maintenance of methanoogenic
conditions. With the application of high up-flow velocity bioreactors can achieve high soluble COD removal even at lower temperatures.

- applying a two-step system consisting of a UASB and EGSB bioreactor for the treatment of raw wastewater at low-temperature (Wang, 1994). In the first step the UASB bioreactor is used to remove suspended COD where its partial hydrolysis and acidification will occur. At low-temperatures accumulated solids need to be frequently removed, resulting in a low SRT. This wasted sludge is unstable (Zeeman et al., 1997; Elmitwalli et al., 2002) and need to be further stabilised in a separate, heated sludge digester (van Haandel and Lettinga, 1994; Wang, 1994; Mahmoud, 2002). The removal of the majority of solids in stage one facilitates high methanogenic capacity in the second stage where the conversion of dissolved COD into methane take place.

- applying a two-step system consisting of anaerobic filter (AF) and anaerobic hybrid bioreactor (AH) (Elmitwalli et al., 2001). A similar approach to that outlined above. In the first step the fixed bed AF retains suspended solids, while the second step AH degrades soluble COD. The filter material of the AH plays an important role in the physical retention of biomass providing further biological COD removal.

- applying one stage UASB system supplemented by a sludge digester (Mahmoud, 2002). This combined system enables both treatment of wastewater and sludge stabilisation. Suspended solids in the influent are partially captured in a UASB bioreactor operating at ambient temperature and conveyed as a concentrated sludge to a sludge digester operating at optimal (mesophilic) temperature. Methanogen enriched digested sludge is recirculated to the UASB to improve its methanogenic capacity. In this approach biodegradable solids attached to sludge flocs in the UASB are degraded, thus avoiding accumulation in the sludge bed and enhancing substrate transport to the biomass. In addition wasted sludge from the UASB is well stabilised. Biogas harvested from the digester is reused for heating the digester (Mahmoud, 2002).
1.5 New Perspectives in Anaerobic Municipal Wastewater Treatment

1.5.1 Anaerobic Membrane Bioreactors (AMBRs)

Recently, membrane bioreactors (MBRs) have become increasingly popular in wastewater treatment applications, especially as membrane costs have dramatically decreased (Furukawa, 2008). MBRs can provide high quality effluent compared to conventional biological systems in addition to their promising role in water reuse applications (Daigger et al., 2005). With ever growing application experiences from aerobic membrane bioreactors (MBRs) (Santos et al., 2011), anaerobic membrane bioreactor (AMBR) technology has also started to be researched for municipal wastewater treatment as a possible alternative to the conventional anaerobic treatment processes (Martin et al., 2010). The development of AMBRs which produce methane for energy production, generate little biomass waste, and are capable of meeting local discharge standards. AMBRs for secondary treatment have also been gaining industry interest (Wen et al., 1999; Baek and Pagilla, 2006; Hu and Stuckey, 2006; Ho et al., 2007). Laboratory-scale studies have demonstrated the effectiveness of AMBRs for the treatment of high-strength (Kimura, 1991; Ahring et al., 1992; Bailey et al., 1994; Fuchs et al., 2003; He et al., 2005; Padmasiri et al., 2007) and low-strength (Wen et al., 1999; Baek and Pagilla, 2006; Hu and Stuckey, 2006; Ho et al., 2007) wastewaters. AMBRs ensure biomass retention by the application of micro-filtration or ultra-filtration processes. This allows complete biomass retention and operation at high sludge concentrations, making AMBRs an attractive prospect with respect to LtAD (Trzcinski and Stuckey, 2010; Martinez-Sousa et al., 2011; Stuckey, 2011). However, with regard to low-temperature AMBRs for direct municipal wastewater treatment, the issue of the hydrolysis rate of suspended solids remains. Furthermore, Martinez-Sosa (2011) reported that increased membrane fouling is a feature of LtAD operation, possibly as a result of suspended solids and soluble COD accumulation and increased viscosity in the bioreactor. However, results obtained by several authors have provided positive experience of utilising AMBRs for the low-
temperature treatment of municipal wastewater (Chu et al., 2005; Ho and Sung, 2010; Smith et al., 2013) with high COD removal efficiencies (>85%) being achieved. However those studies employed synthetic sewage representatives and would need to be validated with actual municipal wastewater. MBRs are energy intensive systems, and much research needs to be carried out into the long term, low cost operation of such membranes; in terms of membrane fouling, cleaning regime, applied flux rates, membrane material and the overall economics of the process. In addition the optimal process configuration, such as anaerobic bioreactor type and the coupling of the bioreactor with the membrane module, has yet to be determined. A comprehensive review of the integration options, limitations and expectations of AMBRs for municipal wastewater is provided by Ozgun et al. (2013).

1.5.2 Source-Separation-Based Sanitation Concept
A new approach to the treatment of municipal and domestic wastewater that is gaining significant attention (particularly in Europe) is the idea of implementing new sanitation concepts based on source separation. For example the separation of domestic wastewater at source results in black water from toilets (faeces and urine) and a less polluted grey water from washing activities such as showers, bath, food preparation and laundry. These source separated wastewater streams differ in quantity and quality and may be treated separately according to their concentrations and composition. The separation of different domestic wastewater streams coupled with targeted treatment of these streams for resource recovery has been recognised as one of the most promising concepts in re-establishing the balance in carbon, nutrient and water cycles (Otterpohl et al., 2003; Wilsenach et al., 2003; Zeeman et al., 2008; Larsen et al., 2009).

Anaerobic treatment in an up-flow UASB bioreactor has been identified as the key technology for energy recovery from the black water portion as it results in the conversion of the organic matter to methane which can be used to generate electricity and heat, while also resulting in low excess sludge generation (Zeeman and Lettinga, 1999; Kujawa-Roeleveld and Zeeman, 2006; Verstraete et al., 2005). As black water contains a significant organic load, typically half the organic load of organic material in domestic
wastewater, in addition to the major fraction of nutrients (Otterpohl et al., 2003; Kujawa-Roeleveld and Zeeman, 2006) the economics of using a heated anaerobic bioreactor are justified for countries with sub-mesophilic temperatures. The volume and concentration of black water depends on the type of toilet and amount of water used to flush, and for example where vacuum toilets are used a more concentrated waste can be produced (de Graaff et al., 2010).

Treatment options used for the grey water portion among others include sequencing batch reactors (Hernandez Leal et al., 2010) and constructed wetlands (Avery et al., 2007). Where land constraints exist an option which has been explored is the construction of constructed wetlands as a green roof (Avery et al., 2007). Further separation options have been investigated to advance the efficiency of the source-separation concept including urine separation from black water. A comprehensive review of anaerobic treatment options and source-separation-based-sanitation concepts is presented by Kujawa-Roeleveld and Zeeman (2006).

1.6 Experimental Approach: Integration of Process Analysis, Physiological Characterisation and Molecular Ecology

Anaerobic bioreactors are still mostly designed and operated as ‘black box’ entities, where organic carbon conversion or effluent quality are the principal measure of the success/failure of the process. Process control strategies do not generally take into account the complex interactions and processes occurring at microorganism level. This had led to a situation where wastewater treatment bioreactors are mainly designed using empirical design criteria, which can lead to over-dimensioning of bioreactors volumes and inefficient or unstable treatment. There is an increasing realisation that this approach is insufficient in the context of optimal process design and performance. Greater knowledge of, and research into the structure, function and biological properties of the microbial communities involved in AD, could not only enable us to fully understand the ecology of the process, but possibly
allow for the advancement and optimisation of the AD treatment process (McHugh et al., 2004). This appears to be a logical approach to take, since the success or failure of an anaerobic treatment approach is entirely dependent on the actions and interactions of complex microbial communities (Wilderer et al., 2002). The identification of key parameters, such as the routes of carbon flow and the identification of key trophic groups may in turn give an improved understanding of the key AD microbially-mediated processes, such as hydrolysis, which is recognized as the rate-limiting step in the AD process (Eastman and Ferguson, 1981; Cirne et al., 2006). An improved understanding may also facilitate more accurate mathematical modelling which has to date been limited by a lack of species differentiation in specific functional roles (Ramirez et al., 2009).

Recently, a more holistic polyphasic approach to bioprocess monitoring through, for example the use of molecular and micro-analytical approaches has begun to elucidate the nature of the anaerobic biofilm communities involved in AD, to a minor extent opening up the ‘black box’ of these engineered systems (Marzorati et al., 2008). This probing of the microbial consortia has furthered our understanding of the complex interactions that underpin the successful operation of high-rate anaerobic systems. Information on the formation of granular biofilms, on the relationship between bioreactor operating conditions and microbial consortia and on the impact of process changes on the microorganisms in bioreactors will, in future, enable the link between the processes occurring at microorganism level (scale ca. 1 µm–1 mm) and the processes occurring within anaerobic bioreactors (scale >1 m) (O’Flaherty et al., 2006). It is anticipated that this will enhance the efficiency and applicability of anaerobic processes and may be of particular relevance for new and emerging AD applications, such as the treatment of low-temperature, low-strength wastewaters.

For this study, in conjunction with traditional bioreactor monitoring tools, such as COD removal efficiency, VFA and CH₄ measurements, physiological characterisation (specific methanogenic activity [SMA] assays) and molecular microbial ecology tools (namely 16S rRNA gene clone library analysis, and real-time [quantitative] polymerase chain reaction assays) were
employed to study the anaerobic communities within LtAD bioreactors with an aim to providing comprehensive information with respect to LtAD of municipal wastewater, potentially enabling the link between microorganism and bioreactor performance. These supplementary analyses are described below in addition to others molecular ecology methods.

1.6.1 Physiological Characterisation
SMA assays assess the activity of the various trophic groups found within anaerobic biomass. Substrates used are 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. The method described by Colleran et al. (1992) monitors the change in biogas pressure within a sealed batch vial in response to substrate degradation and corresponding methane production, and SMA is determined as ml CH₄ gVSS⁻¹ day⁻¹.

1.6.2 Molecular Microbial Ecology
Traditionally, studies of microbial diversity were culture-dependant, and the assumption was that cultivation on an artificial medium yielded the majority of species present in a sample. Indeed this method has allowed many microorganisms to be studied. In fact, many of the bacteria involved in anaerobic digestion were first identified using such a culture-based approach, species such as *Methanosarcina barkeri* and *Methanobacterium formicum* (Zinder and Koch, 1984). However, culture-based approaches are known for their selectivity, and, microscopic analysis of environmental samples have recovered counts of one or two orders of magnitude greater than that which can be cultivated from the same sample, giving rise to the phenomenon known as 'the great plate count anomaly' (Staley and Konopka, 1985). Amann et al. (1995) suggested that only 1% of microorganisms can currently be cultivated and that the number of organisms studied to date may only represent 0.001% to 10% of the total population.

The development of the polymerase chain reaction (PCR) by Kary Mullis in the 1980s opened the door for the development of a range of molecular-based methods, aimed at the culture-independent analysis and
identification of microbes. These new strategies, based on the analysis of DNA directly extracted from environmental samples, replaced the steps of isolation and culturing of microbes, which are renowned for their selectivity, leading to a non-representation of the extent of diversity of the population from which they were obtained (Ranjard *et al.*, 2000). Woese and Fox (1977) pioneered the research into the information contained in the small subunit ribosomal RNA as a method for a more conclusive phylogenetic analysis of all forms of life including microorganisms. The 16S rRNA molecules or the genes which encode them are the molecules of choice for investigating phylogenetic relationships between different microorganisms, because they have many advantages to warrant their use as evolutionary biomarkers: (i) they are present in all organisms and display a high degree of functional constancy (Fox *et al.*, 1980), (ii) they have both conserved and variable regions, which makes possible the targeting of general and specific target sites for PCR primers, (iii) they have adequate sequence information to be used as a phylogenetic marker, (iv) rRNA is a dominant macromolecule, (v) relationships established from rRNA sequences are true evolutionary ones (Pace *et al.*, 1986), (vi) the availability of huge databases for comparative sequence analysis. All of the above advantages display the suitability of 16S rRNA for phylogenetic analysis (Amann and Ludwig, 2000).

### 1.6.2.1 Methods Employed in Molecular Microbial Ecology

Most molecular analysis starts with the extraction of nucleic acids, i.e. DNA and RNA from microbial communities existing in an environmental sample, e.g. anaerobic bioreactor biomass. Many different methods have been described for this task including physical, e.g. bead beating homogenization (Ogram *et al.*, 1987) and chemical, e.g. lysozyme and proteinase K (de Long *et al.*, 1993) means. There are also a number of marketed DNA extraction kits available, some specialising in extraction from environmental samples. In general, physical or mechanical methods result in higher nucleic acid yields than chemical methods, but there is an increased risk of shearing or fragmenting the DNA. Once the DNA has been extracted it can be used as target DNA in PCR (Saiki *et al.*, 1985) to amplify specific genes of interest. By choosing specific primers for specific microbial groups, PCR enables a
particular microbial group to be studied independently of the other groups present. Primer combinations, both universal and domain-specific have been developed making it possible to amplify near full length regions or partial regions of the 16S rRNA gene. Following amplification by PCR, a number of procedures can be undertaken to investigate the diversity of gene type and therefore microbial species, present within the sample under study. The techniques employed in this study include clone library generation, amplified rDNA restriction analysis (ARDRA) and 16s rRNA gene sequencing. In addition real-time (quantitative) PCR was used for the direct and quantitative amplification of variable regions of the 16S rRNA gene, unique to targeted microbial species.

1.6.2.2 Cloning, ARDRA and Gene Sequencing

Cloning and direct sequencing of 16S rRNA genes isolated from an environmental sample is probably the most powerful method for identifying the microorganisms present in that sample. The process begins with extraction of all nucleic acids present in the sample under study and the amplification of the 16S rRNA genes by PCR, with universal or domain-specific primers. Cloning of the PCR products is the next step, whereby one 16S rDNA sequence is cloned into a host *Escherichia coli* (*E.coli*) cell using a vector molecule, such as a plasmid. Thus each *E.coli* cell contains one 16S rDNA product from the PCR amplification, and a clone library is essentially an inventory of the 16S DNAs present in the original sample (Toomey, 2002). Following cloning, the 16S rDNA gene library can be screened using ARDRA, which involves the restriction of the 16S rDNA with an endonuclease enzyme, which basically cuts the sequences into smaller-sized fragments. These fragments are separated by electrophoresis to produce a profile, which is unique for that 16S rDNA sequence. Different sequences will therefore produce different profiles. Similar profiles are grouped into operational taxonomic units (OTUs; Moyer *et al.*, 1994) and representatives can then be sequenced, as opposed to sequencing of all clones in the library.
1.6.2.3 Real-Time (Quantitative) PCR
In conjunction with primers designed to amplify the targeted gene sequences, this approach employs an additional fluorescent probe, which hybridises with a specific site within the targeted region. Each sequence of the primer and probe is designed to be specific for a target organism or microbial group, which equates to increased sensitivity and specificity with three oligonucleotides complementary to the target DNA sequence simultaneously employed (Yu et al., 2005). The amount of emitted fluorescence is directly proportional to the amount of the targeted PCR product.

1.6.2.4 Next-Generation Sequencing
The demand for low-cost sequencing has led to the development of high-throughput or next-generation sequencing technologies that produce thousands or millions of sequences concurrently (Hall, 2007; Church, 2006). High-throughput sequencing technologies have significantly lowered the cost of DNA sequencing beyond what is possible with standard dye-terminator methods (Schuster, 2008). In ultra-high-throughput sequencing as many as 500,000 sequencing-by-synthesis operations may be run in parallel (Bosch and Grody, 2008; Tucker et al., 2009). These next-generation sequencing technologies such as the Roche 454 pyrosequencing platform and Illumina deep sequencing platform incorporate a sequencing-by-synthesis approach (based on the detection of nucleotide incorporation) which can be applied for PCR amplicon (16s rRNA, etc.) or whole genome sequencing. Werner et al. (2011) highlighted the potential for deep sequencing to overcome the limitations of fingerprinting methods with respect to resolution and to shed new light on the nature of the bacterial communities in AD.

1.6.2.5 PCR-Independent Techniques
PCR-based methods employed for molecular microbial ecology studies are not without limitations. It is known that these methods may be affected by preferential or differential amplification (Reysenbach et al., 1992; Walsh et al., 1992), which may hinder the detection of some genotypes when analysing DNA extracted from an environmental sample. In addition, when PCR is carried out on complex bulk DNA extracts a number of issues can be
encountered, such as (i) the formation of heteroduplexes; where PCR products can compete with primers for annealing, and (ii) chimeric amplicon formation; where incompletely extended primers may compete with the original primer for annealing (Kanawaga, 2003). All these artifacts can generate additional signals that do not correspond to genotypes in the sample. These issues can vary between applications and many suggestions have been put forward to minimise these PCR artifacts in environmental profiling and fingerprinting approaches.

PCR-independent methodologies are gaining significant focus as they can facilitate the recovery of important cellular information and recent developments have primarily focused on the functional characterisation and quantification of gene products (mRNA, proteins and metabolites), as well as their interactions. In future, integrative studies that combine deep sequencing or metagenomics with functional ‘omic’ approaches such as metabolomics, metaproteomics and mathematical models (Lauro et al., 2011) may well allow the resolution of metabolic processes, even in complex AD consortia, in the context of community structure. With such powerful ecosystem level information, the ability to eco-engineer communities with superior AD performance may soon be a realisable goal.

1.6.2.6 Microscopy Techniques
Molecular techniques rely on nucleic acid extraction therefore conferring a structurally invasive approach for the analysis of microbial community structure, for example, in anaerobic biofilms. Microscopy methodologies such as fluorescent in situ hybridisation (FISH) offer a sensitive approach for quantitative analysis, as single cells can be specifically detected and counted under a microscope (Wintzingerode et al., 1997). FISH approaches based on the 16S rRNA gene have provided new insights into the microbial consortia of anaerobic bioreactors enabling the identification and quantification of microorganisms at various levels of specificity in complex microbial communities without prior cultivation (Raskin et al., 1994; Sekiguchi et al., 1999; Collins et al., 2005).

However, FISH methods are inadequate to determine reliably the general physiological activity and ecophysiological traits of the detected
microbial cells (Wagner et al., 2003). A combination of microautoradiography with FISH (MAR-FISH) is a technique which is widely applied to study the physiological properties of microorganisms in their natural environment and may even be used to obtain single cell resolution (Lee et al., 1999; Wagner et al., 2006). By applying this technique, the phylogenetic identity and specific activity of targeted microorganisms can be simultaneously examined \textit{in situ} within a complex microbial community (Okabe et al., 2004). By applying a labelled substrate, \textit{in situ} metabolic function can be distinguished without prior need for isolation and enrichment.

### 1.7 Thesis Scope

The main objective of this study was to investigate the feasibility and process considerations of direct anaerobic treatment of municipal wastewater under Irish conditions. The overall aim being to demonstrate successfully the direct high-rate anaerobic treatment of municipal wastewater with performance comparable to that of existing municipal wastewater treatment, primarily CAS. The measure of success for the proposed approach was the recovery of a high quality effluent which complies with local discharge standard with respect to COD while also demonstrating stable bioprocess performance. The various bioreactor trials were designed to simulate the typical fluctuations and changes in influent strength and composition which are associated with this wastewater type. The optimal loading rates for LtAD systems treating municipal wastewater was also a key consideration during this study.

To that end a polyphasic approach to bioprocess monitoring was employed; process technology was studied in combination with microbial physiological analyses (namely specific methanogenesis activity and digestibility batch assays) and 16S rRNA gene fingerprinting techniques (clone library and quantitative PCR analyses) to provide a greater insight into the effect of low-temperature operation, applied substrate (municipal wastewater) and environmental changes on the microbial consortia within the anaerobic systems.

\textbf{Chapter 2} investigates a comparative long-term trial where three laboratory-scale EGSB-AF hybrid bioreactors were seeded with a mesophilic
inoculum and used to treat synthetic sewage at 37°C, 15°C and 10°C at high (750 mg COD l⁻¹) and low (250 mg COD l⁻¹) concentrations with varying loading rates. The efficiency, and limitations of the process was monitored in terms of COD removal, methane biogas quality and yield and physiological profiling. The ability of a mesophilic seed inoculum to adapt to low-temperature conditions, the start-up times achievable and the potential role of an AF in such bioreactor systems were also investigated.

Chapter 3 describes the use a novel bioreactor offering improved operational performance and stability to that achieved using the conventional anaerobic hybrid bioreactors employed for the trials described in Chapter 2 during a long-term trial treating synthetic sewage (500 mg COD l⁻¹) at 12°C. In conjunction to process monitoring, a combination of clone library and real-time PCR were used to monitor the development of metabolically diverse microbial populations. SMA and digestibility assays were employed to monitor the maintenance of a methanogenically active biomass throughout the trials and to demonstrate the development of a psychrotolerent/psychroactive sewage degrading biomass.

Chapter 4 sought to substantiate the results achieved in chapters 2 and 3 with actual municipal wastewater as opposed to a synthetic sewage representation. Raw sewage and settled sewage (primary effluent; the liquid fraction following settlement of primary sludge) was treated directly at organic loading rates of 0.2-2.4 kg COD m⁻³ d⁻¹, and hydraulic loading rates of 3-6 h. SMA assays were used to verify the maintenance and development of a methanogenically active biomass. Clone library analysis examined the bacterial and archaeal communities which had developed from a mesophilic seed inoculum cultivated under low-temperature conditions to treat municipal wastewater. In addition, the potential of an additional membrane-based module for further enhancement of effluent quality was investigated by the coupling of a tangential flow filtration unit with the EGSB-AF bioreactor treating raw sewage (R6).

Chapter 5 presents the principle conclusions of this research, along with future recommendations.
1.8 References


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HIGH-RATE ANAEROBIC DIGESTION OF SYNTHETIC SEWAGE IN HYBRID EGSB BIOREACTORS AT 10°C, 15°C AND 37°C: BIOPROCESS PERFORMANCE AND PHYSIOLOGICAL CHARACTERISTICS
Abstract

The feasibility of long-term (>1 year), low-temperature (10-15°C) anaerobic bioreactor operation, for the treatment of synthetic sewage, was investigated. Three hybrid, expanded granular sludge bed-anaerobic filter bioreactors were seeded with a mesophilic inoculum and employed for the mineralization of low-strength (<1000 mg l\(^{-1}\) chemical oxygen demand [COD]) synthetic sewage wastewater at 37°C (control), 15°C and 10°C (experimental). Bioprocess performance was assessed in terms of COD removal efficiency and methane biogas concentration. Batch specific methanogenic activity assays were performed to physiologically characterise bioreactor biomass. Despite transient disimprovements, mean total COD removal efficiency and methane biogas concentrations exceeded 70% and 50%, respectively, for all bioreactors. Successful treatment was achieved at organic loading rates of 0.5-6 kg COD m\(^{-3}\) d\(^{-1}\) and hydraulic retention times of 1.5-12 h, with comparable performance at all temperatures tested. Specific methanogenic activity testing indicated that a psychroactive biomass developed in the low-temperature bioreactors. The observed efficient and stable bioprocess performance demonstrates the potential for long-term, low-temperature anaerobic treatment of dilute wastewaters.
2.1 Introduction

Full-scale anaerobic digestion (AD) is now an established and proven technology under mesophilic and thermophilic conditions for the treatment of a wide range of industrial wastewaters (Driessen & Yspeert, 1999; Macarie, 2000; Bouallagui et al., 2005; Enright, 2006; Rincón et al., 2006). The most widely applied anaerobic wastewater applications are based on granular sludge-based systems (McKeown et al., 2012). Application of the AD process has recently been extended to the treatment of dilute industrial wastewaters, and even anaerobic sewage treatment is thought to be achievable under certain conditions (Raskin et al., 1994). A key disadvantage of established AD solutions is the requirement for process heating to mesophilic (30-37°C) or thermophilic (>45°C) temperature ranges, however most industrial wastewaters are released for treatment at temperatures below 18°C (Lettinga et al., 2001), this is similarly the case for municipal wastewaters in temperate regions. Therefore, while anaerobic treatment of low-strength wastewaters is feasible in tropical regions, where the ambient temperature is sufficiently high to support mesophilic treatment, in temperate regions the heating requirement has restricted the application of AD for the treatment of lower-strength wastewaters (<1,000 mg/L COD), such as those produced by many food-processing and dairy industry, and municipal wastewater, because the energy required to heat the process exceeds the energy produced per unit volume of wastewater.

Conventional biological treatment of municipal wastewater is in general underpinned by aerobic biological processes as core technologies. The feasibility of anaerobic treatment of sewage and municipal wastewaters is increasingly attracting the attention of sanitary engineers and decision-makers (Seghezzo et al., 1998), and has been applied with encouraging results in tropical and subtropical zones (Seghezzo et al., 1998; Elmitwalli et al., 2001; Lettinga et al., 2001; Aiyuk et al., 2004; Sarti et al., 2006). However a challenge remains in countries where lower ambient temperatures prevail; a significant energy input would be required in order for the process to proceed within the mesophilic temperature range. The potential of anaerobic treatment of wastewaters at ambient or low-temperatures has not yet been fully realised,
as significant physical, chemical and biological challenges need to be overcome to apply high-rate AD under low-temperature conditions (Lettinga et al., 2001, Collins et al., 2006). However the social and economic requirements for low-cost wastewater treatment systems have prompted more advanced study into the area. The development of novel bioreactor designs has helped to overcome one of the key issues associated with low-temperature AD (LtAD); the retention of psychroactive biomass. Hybrid bioreactors, incorporating an anaerobic filter (AF) section have helped to overcome this problem as it enables slow-growing psychroactive biomass lost from the granular sludge bed to be entrapped and retained as fixed-film growth on a support matrix (Collins et al., 2006; McKeown et al., 2009). In addition, membrane-assisted physical separations can be used to underpin sludge retention. Anaerobic membrane bioreactors (AMBRs) ensure biomass retention through the application of micro/ultra-filtration processes. This allows complete biomass retention and operation at high sludge concentrations, making AMBRs an attractive approach for LtAD (Trzcinski and Stuckey, 2010; Martinez-Sousa et al., 2011; Stuckey, 2011). Anaerobic baffled reactors (ABRs), which separate biomass into compartments, have also been demonstrated to be suitable for LtAD, and particularly for treatment of wastewaters with variable flows and those containing toxicants (Feng et al., 2009). The biotechnological application of LtAD represents an economically-attractive waste treatment strategy, which confers considerable advantages over conventional mesophilic (>20°C) and thermophilic (>45°C) treatments (Lettinga et al., 2001), primarily due to increased net energy yields. LtAD does not require process heating, and, thus, overcomes a key technological limitation. Adoption of low temperature or unheated AD as a core technology in municipal wastewater treatment could result in more sustainable management of this abundant wastewater and provide several benefits for sanitary engineering, including reduced demand on fossil fuels, lower capital and energy expenditure, reduced sludge production, and the generation of renewable energy.

The present study investigated the direct anaerobic treatment of a synthetic sewage representative wastewater; SYNTHES (Aiyuk and Verstraete, 2004) using expanded granular sludge bed-anaerobic filter
Chapter 2

(EGSB-AF) bioreactors under low-temperature conditions in terms of system performance and physiological characteristics of bioreactor biomass. The hypothesis being tested was that anaerobic bioreactors operating under low-temperature conditions (15°C and 10°C) could successfully treat a synthetic sewage at varying concentrations and loading rates over a long period (>500 days) with performance comparable to a bioreactor operating in the mesophilic temperature range (37°C). The ability of a mesophilic seed inoculum to adapt to low-temperature conditions, the start-up times achievable and the potential role of an AF in such bioreactor systems was also investigated.

2.2 Materials and Methods

2.2.1 Source of Biomass
Anaerobic sludge granules were obtained from a full-scale, mesophilic (37°C), internal circulation (IC) bioreactor at Carbery Milk Products (Ballineen, County Cork, Ireland) used to treat ethanol production wastewater. The volatile suspended solids (VSS) concentration of the granules was 102.8 g VSS l⁻¹. The anaerobic granules (Ø c. 1-3 mm) were of a regular, spherical shape and a grey-green colour.

2.2.2 Bioreactor Design and Operation
Three identical glass, laboratory-scale (3.5 l; active liquid volume, 3.0 l), expanded granular sludge bed-anaerobic filter (EGSB-AF) hybrid bioreactors similar to that previously described by McHugh et al. (2004), R1-R3, were used in the present study. The upper, ‘fixed-film’ anaerobic filter (AF) section comprised polyethylene rings (Ø, c. 25 mm) randomly packed in a cylindrical, wire-mesh cage. R1 was inoculated with 11 g VSS l⁻¹ of the sludge granules and was operated at 37°C (control). R2 and R3 were each inoculated with 20 g VSS l⁻¹ and were operated at 15°C and 10°C, respectively. R1-R3 were employed to treat a synthetic sewage wastewater (SYNTHES [Table 2.1]; Aiyuk and Verstraete, 2004) over three distinct operational Phases (Phase 1-Phase 3) characterised by the strength of the influent: 750 mg total chemical oxygen demand (COD_{tot}) l⁻¹, (375mg l⁻¹ suspended solids) in Phase 1 (dry
season concentrated wastewater); 250 mg COD$_{\text{tot}}$ l$^{-1}$ (125 mg l$^{-1}$ suspended solids) in Phase 2 (wet season dilute wastewater) and 750 mg COD$_{\text{tot}}$ l$^{-1}$, (375 mg l$^{-1}$ suspended solids) in Phase 3. The trial was divided into 11 operating periods (PI-PIXI), which were characterised by hydraulic retention times (HRT) and consequent organic loading rate (OLR). Effluent was re-circulated through the respective bioreactors to give a liquid up-flow velocity of 5 m h$^{-1}$. Phase and period characteristics are outlined in Table 2.2.

### 2.2.3 Effluent and Biogas Analyses

Samples of bioreactor effluent and biogas were taken thrice weekly for determination of total COD (COD$_{\text{tot}}$) and soluble COD (COD$_{\text{sol}}$) removal efficiency, pH and biogas determination according to Standard Methods (American Public Health Association (APHA) 1998). Volatile fatty acid (VFA) concentrations were determined by GC-MS (Varian, USA).

#### Table 2.1 Composition of SYNTHES at applied COD$_{\text{total}}$ concentrations.

<table>
<thead>
<tr>
<th>Component</th>
<th>COD$_{\text{total}}$ concentration and component amount (mg l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250 mg l$^{-1}$</td>
</tr>
<tr>
<td><strong>Chemical compounds</strong></td>
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</tr>
<tr>
<td>Urea</td>
<td>50</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
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</tr>
<tr>
<td>Na-acetate·3H$_2$O</td>
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</tr>
<tr>
<td>Peptone</td>
<td>9.4</td>
</tr>
<tr>
<td>MgHPO$_4$·3H$_2$O</td>
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</tr>
<tr>
<td>K$_2$HPO$_4$·3H$_2$O</td>
<td>12.5</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>3.1</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>Food ingredients</strong></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>65.6</td>
</tr>
<tr>
<td>Milk Powder</td>
<td>62.5</td>
</tr>
<tr>
<td>Dried yeast</td>
<td>28.1</td>
</tr>
<tr>
<td>Soy oil</td>
<td>15.6</td>
</tr>
<tr>
<td><strong>Trace metals</strong></td>
<td></td>
</tr>
<tr>
<td>Cr(NO$_3$)$_3$·9H$_2$O</td>
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</tr>
<tr>
<td>CuCl$_2$·2H$_2$O</td>
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</tr>
<tr>
<td>MnSO$_4$·H$_2$O</td>
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</tr>
<tr>
<td>NiSO$_4$·6H$_2$O</td>
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</tr>
<tr>
<td>PbCl$_2$</td>
<td>0.1</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>0.2</td>
</tr>
</tbody>
</table>
### Table 2.2 Operational conditions and parameters applied during Phases 1-3 of operation.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Period</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
<th>IX</th>
<th>X</th>
<th>XI</th>
</tr>
</thead>
<tbody>
<tr>
<td>I COD\textsubscript{tot}\textsuperscript{a}</td>
<td></td>
<td>750 ±50</td>
<td>750 ±50</td>
<td>750 ±50</td>
<td>750 ±50</td>
<td>250 ±40</td>
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<td>250 ±40</td>
<td>250 ±40</td>
<td>750 ±50</td>
<td>750 ±50</td>
<td>750 ±50</td>
</tr>
<tr>
<td>I COD\textsubscript{sol}\textsuperscript{b}</td>
<td></td>
<td>240 ±30</td>
<td>240 ±30</td>
<td>240 ±30</td>
<td>240 ±30</td>
<td>80 ±20</td>
<td>80 ±20</td>
<td>80 ±20</td>
<td>80 ±20</td>
<td>240 ±30</td>
<td>240 ±30</td>
<td>240 ±30</td>
</tr>
<tr>
<td>I SS\textsuperscript{c}</td>
<td></td>
<td>375</td>
<td>375</td>
<td>375</td>
<td>375</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>375</td>
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</tr>
<tr>
<td>HRT\textsuperscript{d}</td>
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<td>6</td>
<td>3</td>
<td>12</td>
<td>12</td>
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<td>1.5</td>
<td>12</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>OLR\textsuperscript{e}</td>
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<td>3</td>
<td>6</td>
<td>1.5</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>1.5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>VLR\textsuperscript{f}</td>
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<td>4</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>4</td>
<td>8</td>
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<tr>
<td>SLR\textsuperscript{g} R1(37°C)</td>
<td></td>
<td>0.14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R2(15°C), R3(10°C)</td>
<td>0.075</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SLR\textsuperscript{h} R1(37°C)</td>
<td></td>
<td>0.19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R2(15°C), R3(10°C)</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UV\textsuperscript{i}</td>
<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
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<td>5</td>
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<td>5</td>
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</tr>
</tbody>
</table>

\textsuperscript{a}Influent chemical oxygen demand, total (mg l\textsuperscript{-1}); \textsuperscript{b}Influent chemical oxygen demand, soluble (mg l\textsuperscript{-1}); \textsuperscript{c}Influent suspended solids (mg l\textsuperscript{-1}); \textsuperscript{d}Hydraulic retention time (h); \textsuperscript{e}Organic loading rate (kg COD m\textsuperscript{3} d\textsuperscript{-1}); \textsuperscript{f}Volumetric loading rate (m\textsuperscript{3} Wastewater m\textsuperscript{3} Reactor d\textsuperscript{-1}); \textsuperscript{g}Sludge loading rate (kg COD kg [VSS] d\textsuperscript{-1}); \textsuperscript{h}Sludge loading rate (m\textsuperscript{3} Wastewater kg [VSS] d\textsuperscript{-1}); \textsuperscript{i}Up-flow velocity (m h\textsuperscript{-1}); Values for \textsuperscript{a} and \textsuperscript{b} display standard deviation of bioreactor influent.
2.2.4 Specific Methanogenic Activity Assays

The specific methanogenic activity (SMA) of the seed biomass (day 0), and of biomass from R1-R3 on days 405 and 544, was assayed at each of 10, 15 and 37°C according to Colleran et al. (1992) and Coates et al. (1996). Additionally, R1-R3 biomass samples from days 124, 201, 230, 285, 325 and 371 were tested at the operating temperature of the respective parent bioreactors. Fixed-biofilm samples recovered from the AF of R1-R3 at the conclusion of the trial (day 544) were also tested at the respective operating temperature of the parent bioreactors. SMA assays are batch tests that employ a pressure transducer technique to measure the rate of methane produced over time. SMA assays assess maximum potential activity against intermediates of the AD process. SMA of biomass samples were separately assayed against the direct methanogenic substrates acetate (30 mM) and H₂/CO₂ (80:20, v/v) and higher carbon intermediates butyrate (15 mM),
propionate (30 mM), and ethanol (30 mM) as previously described by Collins et al. (2003). Biomass samples were washed in 1X PBS and were transferred under anaerobic conditions to sterile 20-ml (soluble substrates) or 60-ml (gaseous substrates) hypovials to achieve a final biomass concentration of 2-5 g VSS l⁻¹ in a total volume of 10 ml anaerobic activity test medium. Vials without substrate, or with a pressurised N₂/CO₂ headspace only, were used as controls. Rates of activity are expressed as ml CH₄ g[VSS]⁻¹ d⁻¹.

2.3 Results

2.3.1 Bioreactor Performance

Bioreactor performance during the anaerobic treatment of the synthetic sewage representative wastewater (SYNTHERS) is summarised in Table 2.3. COD removal efficiency total/soluble (CODREtot/sol), biogas methane content (%), and effluent acetic acid concentrations are presented in Figures 2.3-2.5. Operational Phase conditions and parameters are previously outlined in Table 2.2.

2.3.1.1 Phase 1

A short start-up was achieved for each of the bioreactors at the applied OLR of 1.5 kg COD m⁻³ d⁻¹ (PI), with CODREtot+sol of c. 70% achieved by day 13 (Fig. 2.3a and b). This performance continued throughout PI with R2 (15°C) routinely outperforming R1 and R3. Biogas yields for PI of 300-390 ml d⁻¹ were comparable from all three bioreactors, with highest production observed in R2 (Table 2.3). PII was characterised by a decrease in the HRT (consequently increasing OLR to 3 kg COD m⁻³ d⁻¹), and resulted in some biomass washout from each bioreactor. This was associated with gradually decreasing CODRE over 10 d, leading to a sustained period (days 138-170) of poor performance in R1-R3, with mean CODREtot of 44% (Fig. 2.3a). Improved performance was exhibited for R1-R3 for the remainder of PII, with CODREtot and CODREsol of >70% and >50% respectively being recorded (Fig. 2.3a and b). Biogas quality for this period averaged >71% for R1 and R2 and 63% for R3 (Fig. 2.4), however these figures are somewhat misleading as gas chromatography measurements were unavailable during
days 125-159, a time of poor performance for R1-R3 (Fig. 2.3). The introduction of PIII had no adverse effect on performance, with CODREs for R1-R3 showing gradual improvement throughout the period. The final period (IV) of Phase 1 involved a return to the initial (PI) operational conditions. Performance remained stable from that observed at the completion of the previous period. Biogas analyses carried out throughout Phase 1 displayed comparable results for R1 and R2 with R3 showing slightly reduced biogas quality (Fig. 2.4; Table 2.3). Acetic acid concentrations remained low throughout Phase 1 for all three bioreactors (0-11.3 mg l\(^{-1}\)), with mean concentrations of < 7 mg l\(^{-1}\) (Fig. 2.5).

2.3.1.2 Phase 2
The reduction in influent COD (mg l\(^{-1}\)) concentration of Phase 2 (Table 2.2) had an immediate effect on performance and by day 4 of PV CODRE\(_{\text{tot}}\) had dropped to 54% (R1) and 31% (R2, R3; Fig. 2.3a). Biogas quality for R1-R3 reflected this drop in removal efficiencies (Fig. 2.4). The remainder of PV and PVI was characterised by erratic performance for R1-R3 (Fig. 2.3a and b). However, the mean CODREs for PVI were >72% COD\(_{\text{tot}}\) and >67% COD\(_{\text{sol}}\) (Table 2.3). Biogas quality improved in tandem with the improved bioreactor performance with average CH\(_4\) content of biogas c. 50% (Fig. 2.4). The erratic removal rates observed for all bioreactors for PV-PVI were replaced by more stable operation during PVII (OLR, 2 kg COD m\(^{-3}\) d\(^{-1}\)), with mean CODRE\(_{\text{tot} + \text{sol}}\) at 83-90% (Fig. 2.3a and b). The final operational change (PVIII) of Phase 2 involved a reduction in HRT to 1.5 h resulting in an OLR of 4 kg COD m\(^{-3}\) d\(^{-1}\). The increased rate of influent resulted in a decrease in CODRE (Fig. 2.3a and b). Nevertheless, mean CODREs were >75% COD\(_{\text{tot}}\) and >81% COD\(_{\text{sol}}\) for all bioreactors (Table 2.3). Mean biogas quality for periods VII and VIII were comparable for all bioreactors, 47-55% (Fig. 2.4; Table 2.3). Effluent acetic acid concentration was consistent with that observed during Phase 1; (0-11.5 mg l\(^{-1}\)) with a mean for R1-R3 of <6 mg l\(^{-1}\) (Fig 2.5).
Table 2.3 Performance characteristics during Phases 1-3 of operation.

| Phase Period | 1       | 2       | 3       | 4       | 5       | 6       | 7       | 8       | 9       | 10      | 11      | 12      | 13      | 14      | 15      | 16      | 17      | 18      | 19      | 20      | 21      | 22      | 23      |
|--------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| CODRE<sub>tot</sub><sup>a</sup> | R1(37°C) | 74.5 ±6 | 60 ±11.9 | 68.2 ±9.1 | 80 ±10.9 | 62.3 ±14.2 | 73.8 ±13.6 | 84.9 ±4.9 | 75.1 ±3.5 | 74.7 ±11.1 | 77.8 ±10 | 67.4 ±8 |
| R2(15°C)    | 73.7 ±8.1 | 59.8 ±12.4 | 71.3 ±7.1 | 79.2 ±8.9 | 64.9 ±14.8 | 76.4 ±11.9 | 83.1 ±8.7 | 76.1 ±1.5 | 68.4 ±12 | 66.9 ±7.4 | 75.3 ±2.5 |
| R3(10°C)    | 72.1 ±5.7 | 55.6 ±11.1 | 66.3 ±7.1 | 77.1 ±11 | 63.8 ±10.2 | 72.2 ±13.2 | 86 ±5.4 | 77.8 ±3.5 | 65.5 ±12.5 | 72.8 ±8.8 | 70.7 ±14.2 |
| CODRE<sub>sol</sub><sup>b</sup> | R1(37°C) | 74.6 ±6.3 | 45 ±17.6 | 47.9 ±12.9 | 66.5 ±8.6 | 63.7 ±12.1 | 68 ±10.2 | 90.9 ±4.8 | 87.7 ±16.2 | 71 ±13.2 | 78.9 ±7.2 | 70.9 ±10.3 |
| R2(15°C)    | 79.1 ±7.9 | 42.3 ±16.5 | 52.2 ±14.2 | 69.8 ±8.9 | 58.4 ±19.1 | 68.8 ±17.4 | 89.4 ±6.6 | 87.7 ±15.7 | 64.4 ±13.5 | 67.7 ±10.9 | 73.8 ±13 |
| R3(10°C)    | 73.6 ±6.5 | 35.8 ±15.6 | 46.4 ±10.6 | 62.8 ±11.3 | 53.1 ±17.8 | 67.6 ±17.3 | 89.4 ±6.6 | 81.1 ±13.2 | 64.2 ±13.6 | 76.4 ±8.7 | 72.4 ±14.2 |
| CH<sub>4</sub><sup>c</sup> | R1(37°C) | 65.1 ±9 | 71.7 ±3.7 | 58.2 ±7.3 | 60.4 ±3 | 50.4 ±0 | 51.2 ±1.5 | 56.7 ±1.8 | 55 ±4.1 | 63.9 ±10.4 | 65.9 ±3.8 | 72.7 ±2 |
| R2(15°C)    | 51.9 ±9.2 | 71.3 ±2.7 | 67.4 ±1.6 | 54.8 ±4.4 | 14.3 ±0 | 51.6 ±3 | 49.6 ±0.9 | 49.1 ±1.3 | 58.4 ±7.2 | 59.1 ±6.1 | 66.6 ±1.4 |
| R3(10°C)    | 50.4 ±11.4 | 63.1 ±9.6 | 55.1 ±0.6 | 41.6 ±3.1 | 12.6 ±0 | 48.3 ±2.5 | 47.5 ±0.6 | 45.5 ±1.8 | 55.9 ±10.2 | 49.3 ±6.3 | 71.2 ±6 |
| Biogas<sup>d</sup> | R1(37°C) | 369 ±47 | 978 ±89 | - | - | - | - | - | - | - | - |
| R2(15°C)    | 390 ±34 | 942 ±103 | 562 ±38 | 569 ±0 | - | - | - | - | - | - |
| R3(10°C)    | 306 ±42 | 505 ±72 | 379 ±0 | 500 ±0 | - | - | - | - | - | - |

<sup>a</sup> Chemical oxygen demand removal efficiency, total (%); <sup>b</sup> Chemical oxygen demand removal efficiency, soluble (%); <sup>c</sup> Methane in biogas (%); <sup>d</sup> Biogas production (ml d<sup>-1</sup>); All values are the period mean ± period standard deviation.
2.3.1.3 Phase 3

The final phase of operation saw a return to the initial influent concentration of 750 mg l\(^{-1}\) COD\(_{\text{tot}}\). PIX-XI re-created the operational parameters applied in PI-III of Phase 1 although over a shorter timeframe. The re-introduction of the higher strength influent resulted in an immediate effect on CODREs. A sharp decrease in performance from day 440 heralded a phase of erratic performance, CODRE\(_{\text{tot}}\) R1-R3 (30-94%; Fig. 2.3a) with CODRE\(_{\text{sol}}\) displaying similar inconsistency (Fig. 2.3b). Performance recovered and stabilised over the latter half of PIX. The inconsistent performance which was a feature of PIX was replaced by more consistent removal during PX with CODRE\(_{\text{tot}}\) of >87% being achieved for all bioreactors by completion of the period (Fig. 2.3a). CODRE\(_{\text{sol}}\) displayed similar consistency, with R1-R3 mean values of 79%, 68% and 76% respectively (Fig. 2.3b). The final operational change of the current study involved a decrease of HRT to 3hrs resulting in an OLR of 6 kg COD m\(^{-3}\) d\(^{-1}\) (Period XI). The increased loading rate had no significant adverse effect on performance (Fig. 2.3a and b). Biogas quality improved during Phase 3 as a result of the increased influent strength, R1 68%, R2 61% and R3 59% mean biogas quality (Fig. 2.4). Greater fluctuations were observed in acetic acid concentrations (0-18.9 mg l\(^{-1}\)) than recorded for the previous phases however mean values for all bioreactors remained low (<5 mg l\(^{-1}\), Fig. 2.5).
Figure 2.3 Chemical oxygen demand removal efficiency; (a) total and (b) soluble; R1 (□), R2 (△) and R3 (●).
**Figure 2.4** Biogas methane concentration; R1 (□), R2 (△) and R3 (●).

**Figure 2.5** Effluent VFA (acetic acid only detected) concentration; R1 (□), R2 (△) and R3 (●).
2.3.2 Physiological Characterisation of Biomass
The values obtained from specific methanogenic activity testing of the seed inoculum (day 0), and bioreactors (R1, R2 and R3) biomass throughout operation are outlined in Table 2.4 and presented Figures 2.6-2.7.

2.3.2.1 Seed Inoculum Specific Methanogenic Activity (day 0)
SMA values obtained for the seed biomass at the lower test temperatures contrasted greatly with those observed at 37°C indicating the higher potential activity at 37 °C (Table 2.4). All substrates showed a marked decrease in activity at 15°C and a further decrease at 10°C. Indirect substrates displayed significant decreases in potential activity to those obtained at 37°C with 10 and 13 fold decreases in activity against ethanol, 4 and 6 fold decreases against propionate and 4 and 12 fold decreases against butyrate being recorded at 15°C and 10°C respectively (Table 2.4). Values against direct methanogenic substrates showed a similar trend with 7 and 8 fold decreases against acetate and 2 and 8 fold decreases against H₂/CO₂ at 15°C and 10°C respectively (Table 2.4; Fig. 2.7). Recorded values for the direct methanogenic precursors, acetate and H₂/CO₂ indicated the dominance of acetoclastic methanogens. This was the case for all test temperatures, with an almost 6:1 ratio in activity of acetoclastic:hydrogenotrophic methanogens being recorded at 37°C and 10°C (Table 2.4; Fig. 2.6).
Table 2.4 Specific methanogenic activity (ml CH₄ g [VSS]⁻¹ d⁻¹) of seed inoculum and bioreactor biomass.

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<th>Day</th>
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<th>Test temp. (°C)</th>
<th>Ethanol (ml g⁻¹ d⁻¹)</th>
<th>Acetate (ml g⁻¹ d⁻¹)</th>
<th>Propionate (ml g⁻¹ d⁻¹)</th>
<th>Butyrate (ml g⁻¹ d⁻¹)</th>
<th>H₂/CO₂ (ml ml⁻¹)</th>
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<td>6 ±1</td>
<td>13 ±1</td>
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</table>

GSB—granular sludge bed biomass; AF—anaerobic filter biomass; NAD—no activity detected; All values are the mean of triplicates ± standard deviation; dashed lines indicate operational phases.
2.3.2.2 Bioreactor Methanogenic Activity (Phase 1)

A significant alteration in the structure of the methanogenic community of biomass samples from each bioreactor was observed on day 124 with a marked increase in hydrogen-utilising activity being observed, these increases were coupled with decreases against acetate. This trend was most striking for the R1 (37°C) biomass (Fig. 2.4a). The structure of the R1 methanogenic community following the initial shift indicated from SMA testing on day 124 averaged 2:1 hydrogenotrophic:acetoclastic methanogenic activity (days 201, 230, 285; Table 2.4, Fig. 2.6a). This hydrogenothrophic methanogen dominance of R1 was not a feature of the lower temperature R2 (15°C) and R3 (10°C) biomass (Figs. 2.6b and c). By the conclusion of Phase 1, SMA values obtained for the indirect substrates ethanol, propionate and butyrate of all bioreactors were lower than those recorded for the seed inoculum. These decreases were most distinct for assays against propionate with a 78, 86 and 92% decrease in potential activity for R1, R2 and R3 respectively from values obtained for the seed inoculum (Table 2.4).

2.3.2.3 Bioreactor methanogenic activity (Phase 2)

SMA testing on completion of the initial period (V) of Phase 2 (day 325) displayed decreases by all bioreactors for both the acetate and H2/CO2 assays (Fig. 2.6), with the exception of the R2 biomass against H2/CO2 (Fig. 2.6b). This downward trend ceased and values recovered to a degree from this point to the completion of the phase, with the R2 biomass again opposing the trend. After an initial increase in activity against H2/CO2 reduced values were recorded for R2 on days 372 and 405 (Fig. 2.6b). On completion of Phase 2 decreased values were recorded against both direct methanogenic substrates (acetate and H2/CO2) for all bioreactors from values obtained on completion of Phase 1. Fluctuations were observed against indirect substrates however on completion of Phase 2 most potential SMA values had recovered to pre-phase rates (Fig. 2.6). R2 and R3 on day 405 returned higher potential activity values against propionate and butyrate that those recorded at the commencement of Phase 2 (Figs. 2.6b and c). R2 and R3 potential activity against butyrate and H2/CO2 displayed higher activity values than those obtained from the seed inoculum indicating the development of a low-
temperature tolerant mesophilic biomass (Fig. 2.6, Table 2.4). On day 405, the completion of Phase 2 SMA testing of biomass from each bioreactor was carried out at 37, 15 and 10°C. All samples displayed highest potential activity at 37°C (Fig. 2.7, Table 2.4) indicating that the biomass had retained its mesophilic nature despite 405 days operation at 15°C and 10°C in the case of the R2 and R3 biomass respectively. R1 returned no activity against the indirect methanogenic substrates ethanol, propionate and butyrate at 10°C (Table 2.4).

### 2.3.2.4 Bioreactor Methanogenic Activity (Phase 3)

SMA testing of bioreactor biomass samples at their respective operational temperature on completion of Phase 3 (day 544) displayed a significant increase in acetoclastic methanogenic activity for all samples, with R2 and R3 displaying a 2 fold increase in potential activity against acetate (Figs. 2.6b and c). Decreases in activity against H₂/CO₂ were exhibited for the R1 and R3 biomass while R2 showed a slight increase against H₂/CO₂ (Figs. 2.6 and 2.7). Values against indirect substrates generally displayed a decrease in activity from values obtained on day 405, with the exception of R2 and R3 activity against ethanol, R3 showed a >3 fold increase (Fig. 2.6). Once again when bioreactor biomass samples from R2 and R3 were tested at increased temperature, activity dramatically increased (Fig. 2.7, Table 2.4). SMA testing was carried out on the fixed film biomass recovered from the AF at bioreactor operational temperature. AF SMA against all substrates was similar to the granular sludge bed biomass for the lower temperature R2 and R3 (Fig 2.6). R1 displayed reduced activity against indirect substrates with the exception of butyrate, a 50% reduced activity against acetate was also observed. Greater activity against H₂/CO₂ was recorded, however, within R1 AF biofilm compared with the sludge bed biomass (Fig. 2.6a; Table 2.4).
Figure 2.6 Temporal SMA of biomass at bioreactor operating temperature;
(a) R1 (37°C), (b) R2 (15°C) and (c) R3 (10°C); AF – anaerobic filter.
Figure 2.7 Effect of temperature on SMA profiles of sludge bed biomass against the direct methanogenic substrates; (a) R1, (b) R2 and (c) R3; highlighted bars indicate biomass at bioreactor operational temperature.
2.4 Discussion

2.4.1 Bioreactor Performance

The long-term direct treatment of synthetic sewage in both the mesophilic temperature range (R1) and under low-temperature conditions (R2 and R3) was demonstrated successfully at applied OLRs of 0.5-6 kg COD m\(^{-3}\) d\(^{-1}\) with HRTs as low as 1.5 h. Mean total CODREs of 70% were achieved by all three bioreactors (Fig. 2.3a). The performance observed is comparable to previous studies of both mesophilic (>20°C) and LtAD applications of both synthetic (Domingues Rodrigues et al., 2003; Aiyuk and Verstraete, 2004) and raw (van de Last and Lettinga, 1992; Yu and Anderson, 1996; Collins et al., 1998; Rebac et al., 1999; Aiyuk et al., 2004) sewage. The results obtained for R3 at 10°C compare favourably with similar studies (Grin et al., 1983; de Man et al., 1986; Derycke and Verstraete, 1986; Maaskant et al., 1991; Rebac, 1998; Bodik et al., 2000). In general, the overall operation and performance of all bioreactors between operational changes was stable, and satisfactory treatment was maintained. The dramatic decline in removal efficiency observed during the initial half of PII can most likely be attributed to the halving of the retention time and consequent doubling of the organic loading rate. A more protracted or step-wise increase in organic loading rate may have proved to be a more prudent approach helping to lessen the inhibitory effect on the biomass, and providing time for community adaptation; the drop in COD removal efficiencies experienced appears to have been an overwhelmed biomass response.

The hydrolysis of suspended solids contained in sewage is considered the main challenge for LtAD of municipal wastewater. Hydrolysis is the rate limiting step in the AD process and is greatly influenced by process temperature and solids retention time (SRT) (Pavlostathis and Giraldo-Gomez, 1991; Miron et al., 2000; Sanders, 2001). Interestingly, temperature did not appear to affect hydrolysis rates or be a determining factor for the anaerobic treatment of synthetic sewage with all bioreactors performing comparably and displaying similar responses to the various operational conditions applied. Zeeman and Lettina (1999) demonstrated that the lower the operational temperature the longer the SRT required to degrade sewage
solids. However in this study the applied operational parameters, HRT and OLR appeared to exert a greater effect on performance than operating temperature. Indeed R2 routinely outperformed the mesophilic bioreactor (R1) in terms of CODRE and biogas quality, particularly during the first phase of operation. During stable operation biogas quality was comparable for R1-R3. Considerably reduced methane content in biogas was observed throughout Phase 2 (days 286-405), which was most likely a reflection of the more dilute nature of the sewage (which was used to simulate wet-weather (winter) conditions). However, comparable methane production, indicative of active anaerobic conversion of organic matter, was obtained by R1-R3 to that reported by Syutsubo et al. (2008).

Overall, results indicated that a well-balanced, psychroactive methanogenic consortium developed during the direct LtAD of synthetic sewage. Temperature influences the rate and pathways of carbon flow during methanogenesis, by affecting the activity of particular microbial groups and the structure of the consortia (Glissmann et al., 2004). However optimum growth temperatures under low-temperature conditions are not a pre-requisite for cold-adapted organisms; activity at low-temperatures is the most important characteristic (Cavicchioli, 2006). The stable operation observed for the three bioreactors and in particular the 15°C bioreactor (R2) shows the potential for the low-temperature anaerobic treatment of sewage in countries with a temperate climate, such as Ireland.

2.4.2 Microbial Physiology
The mesophilic nature of the seed inoculum confirmed through SMA testing was as expected as the seed biomass was sourced from a full-scale bioreactor operating in a mesophilic temperature range, this was not of particular concern as the development of low-temperature activity following exposure to low-temperature conditions in bioreactors has been previously documented (Enright et al., 2005). Profiling of the seed inoculum suggested that acetoclastic methanogens were the dominant methane producers. Subsequent SMA testing on day 124 identified the emergence and indeed apparent dominance of a hydrogenotrophic methanogenic community in the biomass of R1-R3; this was coupled together with a decrease in activity against acetate
at all temperatures (Fig. 2.6; Table 2.4), this despite findings which suggest methane production during LtAD is dominated by acetate mediated methanogenesis (Akila & Chandra, 2007). However the dominance of hydrogenotrophic methanogens in LtAD is not uncommon or indeed is a shift towards hydrogenotrophic methane production under low-temperature conditions (McHugh et al., 2003, 2004; Enright et al., 2005; Collins et al., 2005; Connaughton et al., 2006; Syutsubo et al., 2008; McKeown et al., 2009, O’Reilly et al., 2010). Indeed, trophic interactions within low-temperature methanogenic communities are governed by the thermodynamics of the respective biochemical conversions (Koysyurbenko, 2005) and at lower temperatures, hydrogenotrophic methanogenesis requires less energy (Lettinga et al., 2001). Methanogenesis from H₂/CO₂ is thermodynamically more favourable at low-temperatures (Conrad and Wetter, 1990), as hydrogen thresholds decreases and gas solubility increases with reduced temperature, possibly leading to increased levels of hydrogen in bioreactor liquor (Lettinga et al., 2001). This marked dominance persisted in R1 throughout Phases 1 and 2; however the shift towards hydrogenotrophic methanogenesis appeared only transient for R2 and R3 with subsequent SMA analyses against the direct methanogenic precursors pointing to a more balanced methanogenic community structure. This rebalancing of methanogens in R2 and R3 may suggest an alternative fate for hydrogen within the granular sludge bed, through the action of homoacetogenic bacteria converting the hydrogen to acetate thus providing ample substrate for acetoclastic methanogens (Kelly et al., 2010). Homoacetogenic bacteria have been shown to be strong competitors of methanogens for hydrogen at low-temperature (Conrad et al., 1989; Kotsyurbenko et al., 1996). However, hydrogenotrophic methanogenesis has also been shown to play an important role in cold terrestrial habitats (Lansdown et al., 1992; Horn et al., 2003; Kotsyurbenko et al., 2007) and in waste treatment bioreactors (McKeown et al., 2009).

The period of poor performance coupled with washout of bioreactor R1-R3 biomass experienced during PII may be attributed to the diminished acetoclastic methanogenic activity observed on day 124; McHugh et al. (2003, 2004) proposed that hydrogenotrophic methane production was a feature of bioreactors operating under stressed environments. The subsequent
re-establishment of acetoclastic methane production appears to have led to more stable operation and granule integrity; a robust architecture to bioreactor granules was retained throughout the remainder of the trial in R1-R3. The importance of maintaining a stable acetoclastic methanogenic community for the successful operation of anaerobic digesters in terms of both methane production (Lettinga 1995), and granule integrity has been previously described (O’Flaherty et al., 1997 and Enright et al., 2008). Testing on completion of the trial (day 544) displayed the return of acetoclastic methanogens as the dominant methanogenic population, similarly observed for the seed inoculum.

SMA testing carried out on day 405 and 544 (completion of Phase 2 and Phase 3 respectively) at 37°C on biomass from bioreactors R2 and R3 returned greater activity for all substrates at the elevated temperature indicating that the development of a truly low-temperature/psychrophilic microbial population did not occur within either biomass. This is in agreement with similar reports of LtAD applications (Rebac et al., 1999; Collins et al., 2003, 2005), who also agree that the development of a psychrophilic population is not essential for effective wastewater treatment at low-temperatures. SMA testing at various stages throughout the bioreactor trials recorded increased methanogenic activity for both experimental bioreactors (R2 and R3) from that observed at their operational temperature on day 0 (seed inoculum) against all substrates with the exception of propionate. Lettinga et al., (1999) found that propionate degradation is sensitive to low-temperature incubation. Propionate aside, SMA values indicated that the successful enrichment of both acetogenic and methanogenic communities had occurred under low-temperature conditions, this was particularly evident for the R2 biomass cultivated at 15°C, and is similar to the findings of Collins et al. (2006) and McKeown et al. (2009). This reaffirms the findings that a mesophilic seed inoculum can be successfully adapted to the conditions of LtAD. It is unknown if the increased low-temperature activity was due to the growth of new organisms or a response of the existing microbial consortia present in the seed inoculum; where according to Rebac et al. (1999) mesophilic consortia are able to grow and metabolise under reduced temperatures. SMA profiles for the AF media biofilm of the low-temperature
bioreactors indicated that this biomass was as active and important, with respect to COD removal, as that in the granular sludge section. This was not the case, to as great an extent, in the mesophilic R1 bioreactor.

Values obtained for R1 AF suggest distinct methanogenic niches had developed within the bioreactor; with a predominantly acetoclastic methanogenic community in the sludge bed and the AF biomass apparently dominated by hydrogenotrophic methanogens. This displays the capacity for an alternative community structure to develop within the AF, having essentially being seeded from up-flow of the granular sludge bed biomass.

2.5 Conclusions

The results presented in the current study demonstrate the potential for low-temperature anaerobic treatment of sewage. Despite the fluctuations in COD removal, the comparable performance between mesophilic and low-temperature operation suggest that improved performance is a matter for process optimisation, rather than a function of temperature limitation. This study also demonstrated that:

- LtAD of synthetic sewage is feasible at applied OLRs of 0.5-6 kg COD m\(^{-3}\) d\(^{-1}\) and HRTs of 1.5-12 h;
- mesophilic inocula can adapt to sub-optimal temperatures;
- The SMA profiles for the anaerobic filter sections of the low-temperature bioreactors indicated that the biomass was as active and important, with respect to COD removal, as that in the granular sludge section. This was not the case, to as great an extent, in the mesophilic reactor;
- The EGSB-AF style bioreactor configuration has potential for the treatment of municipal wastewater at both mesophilic and low-temperatures;
- Retention of bioreactor biomass may be an issue for full-scale low-temperature bioreactor operation; the use of inert support matrices and/or non-granular-based systems should be investigated;
The results presented in this study suggest that a modified bioreactor configuration, with an enhanced AF section allowing for greater biomass retention and biofilm formation could benefit the performance and efficiency of LtAD of municipal wastewater.
2.6 References


CHAPTER 3

HIGH-RATE LOW-TEMPERATURE ANAEROBIC TREATMENT OF SYNTHETIC SEWAGE IN EGSB-AF BIOREACTORS: REPRODUCIBILITY OF BIOPROCESS PERFORMANCE PHYSIOLOGICAL AND MOLECULAR CHARACTERISTICS
Abstract

The results presented in chapter 2 indicated that the direct anaerobic treatment of synthetic sewage is feasible under low-temperature conditions. This chapter presents the results of a trial to investigate the use of an alternative anaerobic filter fixed-film matrix material; granulated pumice stone, to enhance process efficiency and stability. In addition the reproducibility of low-temperature anaerobic biological wastewater treatment trials was evaluated. Two identical expanded granular sludge bed-anaerobic filter hybrid bioreactors (R4 and R5) were used to treat synthetic sewage wastewater at 12°C to investigate the effect of operational changes and influent on bioreactor performance and microbial community composition, which were assessed by chemical oxygen demand (COD) removal, effluent volatile fatty acid determination and clone library analysis, respectively. Methanogenic activity and community dynamics were monitored using specific methanogenic activity and quantitative polymerase chain reaction (PCR) assays. Bioreactor performance and microbial community dynamics were each well replicated between bioreactors R4 and R5. Successful treatment by both bioreactors was achieved at organic loading rates of 0.5-6 kg COD m\(^{-3}\) d\(^{-1}\), and hydraulic retention times of 2-24 h. Bacterial communities, as deduced from clone library analysis at the conclusion of the trial, were phylogenetically diverse and dynamic, consisting of key metabolically diverse fermentative and hydrolytic bacterial populations. Archaeal methanogenic dynamics indicated that acetoclastic methanogenic activity directly correlated to bioreactor performance.
3.1 Introduction

Anaerobic digestion (AD) is a well-established and attractive treatment approach for a wide variety of waste and wastewater streams (Sekiguchi et al., 2001) and is now widely recognised as the core technology for the sustainable management of waste (Zeeman and Lettinga, 1999; Hammes et al., 2000). In the last two decades, the advancement of anaerobic treatment technology has resulted in the successful harnessing of this technology for the treatment of a wide variety of wastewater streams, mostly consisting of soluble and easily degradable organic substances, and typically at high concentrations (Uemura and Harada, 1999). The successful utilisation of AD for wastewater treatment can be largely attributed to the development of existing bioreactor designs and new and innovative designs, i.e., anaerobic fluidized and fixed bed bioreactors such as upflow anaerobic sludge bed (UASB), upflow anaerobic hybrid reactors (UAHR), and expanded granular sludge bed (EGSB) bioreactor designs. The next challenge in the application of AD treatment is to assess the capability of the technology to treat waste streams which consist of material which is not as readily degradable or soluble as previously treated influents. These types of wastewater streams include lower-strength wastewaters such as municipal wastewater; sewage and domestic wastewater (van der Last and Lettinga, 1992). The treatment of municipal wastewater in developed countries is currently characterised by a disposal based linear system, incorporating aerobic microbiological treatment as the core technology. The consequences of utilising aerobic systems such as activated sludge plants is a large capital expenditure during installation, and operation through the use of large amounts of fossil fuels. In addition the generation of large quantities of secondary sludge requiring treatment before re-use/re-cycle, demonstrate the need for a radical shift in the way society and the established sanitary wastewater-engineering world looks at the treatment of municipal wastewater treatment. The advent of a sustainable system for the treatment of such waste would constitute a significant advance in environmentally sustainable engineering. This concept of sustainability could be facilitated through a shift from aerobic to anaerobic microbiology as the core technology for the treatment of municipal wastewater (O’Flaherty et al.,
However the anaerobic treatment of municipal wastewater/sewage is a real challenge because sewage belongs to the ‘complex’ wastewater category. The reasons for this include: (i) it contains a high fraction of particulate COD, (ii) the biodegradability of the typical COD fractions is moderate, (iii) it's a relatively low-strength wastewater, but concentrations can vary, (iv) the sewage can be discharged at relatively low-temperature (Lettinga et al., 2001). To date, the vast majority of full-scale applications of anaerobic treatment have been restricted to concentrated wastewater streams at temperatures exceeding 18°C. Therefore, while anaerobic treatment of sewage has been exploited at full scale in several tropical countries (e.g. Brazil, Mexico, Columbia, Uruguay; van Haandel and Lettinga 1994), where the ambient temperatures are sufficiently high enough to assist the process, anaerobic treatment of sewage is so far not applied at full-scale in countries with lower ambient temperatures (Elmwitalli et al., 2001).

In addition to process considerations relatively little is known regarding the structure, function and biological properties of the microbial communities involved in the AD process (McHugh et al., 2004). Because of the limitations of traditional culture-dependent methods, it has not been possible to identify and characterise most of the microorganisms in complex anaerobic communities (Wagner & Loy, 2002). However, the emergence in recent years of molecular microbiological techniques has revealed new levels of diversity within the microbial populations present in anaerobic bioreactors (Sekiguchi et al., 1998; Collins, 2003; McHugh, 2003). Microbial communities are in general complex assemblages of populations with diverse phylogenies and physiologies. Previous studies have focussed on investigating the community structure of methanogens because they are directly responsible for methane production (Yu et al., 2005). However, the overall process should be viewed in light of the behaviour of the methanogens, acetogens and acidogens thus providing new insights to the microbial consortia underpinning AD bioreactors.

The principal hypothesis being tested was that long term, low-temperature (12°C), anaerobic treatment of synthetic sewage could be successfully achieved, with improved operational performance and stability to that achieved using the conventional anaerobic hybrid bioreactors.
employed for the trials described in Chapter 2, by employing an improved novel anaerobic filter (AF) section. In addition this chapter describes the replicated high-rate, anaerobic biological treatment of synthetic sewage wastewater at low-temperatures by two identical hybrid EGSB–AF bioreactors. The response of the digesters to the application of various operational parameters was examined in terms of bioreactor performance and physiological characteristics. In addition the hydrolytic capacity of the sewage-degrading consortia was investigated by incubation batch assays. Molecular ecology techniques were also applied through the use of clone library and real-time PCR analysis in order to monitor the dynamics of the archaeal and bacterial populations of both systems. The duplicate nature of the experiment attempts demonstrably to replicate low-temperature AD (LtAD) trials. The schedule of analysis carried out on seed inoculum and bioreactor biomass is outlined in Table 3.2.

3.2 Materials and Methods

3.2.1 Source of Biomass
Anaerobic sludge granules were obtained from a full-scale, mesophilic (37°C), internal circulation (IC) bioreactor at Carbery Milk Products (Ballineen, County Cork, Ireland) used to treat ethanol production wastewater. The volatile suspended solids (VSS) concentration of the granules was 94 g VSS l⁻¹. The anaerobic granules (Ø c. 1-3 mm) were of a regular, spherical shape and a grey-green colour.

3.2.2 Bioreactor Design and Operation
Two identical glass laboratory-scale expanded granular sludge bed-anaerobic filter (EGSB-AF) hybrid bioreactors (active liquid volume, 2.9 l) similar to that previously described by McHugh et al. (2004) were used (Fig. 3.1). The upper ‘fixed-film’ anaerobic filter (AF) section consisted of granulated pumice stone (Ø c. 1-4 mm) tightly packed in a low density polyethylene (LDPE) cylinder (Fig. 3.2). The lower ‘sludge bed’ section of the bioreactors (R4 and R5) were inoculated with 20 g VSS l⁻¹ (58 g VSS; .62 l sludge) of the granular seed biomass. The bioreactors were operated at 12°C and
employed to treat a synthetic sewage wastewater (SYNTHES; Aiyuk and Verstraete, 2004) at an influent strength of 500 mg l\(^{-1}\) total COD over 282 days. A superficial upflow velocity of 2.5 m h\(^{-1}\) was applied to the bioreactors for the duration of the trial, by continuous recirculation of effluent through the bioreactors. The trial was divided into six operational phases, Phase 1-6, which were characterised by changes applied to hydraulic retention time (HRT) and consequent changes in organic loading rate (OLR). Phase conditions are outlined in Table 3.1.

**Table 3.1** Operational conditions and parameters applied to bioreactors R4 and R5 during operation.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
<th>1 (0-34)</th>
<th>2 (35-120)</th>
<th>3 (121-162)</th>
<th>4 (163-226)</th>
<th>5 (227-267)</th>
<th>6 (268-282)</th>
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<tbody>
<tr>
<td>T (^{a})</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>I COD(_{\text{tot}}) (^{b})</td>
<td>496.5 ±18.2</td>
<td>503.9 ±25.2</td>
<td>526 ±51.9</td>
<td>512.2 ±30</td>
<td>496.1 ±18.1</td>
<td>515.5 ±22.4</td>
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</tr>
<tr>
<td>I COD(_{\text{sol}}) (^{c})</td>
<td>293.2 ±11.9</td>
<td>280 ±22.6</td>
<td>302.1 ±26.3</td>
<td>281.6 ±31.3</td>
<td>273.5 ±33.6</td>
<td>286.4 ±23.9</td>
<td></td>
</tr>
<tr>
<td>HRT (^{d})</td>
<td>24</td>
<td>12</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>OLR (^{e})</td>
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<td>1</td>
<td>1.5</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>VLR (^{f})</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>SLR (^{g})</td>
<td>0.025</td>
<td>0.05</td>
<td>0.075</td>
<td>0.10</td>
<td>0.15</td>
<td>0.30</td>
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</tr>
<tr>
<td>SLR (^{h})</td>
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<td>0.1</td>
<td>0.15</td>
<td>0.2</td>
<td>0.3</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>UV (^{i})</td>
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<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Temperature (°C); \(^{b}\)Influent chemical oxygen demand, total (mg l\(^{-1}\)); \(^{c}\)Influent chemical oxygen demand, soluble (mg l\(^{-1}\)); \(^{d}\)Hydraulic retention time (hrs); \(^{e}\)Organic loading rate (kg COD m\(^{-3}\) d\(^{-1}\)); \(^{f}\)Volumetric loading rate (m\(^{3}\) Wastewater m\(^{-3}\) Reactor d\(^{-1}\)); \(^{g}\)Sludge loading rate (kg COD kg [VSS]\(^{-1}\) d\(^{-1}\)); \(^{h}\)Sludge loading rate (m\(^{3}\) Wastewater kg [VSS]\(^{-1}\) d\(^{-1}\)); \(^{i}\)Up-flow velocity (m h\(^{-1}\)); Values for \(^{b}\) and \(^{c}\) are the phase mean ± phase standard deviation; Values for \(^{g}\) and \(^{h}\) are calculated at an influent of 500 mg l\(^{-1}\) total COD.
Figure 3.1 EGSB-AF Bioreactors, R4 and R5 employed during the study.

Figure 3.2 Anaerobic filter units.

3.2.3 Effluent and Biogas Analyses
Samples of bioreactor effluent and biogas were taken thrice weekly for determination of total COD (COD_{tot}), soluble COD (COD_{sol}), pH and biogas determination according to Standard Methods (American Public Health Association (APHA) 1998). Volatile fatty acid (VFA) concentrations were determined by GC-MS (Varian, USA). Total phosphorus (expressed as PO_4^{3-} ) analysis of bioreactor influent and effluent were measured by colorimetric spectrophotometry (HACH DR4000), according to the HACH
molybdovanadate method with acid persulfate digestion (HACH Lange, USA). All samples were filtered through 0.45µm filter prior to PO₄³⁻ analysis.

3.2.4 Specific Methanogenic Activity Assays

The specific methanogenic activity (SMA) of biomass samples was obtained according to Colleran et al. (1992) and Coates et al. (1996). SMA values were determined against the direct methanogenic substrates acetate (30mM) and H₂/CO₂ (80:20 v/v), and the indirect substrates propionate (30mM), butyrate (15mM) and ethanol (30mM) as previously described by Collins et al. (2003). Briefly, biomass samples were washed in 1X PBS and were transferred to sterile 20-ml (soluble substrates) or 60-ml (gaseous substrates) hypovials to achieve a final biomass concentration of 2-5 g VSS l⁻¹ in a total volume of 10 ml anaerobic activity test medium. Vials without substrate, or with only pressurised N₂/CO₂ headspace, were used as controls. Assays were carried out at 12°C and 37°C.

3.2.5 Digestibility Assays

The digestibility of the bioreactor influent SYNTHES by the seed inoculum at the start of the trial (day 0) and bioreactor biomass on trial completion (day 282) was investigated by employing both SMA and batch assays. Digestibility assays – an adaptation of the SMA assay described above; biomass samples were washed 3 times in 1X PBS to remove any residual substrate or VFAs and transferred to sterile 20-ml hypovials to achieve a final biomass concentration of 2-5 g VSS l⁻¹ in a total volume of 9 ml anaerobic activity test medium, 1 ml of SYNTHES at 5000 mg l⁻¹ total COD was added in order to give an in-vial COD concentration of 500 mg l⁻¹. These assays were carried out at 12°C and 37°C. Concurrently vials (20-ml) were set up as above for the SMA assay with representative vials (x 3) of each biomass sacrificed along a series of time points (every 3 h over a 48 h period) for further analyses, in an attempt to elucidate the degradation of the trial influent; the hydrolysis of the high molecular weight constituents (i.e. carbohydrate and protein) of SYNTHES and to further demonstrate the adaptation of the bioreactor(s) biomass to the influent at the bioreactor operational temperature of 12°C. The sacrificed vials were analysed for total
carbohydrate (Dubois et al., 1956; Sims, 1978) and protein (Bradford, 1976) concentration, VFAs (GC-MS (Varian, USA)) present, and methane (Standard Methods (American Public Health Association (APHA) 1998)).

3.2.6 DNA Extraction
Biomass total genomic DNA was obtained using the MoBio Soil DNA Extraction kit (Cambio, Cambridge, UK), according to the manufacturer’s instructions. All granular sludge samples for molecular analysis were first mechanically crushed by grinding using a pestle and mortar and 0.5g of sludge granules was used for extractions. DNA was extracted in triplicate and pooled for each sample prior to analysis. Extracted DNA and downstream PCR reactions were electrophoresed on 1% (w/v) agarose gels in 1X TAE, containing 1 µg ethidium bromide ml⁻¹ (Maniatis et al., 1989), with visualization by UV excitation to determine DNA integrity or to check PCR amplicon size.

3.2.7 Generation of Clone Libraries, Amplified rDNA Restriction Analysis (ARDRA) and Phylogenetic Classification
Archaeal and bacterial clone libraries were generated through PCR amplification of 16S rRNA genes from total genomic DNA using the archaeal primers 21F (5′-TTCCGGTTGATCCYCGCGGA-3′) Stackebrandt & Goodfellow, 1991) and 958R (5′-YCCGGCGTTGAMTCCAATT-3′; DeLong, 1992) and the bacterial primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′) DeLong 1992) and 1392R (5′-ACGGGCGGTGTGTRC-3′) Lane et al., 1985). Amplicons were ligated into plasmid vector pCR 2.1-TOPO® XL (Invitrogen) and used to transform chemically competent Escherichia coli cells as described by McHugh et al. (2003b). Amplified rDNA restriction analysis (ARDRA) was performed, whereby clones were categorized into distinct operational taxonomic units (OTUs) based on restriction with the tetrameric restriction enzyme Hae III (McHugh et al., 2004). Unique clonal restriction patterns were identified, and representatives of these sequenced. The retrieved sequences were compared to previously identified sequences using the web-based Basic Local Alignment Search Tool, (BLASTn) (www.ncbi.nlm.nih.gov/BLAST) and the
Ribosomal Database Project (RDP) (Maidak et al., 2000) and aligned using the ARB database software programme’s (Ludwig et al., 2004) Fast Aligner Tool. Phylogenetic trees were constructed using the neighbor joining (Jukes–Cantor correction) (Saitou & Nei, 1987) algorithms implemented in ARB.

3.2.8 Accession Numbers
The partial 16S rRNA gene sequences determined in this study through clone library analysis were deposited in the GenBank database and assigned the following accession numbers: Archaea - FJ005019-FJ005025 (seed inoculum); HQ008058-HQ008069 (R4, day 282); HQ008070-HQ008077 (R5, day 282). Bacteria - GQ423770-GQ423785 (seed inoculum); HQ008078-HQ008110 (R4, day 282); HQ008111-HQ008145 (R5, day 282).

3.2.9 Quantitative Polymerase Chain Reaction Assays
Quantitative real-time PCR (qPCR) was carried out on biomass DNA employing four qPCR primer/probe sets, representing four target methanogenic groups, i.e., two hydrogenotrophic orders (Methanobacteriales (MBT) and Methanomicrobiales (MMB)) and two acetoclastic families (Methanosarcinaceae (Msc) and Methanosaetaceae (Mst)), described in detail by Yu et al. (2005) and Lee et al. (2009), were applied. The PCRs were performed using a LightCycler 480 (Roche, Germany). Each reaction mixture was prepared using the LightCycler 480 Probes Master kit (Roche): 10 µl LC 480 reaction mix 2X solution, 1 µl of each primer (final concentration, 500 nM), 2 µl of the probe (final concentration, 200 nM), 2 µl PCR-grade pure water and 5 µl template DNA (Lee et al., 2009). All DNA templates were analyzed in triplicate. Two-step amplification of the target DNA, combining the annealing and the extension steps, was performed applying the following conditions: an initial 10-min pre-denaturation step at 94°C for FastStart Taq DNA polymerase activation; 40 cycles of denaturation at 94°C for 10 s; and simultaneous annealing and extension at 60°C for 30 s. The annealing and extension for the MMB-specific set was performed at 63°C (Lee et al., 2009; Yu et al., 2005). Quantification standard curves were constructed as previously described (Yu et al., 2006) using the representative strains corresponding to each primer/probe sets used. The hydrogenotrophic
methanogen representative strains used were *Methanobacterium formicicum* M.o.H. (DSM 863) and *Methanobrevibacter arboriphilicus* DH1 (DSM 1536) for the MBT set, while * Methanospirillum hungatei* JF1 (DSM 864) and *Methanomicrobium mobile* BP (DSM 1539) were used for the MMB set. Acetoclastic methanogen representative strains used were *Methanosarcina acetivorans* C2A (DSM 2834) * Methanosarcina barkeri* MS (DSM 800) * Methanosarcina mazei* Go1 (DSM 3647) for the Msc set, with *Methanosaeta concilli* GP6 (DSM 3671) being used for the Mst set. A total of ten standard plasmids containing the full-length 16S rRNA gene sequences of the representative strains, were kindly provided by Prof. S. Hwang, POSTECH, Korea (Yu et al., 2005). The mass concentration of each standard plasmid was measured in duplicate using a Qubit system (Invitrogen) and converted to its copy concentration, as previously described by Yu et al. (2006). A 10-fold dilution series of $10^1$-$10^9$ copies $\mu l^{-1}$ was generated for each standard solution, which were then analysed in triplicate by qPCR with the corresponding primer- and probe-set (Lee et al., 2009). The resulting threshold cycle values ($C_T$) were plotted against the logarithm of their initial copy concentrations. Subsequently, the 16S rRNA gene copy concentrations of each target group were determined against the corresponding standard curves within the linear range ($r^2 > 0.995$).

**Table 3.2** Schedule of analysis and methods undertaken on the seed inoculum and bioreactor (R4 and R5) biomass.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Sampling day</th>
</tr>
</thead>
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<tr>
<td></td>
<td>0 (seed inoculum)</td>
</tr>
<tr>
<td>SMA</td>
<td>✓</td>
</tr>
<tr>
<td>Digestibility</td>
<td>✓</td>
</tr>
<tr>
<td>Clone Libraries</td>
<td>✓</td>
</tr>
<tr>
<td>qPCR</td>
<td>✓</td>
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</table>

### 3.3 Results

#### 3.3.1 Bioreactor Performance

Bioreactor performance during the low-temperature anaerobic treatment of the synthetic sewage representative wastewater (SYNTHEs) is summarised
in Table 3.3. Influent and effluent COD$_{\text{tot}+\text{sol}}$ concentrations, COD removal efficiencies (CODRE$_{\text{tot}+\text{sol}}$), biogas methane content (%), VFA concentrations and phosphorus (P) concentrations (expressed as PO$_4^{3-}$) are presented in Figures 3.3-3.7 respectively. Operational phase conditions and parameters are previously outlined in Table 3.1.

### Table 3.3 Performance characteristics of bioreactors R4 and R5 during operation.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Days</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<td></td>
<td></td>
<td>0-34</td>
<td></td>
<td>35-120</td>
<td></td>
<td>121-162</td>
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<td>I COD$_{\text{tot}}$</td>
<td>496.5 ±18.2</td>
<td>503.9 ±25.2</td>
<td>526 ±51.9</td>
<td>512.2 ±30</td>
<td>496.1 ±18.1</td>
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<td></td>
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<td>E COD$_{\text{tot}}$</td>
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<td>I COD$_{\text{sol}}$</td>
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<td>CH$_4$</td>
<td>R4</td>
<td>10 ±6.5</td>
<td>21.5 ±6.8</td>
<td>33.9 ±10.9</td>
<td>32.5 ±11.4</td>
<td>52.3 ±18.7</td>
<td>57.3 ±12.5</td>
</tr>
<tr>
<td></td>
<td>R5</td>
<td>8 ±9.4</td>
<td>27.8 ±5.3</td>
<td>23.3 ±11</td>
<td>26.7 ±8.3</td>
<td>57.3 ±12.4</td>
<td>58 ±9.3</td>
</tr>
</tbody>
</table>

Influent chemical oxygen demand, total (mg l$^{-1}$); Effluent chemical oxygen demand, total (mg l$^{-1}$); Total COD removal efficiency (%); Influent chemical oxygen demand, soluble (mg l$^{-1}$); Effluent chemical oxygen demand, soluble (mg l$^{-1}$); Soluble COD removal efficiency (%); Influent phosphorus (mg l$^{-1}$ PO$_4^{3-}$); Effluent phosphorus (mg l$^{-1}$ PO$_4^{3-}$); Phosphorus removal efficiency; Effluent VFA (mg l$^{-1}$ acetic acid); Methane in biogas (%);

All values are the phase mean ± phase standard deviation.

A rapid start-up was achieved by both bioreactors, with >80% CODRE$_{\text{tot}+\text{sol}}$ being achieved within 3 days of operation. Stable operation was observed for the remainder of the initial phase, with mean CODRE$_{\text{tot}+\text{sol}}$ of >80 and >70% respectively being recorded (Figs. 3.4a and b), biogas quality also improved.
as the phase progressed (>20%, Fig. 3.5). VFA analysis of bioreactor effluent detected acetic acid only, with concentrations remaining low (<5 mg l⁻¹, Fig. 3.6). Performance was comparable between both R4 and R5. The decrease in HRT to 12 h (OLR, 1 kg COD m⁻³ d⁻¹) associated with Phase 2 resulted in a slight decrease in effluent quality with R5 showing a greater adverse response. This decrease in effluent quality was however transient and by the completion of Phase 2 effluent quality had recovered to the levels observed during Phase 1 (<100 mg l⁻¹ COD_{tot+sol}, Figs 3.3a and b). R4 performance was marginally better than R5, with the exception of biogas quality. Once again effluent VFA concentrations (acetic acid) remained low for both bioreactors. Phase 3 involved a decrease in HRT to 8 h and subsequent increase in OLR to 1.5 kg COD m⁻³ d⁻¹, this alteration resulted in decreased CODREs for both bioreactors, this decrease was not as pronounced for R4, R5 CODRE_{tot} dropped below 70% (day 129). The following 10 days of operation saw a recovery in performance with R4 and R5 both recording >85% CODRE_{tot}. However once it had appeared R4 and R5 had stabilised, both exhibited a sharp decline in performance (60-70% CODRE_{tot}) which persisted for the final 7 days of Phase 3. R4 fared better than R5 in terms of both COD_{tot} and COD_{sol} removal. Fluctuations in effluent acetic acid concentrations were observed for R5 throughout Phase 3. Despite these VFA and COD fluctuations mean CODRE_{tot+sol} was >70% for both bioreactors. The introduction of Phase 4 (HRT 6 h, OLR, 2 kg COD m⁻³ d⁻¹) had no adverse effect on the performance of R4 and R5 and within 30 days of the phase beginning improved and stable operation was witnessed with 90% COD_{tot} and >80% COD_{sol} removal being recorded for both bioreactors. The decrease in performance which marked the completion of Phase 3 was similarly exhibited towards the completion of Phase 4 with the last 12 days of phase operation returning performance similar to that observed during the first stage of Phase 4, however on this occasion R4 and R5 displayed similar COD removal characteristics, and also effluent acetic acid concentrations. Phase 5 saw a further decrease in HRT to 4 hrs (OLR, 3 kg COD m⁻³ d⁻¹), the applied HRT reduction having no effect on CODRE_{tot} 65-75%, CODRE_{sol} was somewhat more erratic (50-75%). With the exception of some instability during days 254-259, performance during Phase 5 was steady, with mean CODRE_{tot+sol}
of >71 and 65% respectively. Effluent acetic acid concentrations displayed more consistency during Phase 5 (mean <6 mg l\(^{-1}\)) with R4 and R5 returning comparable concentrations, simultaneously biogas quality improved during this phase with >70% biogas methane concentration being routinely achieved.

There was no effect on the performance of R4 and R5 observed upon the applied HRT reduction (Phase 6) on day 268 to 2 h (OLR, 6 kg COD m\(^{-3}\) d\(^{-1}\)) from that recorded on completion of Phase 5, indeed mean CODREs were greater than those recorded for Phase 5, CODRE\(_{\text{tot}}\) averaging 75% for both bioreactors. Mean acetic acid concentrations and biogas methane content improved during Phase 6 from those observed during Phase 5. The comparable performance between R4 and R5 in terms of effluent COD concentrations, CODREs and biogas quality recorded towards the completion of Phase 4 was also a feature of Phases 5 and 6.

Phosphorus (PO\(_4^{3-}\)) analysis of R4 and R5 bioreactor effluents was carried out throughout the trial (Fig. 3.7). Values obtained suggested the occurrence of anaerobic phosphorus uptake/accumulation by the bioreactor biomass or an alternative mechanism for P attenuation in the bioreactors. Mean phosphate removal reached 60-70% during Phases 2-5 (Table 3.3), while greater removal observed for R5 during Phase 6 when OLR was highest. This suggests a biological, rather than a purely physical basis for phosphorus attenuation in the system. This finding is clearly significant and represents a further potential advantage of the LtAD approach for sewage treatment.
Figure 3.3 Chemical oxygen demand concentration; (a) total and (b) soluble; influent (▲), R4 effluent (■) and R5 effluent (○).
Figure 3.4 Chemical oxygen demand removal efficiency; (a) total and (b) soluble; R4 (■) and R5 (○).
Figure 3.5 Biogas methane concentration; R4 (■) and R5 (○).

Figure 3.6 Effluent VFA (acetic acid only detected) concentration; R4 (■) and R5 (○).
3.3.2 Physiological Characterisation of Biomass

The values obtained from specific methanogenic activity testing of the seed inoculum (day 0), and bioreactors (R4 and R5) biomass on days 120 and 282 at 12°C and 37°C are outlined in Table 3.4

3.3.2.1 Seed Inoculum Methanogenic Activity (day 0)

SMA values obtained from the mesophilic seed biomass displayed reduced activity against all substrates when tested at 12°C than those recorded at 37°C (Table 3.4). Decreases in methanogenic activity against propionate and butyrate at 12°C were less pronounced than for the other substrates tested. Values obtained against the direct methanogenic substrates acetate and H₂/CO₂ at 37°C suggested the dominance of acetoclastic methanogens, this was in contrast with the values obtained at 12°C which returned greater activity against H₂/CO₂.

Figure 3.7 Total phosphorus concentration; influent (▲), R4 effluent (■) and R5 effluent (○).
Table 3.4 Specific methanogenic activity (ml CH₄ g [VSS]⁻¹ d⁻¹) of seed inoculum and bioreactor biomass.

<table>
<thead>
<tr>
<th>Day</th>
<th>Biomass</th>
<th>Test temp. (°C)</th>
<th>Ethanol</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>H₂/CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Seed</td>
<td>12°C</td>
<td>1.9 ±0.1</td>
<td>2.1 ±0.2</td>
<td>1.3 ±0.2</td>
<td>2 ±0.3</td>
<td>4.5 ±0.1</td>
</tr>
<tr>
<td></td>
<td>Inoculum</td>
<td>37°C</td>
<td>7.1 ±1.1</td>
<td>19.4 ±2.2</td>
<td>1.8 ±0.6</td>
<td>3.2 ±1</td>
<td>11.1 ±0.5</td>
</tr>
<tr>
<td>120</td>
<td>R4 GSB</td>
<td>12°C</td>
<td>6.5 ±0.6</td>
<td>10.2 ±1.3</td>
<td>1.2 ±0.1</td>
<td>2.3 ±0.1</td>
<td>10.9 ±0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>28.6 ±3.4</td>
<td>96.2 ±5.2</td>
<td>7.3 ±2.1</td>
<td>14.1 ±3.2</td>
<td>48.6 ±7.2</td>
</tr>
<tr>
<td>120</td>
<td>R5 GSB</td>
<td>12°C</td>
<td>9 ±0.7</td>
<td>4.7 ±0.5</td>
<td>1.9 ±0.3</td>
<td>3.3 ±1.4</td>
<td>20.5 ±2.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>43.6 ±12.1</td>
<td>51.6 ±3.8</td>
<td>8.3 ±2.7</td>
<td>19.8 ±4.3</td>
<td>109.7 ±6.9</td>
</tr>
<tr>
<td>282</td>
<td>R4 GSB</td>
<td>12°C</td>
<td>24.7 ±2.9</td>
<td>26.3 ±0.5</td>
<td>2.7 ±0.3</td>
<td>3.9 ±0.6</td>
<td>17.6 ±1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>181.2 ±3.2</td>
<td>247.2 ±6.8</td>
<td>16.1 ±3.7</td>
<td>31.6 ±3</td>
<td>163.4 ±25.6</td>
</tr>
<tr>
<td>282</td>
<td>R5 GSB</td>
<td>12°C</td>
<td>20.1 ±1.4</td>
<td>28.3 ±0.3</td>
<td>3 ±0.3</td>
<td>3.7 ±0.5</td>
<td>15.4 ±1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>143.9 ±4.1</td>
<td>278.8 ±17.5</td>
<td>14 ±1.7</td>
<td>22.9 ±1.7</td>
<td>118.7 ±27.6</td>
</tr>
</tbody>
</table>

GSB – granular sludge bed biomass; All values are the mean of triplicates ± standard deviation.

3.3.2.2 R4 and R5 Methanogenic Activity (day 120)

Biomass from both R4 and R5 on day 120 exhibited increased methanogenic activity at both 12°C and 37°C against all substrates from values obtained on the seed inoculum (day 0, Table 3.4). The mesophilic nature of the biomass was maintained with greater activity at 37°C being recorded for all substrates. Bioreactor biomass from R4 and R5 tested at their operational temperature of 12°C against the higher carbon intermediates; propionate and butyrate displayed similar activity, activity against ethanol was greater for the R5 biomass, values obtained at 37°C recorded a similar trend against those substrates. However distinct differences were observed between the R4 and R5 biomass for acetate and H₂/CO₂ at both 12°C and 37°C. R4 biomass tested at 12°C displayed a balanced methanogenic community, while at 37°C acetoclastic methanogens were dominant. Testing of the R5 biomass indicated that hydrogenotrophic methanogens were the foremost methane producers, with greater activity at 12°C and 37°C (4-fold and 2-fold respectively) being recorded against H₂/CO₂.

3.3.2.3 R4 and R5 Methanogenic Activity (day 282)

By the completion of the trial, once again increased activity was recorded for the R4 and R5 biomass against all substrates at 12°C and 37°C. The hydrogenotrophic methanogenic dominance observed in the R5 biomass on
day 120 was reversed with the action of acetoclastic methanogens now appearing to be the preferred route for methanogenesis in both bioreactors. In contrast to the previous test day, values obtained for the R4 and R5 biomass were comparable for all substrates at both 12°C and 37°C, suggesting similar communities at work in both bioreactors. Greater overall activity of the bioreactor biomass at 12°C from that recorded on the seed inoculum at 12°C for ethanol (>10-fold) and in particular for the direct methanogenic precursors acetate and H₂/CO₂ (>12-fold and >3-fold, respectively, Table 3.4) suggest the successful enrichment of syntrophic and methanogenic populations under low-temperature conditions.

### 3.3.3 Biomass Adaptability and Substrate Digestibility

SMA values obtained from the seed inoculum (day 0) and on trial completion (day 282) against the trial influent, SYNTHES revealed greater activity at 37°C (Table 3.5, Fig. 3.8b). Adaptation of the seed biomass to the trial influent was verified on day 282 with a >8-fold increase in activity at bioreactor operational temperature (12°C) being recorded with both bioreactors R4 and R5 returning similar values against the trial influent (Table 3.5, Fig. 3.8a).

#### Table 3.5 Specific methanogenic activity (ml CH₄ g [VSS]⁻¹ d⁻¹) of seed inoculum and bioreactor biomass against the trial influent; SYNTHES.

<table>
<thead>
<tr>
<th>Day</th>
<th>Biomass</th>
<th>Test temp. (°C)</th>
<th>SYNTHES: 500 mg l⁻¹ COD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Seed</td>
<td>12°C</td>
<td>1.1 ±0.1</td>
</tr>
<tr>
<td></td>
<td>Inoculum</td>
<td>37°C</td>
<td>3.5 ±0.7</td>
</tr>
<tr>
<td>282</td>
<td>R4 GSB</td>
<td>12°C</td>
<td>8.7 ±1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>34.1 ±3.6</td>
</tr>
<tr>
<td>282</td>
<td>R5 GSB</td>
<td>12°C</td>
<td>9.1 ±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37°C</td>
<td>35.6 ±2.8</td>
</tr>
</tbody>
</table>

GSB - granular sludge bed biomass; COD - chemical oxygen demand, total;
All values are the mean of triplicates ± standard deviation.
Batch incubations of bioreactor biomass samples (R4 and R5, day 282) with SYNTHES demonstrated that hydrolytic and subsequent methanogenic conversion of SYNTHES occurred within the experimental time frame, however this was not so for the seed inoculum incubations which recorded only miniscule (0.3% CH₄) amounts of methane in the biogas by completion of the experiment (48 h) compared to 5.5% CH₄ for bioreactors (R4 and R5) biomass incubations (Fig. 3.9c). The bioreactor biomass displayed methane formation within the first time point (3 h), indicating substrate conversion. A contrast in the rate of hydrolytic breakdown of the high molecular-weight polymers protein and carbohydrate present in SYNTHES was also evident between the seed inoculum and bioreactor biomass (Figs. 3.9a and b). After 3 h protein concentration of the test vials with bioreactor biomass had decreased approximately 10%, while the seed biomass recorded just a 3% decrease in protein from those concentrations recorded at T=0. By completion of the incubations, mean protein concentration in vials containing the R4 and R5 biomass was 22% lower than that from seed inoculum incubations. Total
carbohydrate profiles displayed a similar trend, the seed inoculum incubations returning transient increases and decreases in relative concentrations of carbohydrate. The fluctuations in relative concentrations of both total protein and carbohydrates apparent during incubations may be as a result of the breakdown of the more complex polymers present in SYNTHES to smaller proteins and monomeric sugars, which were not differentiated by the assays applied; this feature was more evident with seed inoculum incubations. Profiles of detected VFAs varied; contrasting not only between the seed inoculum incubations and the bioreactor biomass incubations but also between the R4 and R5 biomass incubations (Figs. 3.9a-c). Acetic acid concentrations of vials containing seed inoculum biomass varied during the incubations due to acetate production and subsequent utilisation, overall concentrations were higher than those recorded at T=0. Incubations of bioreactor biomass displayed similar trends in terms of VFAs detected and utilised for R4 and R5, however at different rates. While rates of protein and carbohydrate breakdown were similar for both the R4 and R5 biomass, the recorded values and profiles of VFAs particularly within the first 24 h of incubation suggest different communities, rates of hydrolysis and metabolic pathways may be occurring within the two bioreactors. From 24 h both the R4 and R5 incubations displayed similar results for all VFAs detected.
Figure 3.9 Temporal concentrations of detectable (a) total protein; (b) total carbohydrate and (c) biogas methane concentration (%) of seed inoculum (▲), R4 (■) and R5 (○) with (d), (e) and (f) displaying detectable VFAs for seed inoculum, R4 and R5 respectively; acetic acid (♦), propionate (··●··), butyrate (×) and valeric acid (+) within batch incubations containing SYNTHES at 500 mg l⁻¹ COD.
3.3.4 Clone Library Analysis

3.3.4.1 Archaeal 16S rRNA Clone Library Analysis

ARDRA identified 27 different operational taxonomic units (OTUs) from archael clone libraries; 7 OTUs from the seed inoculum, 12 OTUs from bioreactor R4 and 8 OTUs from bioreactor R5 on day 282 (Fig 3.10 and 3.11).

‘Seqmatch’ analysis indicated the seed inoculum archaeal community could be divided into two distinct clusters, namely the *Euryarchaeota* and *Crenarchaeota*. *Euryarchaeotal* clones consisted of two orders *Methanosarcinales* (families; *Methanosaetaceae, Methanosarcinaceae*) and *Methanobacterbiales* (family; *Methanobacteriaceae*) (Figs. 3.10 and 3.11a). *Methanosaeta*-like organisms appeared to dominate the archaeal community of the seed inoculum (72% of clones), this also appeared to be the case for the bioreactor R4 and R5 biomass (73% and 70% respectively). *Methanobacteriales*-like clones (hydrogenotrophic methanogens) showed an increase in relative abundance for both R4 (20%) and R5 (18%) from that observed in the seed inoculum (12%) (Figs. 3.10, 3.11b and c). *Crenarchaeota*-like clones identified were highly similar to members of the Group 1.3 division of the *Crenarchaeota*, or Group 1.3b as proposed by Ochsenreiter et al. (2001). *Crenarchaeota* comprised one OTU in the seed sludge (clone FJ005022), representing 16%. Clonal analysis of the R4 biomass indicated a decrease in the relative abundance of *Crenarchaeota* like clones representing just 5% of the library; while this non-methanogenic *Archaea* persisted to a greater extend in the R5 biomass displaying only a slight decrease at 12% from that recorded in the seed inoculum.
Figure 3.10 Phylogeny of archaeal 16S rRNA gene sequences obtained from the seed inoculum (Seed) and bioreactor (R4 and R5) clone libraries calculated using the Maximum Likelihood algorithm (Felsenstein, 1981). GenBank accession numbers of the reference and clonal sequences from this study are indicated and the relative abundance (%) of each OTU is shown in parentheses.
Figure 3.11 Comparative distribution of archaeal OTUs retrieved from (a) seed sludge (day 0); (a) bioreactor biomass R4 and (b) bioreactor biomass R5 (day 282).

3.3.4.2 Bacterial 16S rRNA Clone Library Analysis
ARDRA identified 27 different operational taxonomic units (OTUs) from bacterial clone libraries; 16 OTUs from the seed inoculum, 33 OTUs from bioreactor R4 and 35 OTUs from bioreactor R5 on day 282 (Figs. 3.12 and 3.13).

The bacterial clone library of the seed inoculum consisted of sequences related to the Bacteroidetes (33%), Firmicutes (27%), Chloroflexi (18%), Actinobacteria (11%), δ-proteobacteria (8%) and Spirochaetes (3%) groups (Fig. 3.12 and 3.13a). The dominant group consisting of Bacteroidetes-like clones was divided into two family subgroups, viz. Porphyromonadaceae and Bacteroidetes. The phylum Firmicutes was represented by sequences related to the obligate anaerobes Clostridia, order Clostridiales. By day 282 the bacterial communities of the R4 and R5 biomass as identified by clone library analysis indicated a much greater diversity of phylogenetic groups from that observed in the seed inoculum (Figs. 3.12, 3.13b and c). While the groups identified in the seed inoculum remained, other groups emerged. Amongst the groups to emerge were representatives of the novel candidate divisions TM7,
Termite Group 1 and OP11, other groups to emerge were the Plantomycetes, Synergistetes and Acidobacteria. With the exception of the Bacteroidetes and Spirochaetes whose relative abundance increased, the remaining groups which were present in the seed inoculum displayed significant decreases in relative abundance in the bioreactor biomass samples, with Actinobacteria-like clones being absent from the R5 biomass. The most marked decrease occurred in the abundance of Firmicutes-like clones, representing just 7% and 8% of the R4 and R5 libraries respectively. The Chloroflexi also showed dramatic decreases. By day 282 the Bacteroidetes remained the dominant group closely followed by the TM7 which emerged as a significant group within the bacterial community of both R4 (29%) and R5 (20%). The Chloroflexi persisted to a greater extent in the R4 biomass, while the Spirochaetes represented a significant group (12%) in the R5 bacterial community. Distinct groups were identified in the R5 biomass which did not arise in the R4 clone library, namely the OP11, Planctomycetes, Synergistetes and Acidobacteria. Overall, the majority of sequences retrieved from clone library analyses of the seed inoculum, R4 and R5 bioreactors were related to uncultured bacteria from diverse anaerobic habitats.
Figure 3.12 (a) Phylogeny of bacterial 16S rRNA gene sequences obtained from the seed inoculum (Seed) and bioreactor (R4) clone libraries calculated using the Maximum Likelihood algorithm (Felsenstein, 1981). GenBank accession numbers of the reference and clonal sequences from this study are indicated and the relative abundance (%) of each OTU is shown in parentheses.
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Figure 3.12 (b) Phylogeny of bacterial 16S rRNA gene sequences obtained from the seed inoculum (Seed) and bioreactor (R5) clone libraries calculated using the Maximum Likelihood algorithm (Felsenstein, 1981). *GenBank* accession numbers of the reference and clonal sequences from this study are indicated and the relative abundance (%) of each OTU is shown in parentheses.
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(a) Firmicutes
- Accession No. % Lineage
  - GQ423771 4 Ruminococcaceae
  - GQ423774 12 Lachnospiraceae
  - GQ423776 7 Eubacteriaceae
  - GQ423782 4 Ruminococcaceae

- Chloroflexi
  - Accession No. % Lineage
    - GQ423777 2 Anaerolineaceae
    - GQ423781 4 Anaerolineaceae
    - GQ423783 9 unclassified
    - GQ423785 3 unclassified

- Actinobacteria
  - Accession No. % Lineage
    - GQ423773 4 Propionibacterales
    - GQ423780 4 unclassified Actinobacteria

- Bacteroidetes
  - Accession No. % Lineage
    - GQ423770 11 unclassified Bacteroidetes
    - GQ423777 16 unclassified Bacteroidetes

(b) Firmicutes
- Accession No. % Lineage
  - HQ008097 1 unclassified Clostridiales
  - HQ008104 1 unclassified Spirochaetes

- Chloroflexi
  - Accession No. % Lineage
    - HQ008103 1 unclassified

- Actinobacteria
  - Accession No. % Lineage
    - HQ008078 3% Conexibacteraceae

- Bacteroidetes
  - Accession No. % Lineage
    - HQ008091 1 Prolixibacter
    - HQ008092 2 unclassified Spirochaetes

- δ-Proteobacteria
  - Accession No. % Lineage
    - HQ008080 1 Polyangiaceae
    - HQ008083 4 TM7 genera incertae sedis

- Spirochaetes
  - Accession No. % Lineage
    - HQ008089 4 Spirochaetaceae
    - HQ008092 2 unclassified Spirochaetes

- Termite Group 1, 2%
  - Accession No. % Lineage
    - HQ008100 1 uncultured Termite Group 1

- Bacteroidales
  - Accession No. % Lineage
    - HQ008087 11 Bacteroidetes
**Figure 3.13** Comparative distribution of bacterial OTUs retrieved from (a) seed sludge (day 0); (a) bioreactor biomass R4 and (b) bioreactor biomass R5 (day 282).

### 3.3.7 Quantitative Polymerase Chain Reaction Assays

16S rRNA genes of the acetoclastic family *Methanosetaeaceae* were dominant in the seed inoculum at $2.94 \times 10^9$ copies g [VSS]$^{-1}$ and indeed in all samples with the exception of R5 on day 226 which showed that *Methanobacteriales* genes were in the ascendancy (Fig. 3.14). Fluctuations in *Methanosetaeaceae* gene copy numbers were observed in the temporal samples, with greater concentrations being recorded in R4 than in R5. Gene copy numbers for the other acetoclastic methanogen probe Msc (*Methanosarcinaceae*) were below the detectable limit. The hydrogenotrophic methanogen probes indicated that *Methanobacteriales* was dominant over *Methanomicrobiales* in the seed inoculum and on day 226 in both bioreactors. Interestingly hydrogenotrophic methanogens were not detected on days 120 in both bioreactors and only *Methanobacteriales* was detected in R4 on day 162. However on day 226 both
Methanobacteriales and Methanomicrobiales genes were detected in both bioreactors with Methanobacteriales being the dominant methanogen in R5 at 3.11 x 10^9 copies g [VSS]^{-1}.

![Temporal 16S rRNA gene copy numbers for Methanobacteriales, Methanomicrobiales, and Methanosaetaceae from bioreactors R4 and R5. Methansarcinaceae 16S rRNA gene copy numbers were below the detection limit for all samples.](image)

**Figure 3.14** Temporal 16S rRNA gene copy numbers for Methanobacteriales (■), Methanomicrobiales ( ), and Methanosaetaceae ( ) from bioreactors R4 and R5. Methansarcinaceae 16S rRNA gene copy numbers were below the detection limit for all samples.

### 3.4 Discussion

#### 3.4.1 Bioreactor Performance

The successful long-term direct treatment of synthetic sewage at applied OLRs of 0.5-6 kg COD m^{-3} day^{-1} was demonstrated at 12°C. Mean CODRE_{tot+sol} of >75% and >70% was achieved in both systems during the 282 day trial. The rapid start-up suggests that the mesophilic biomass adapted quickly to the reduced operating temperature. Therefore, while psychrophilically-cultivated biomass is not readily available, it is clear that mesophilic seed inoculum can be successfully used for the start-up of a LtAD system; this is in agreement with previous findings (Enright *et al.*, 2005, McKeown *et al.*, 2009).
A very short start-up period was observed for each of the bioreactors at the applied OLR of 0.5 kg COD m$^{-3}$ d$^{-1}$ (PI), with COD$_{tot}$ and COD$_{sol}$ removal efficiencies of c. 80% achieved by day 5 (Fig. 3.4). This start-up regime compared favourably to that achieved during the first trials (Chapter 2) and also to literature guidelines for conventional mesophilic AD (McHugh et al. 2003). The results compare well with the results obtained by Aiyuk and Verstraete, (2004), who achieved an average of 84% COD removal while treating the same influent (SYNTHES) at 33°C. The results obtained also compare favourably with previous LtAD of sewage trials, based on the EGSB design (de Man et al., 1986; van der Last and Lettinga, 1992; Wang, 1994; Syutsubo et al., 2008; Chunjuan et al., 2009).

The ability of both bioreactors to withstand and adapt to changes in operational conditions demonstrates the robustness of the systems with respect to changes in OLR, which is an important consideration for full-scale applications, given the variable characteristics of sewage. Despite the stepwise reductions in HRT and the consequent increases in OLR, a stable performance was observed throughout the six operational phases in terms of effluent quality. Langenhoff and Stuckey (2000) have suggested that a surplus of active biomass in anaerobic bioreactors, such as was the case in this study, likely results in greater stability when environmental perturbations such as increased organic load are applied. The stability and reproducibility of LtAD coupled with the systems response to operational changes are critical considerations for the full-scale implementation of this technology.

The hydrolytic pathways within LtAD bioreactors remain poorly understood, especially in the novel EGSB-AF systems utilised in this study. Generally, hydrolysis is considered to be the rate-limiting step in the AD of wastewaters with particulate organic material (Noike et al., 1985; Veeken & Hamelers, 1999). It has been reported when using the UASB to treat raw domestic sewage, an important fraction of excess sludge arises from inception in the sludge bed of poorly degradable solids found in the influent (van Haandel and Lettinga, 1994). The accumulation and slow/moderate hydrolysis of this particulate material has been reported to severely affect bioreactor performance resulting in reduced SMA of the biomass (Zeeman et al., 1997; Mahmoud et al., 2008). Pavlostatis and Giraldo-Gomez (1991)
noted that at low-temperatures in particular the rate of solids conversion to biogas, in most cases, is limited by the rate of hydrolysis. During the 282 days of operation, this did not appear to be an issue. The hydrolytic capabilities of the bacterial populations present in the sewage-degrading consortium were evident through the batch tests carried out on day 282 (Fig. 3.9). In addition, SMA analysis of the bioreactor biomass against key intermediate substrates improved as the trial progressed (Table 3.4), and activity against the trial influent, showed a much improved rate of conversion on the completion of the trial (Table 3.5). Those two investigations indicate the condition of the bioreactor biomass, suggesting that the accumulation of particulate COD was not of major concern. Longer trials would be required to fully ascertain if this is an operational issue which could arise over time.

3.4.2 Microbial Physiology
A methanogenically active biomass developed in both bioreactors during this study with the successful enrichment of psychroactive acetogenic and methanogenic populations during low-temperature cultivation. SMA testing of bioreactor biomass on day 120 returned values which exceeded those recorded on the seed inoculum (day 0) at 12°C for all substrates tested. This low-temperature adaptation was further demonstrated on testing of the takedown biomass. Previous reports have similarly reported the successful adaptation of a mesophilic seed inoculum to low-temperature conditions (Collins et al., 2006; McKeown et al., 2009). SMA assays carried out using bioreactor biomass generally did not indicate the development of truly low-temperature (psychrophilic) microbial communities but rather suggested the development of a low-temperature tolerant mesophilic biomass. Biomass tested from both bioreactors on day 120 and on takedown returned greater activity against all test substrates when tested at 37°C indicating that the seed inoculum had retained its mesophilic nature. Lettinga et al., (1999) found that propionate degradation is sensitive to low-temperature incubation, while Rebac et al., (1999), identified propionate-oxidation as a rate-limiting step for low-temperature bioreactor operation. This was however not the case in the current study where effluent propionate concentrations remained below the detectable limit (≤1 mg l⁻¹) throughout the trial. In conjunction with this by
the trial completion SMA testing of bioreactor biomass at 12°C showed increased potential activity against propionate.

SMA testing on day 120 indicated a balanced methanogenic community had developed in R4, while testing of R5 biomass identified hydrogenotrophic methanogenesis as the predominant methane producers, far succeeding activity levels recorded against acetate. McHugh et al. (2003, 2004) have suggested previously that hydrogenotrophic methane production was a feature of bioreactors operating under stressed conditions. Previous trials have also observed elevated hydrogenotrophic methanogenic activity during periods of stressed or perturbed mesophilic bioreactor operation. This may be due to the inhibition of acetoclastic methanogens and the subsequent oxidation of acetate to H₂ and CO₂, thus providing hydrogen for the reduction of CO₂ to CH₄ by a less sensitive hydrogenotrophic mechanism (Schnürer et al., 1999). The cause of this divergence in methanogenic activity in one of two replicate systems is unknown. The stress reponse shift to hydrogenotrophic methane production may explain this divergence as R5 removal efficiencies were marginally lower than R4. The effect of the decrease in acetoclastic methanogenic activity was translated through temporal increases in R5 effluent acetic acid concentrations and decreased biogas methane concentration during Phases 4 and 5. qPCR data also displayed a significant decrease in gene copy numbers of Methanosetaecae on day 120. Despite this temporal operational issue a rapid recovery in acetoclastic methanogenic activity followed. By Phases 5 and 6 the various operational indicators suggested a recovery in acetoclastic methanogenic activity with parity being observed between both bioreactors in terms of bioreactor performance, effluent acetic acid concentrations and biogas methane concentration. qPCR data similarly indicated an increase in Methanosetaecae gene copy numbers of R5 biomass on day 162. The re-establishment of the acetoclastic methanogenic community of R5 demonstrates the robustness, adaptability and dynamic nature of these low-temperature systems.

By completion of the trial SMA testing suggested that the main route of methangogenesis was through the decarboxylation of acetate. This finding was reinforced by clone library analysis and qPCR data, which showed that
Methanosarcinales dominated the archaeal communities of both bioreactors. Several authors have reported that acetate is the dominant pathway of methanogenesis within methanogenic consortia from natural (Schulz et al., 1997; Fey and Conrad, 2000; Metje and Frenzel, 2007) and engineered (McHugh et al., 2003; Akila and Chandra, 2007) low-temperature environments. Indeed acetoclastic methanogens are considered the most important methanogenic species, as 70% of the total methane generated during the AD of sewage is via this pathway (Grotenhuis 1992; Lettinga 1995). Schulz and Conrad (1996) propose that increased acetate production through homoacetogenic activity may account for this phenomenon under low-temperature conditions. These organisms are capable of both autotrophic and heterotrophic growth, generating acetate as sole end product from either H₂/CO₂ or multi-carbon compounds. Although the role of these bacteria is unclear, there is some evidence to suggest that they may be important in some systems (Zhang & Noike 1994; O’Flaherty et al. 1998; Kotsyurbenko et al. 2001; Ryan et al. 2004). The most common autotrophic homoacetogenic genera are Clostridium and Acetobacterium, while the heterotrophic homoacetogens are distinct in that they are unable to grow on hydrogen and carbon dioxide (Dolfing 1988). While not identified in the seed inoculum bacterial clone library, Clostridiales sp. was identified in the bacterial clone libraries of both bioreactors on day 282. While methanogenesis in both systems was dominated by acetoclastic methanogens on completion of the experiment, there was still significant and notable activity against H₂/CO₂. Previous studies have suggested that a combination of both hydrogenotrophic and acetoclastic methanogens is essential for a balanced and successful anaerobic biological treatment, particularly during perturbations to the system (Leclerc et al., 2001). Nozhevnikova et al., (2000) reported that high densities of both are necessary for efficient degradation of VFAs and methane production at low-temperatures.

3.4.3 Bioreactor Configuration and Biomass Retention
The use of a pumice stone packed AF section was investigated in the current study. The role and effectiveness of filter media for the treatment of sewage both through the use of AF and AH bioreactors are well documented
(Elmitwalli et al., 1999, 2002). The applied packing material satisfied the characteristics of high specific surface area and porosity, large pore size and surface roughness which offered effective performance of AF/AH bioreactors in previous studies (Huysman et al., 1983; Young and Dahab, 1983; Young, 1991; Iwai and Kitao, 1994; Elmitwalli et al., 1999, 2002). The suitability of the current filter/packing material was demonstrated successfully for the direct treatment of synthetic sewage; the AH design and in particular the use of pumice stone as a filter packing material appeared effective in offering physical and bio-physical removal of suspended solids during the treatment of SYNTHES. Yoda et al. (1985) reported that colloidal particles in the influent were very difficult to remove and represented up to 60-70% COD of the effluent of an anaerobic fluidized bed treating sewage, while Wang (1994) found that the removal of colloidal particles was the rate-limiting step in a two-step UASB+EGSB system treating domestic sewage. As reported by Sayed and Fergala (1995) the `entrapment' mechanism involved in removing solids by bioreactor sludge beds is not sufficient for the complete removal of colloidal particles from sewage. The anaerobic hybrid bioreactor utilised combined the advantages of EGSB and AF bioreactors, while minimising their limitations.

The design of the bioreactor enabled the AF section to remove suspended solids (both biomass and suspended COD) from the effluent as it exited the system. The position of the effluent recycle line subsequently allowed the majority of entrapped solids to be drawn from the filter unit and circulated back through the sludge bed. The level of total suspended solids (TSS) recorded in the effluent of both bioreactors R4 and R5 remained low throughout the trial; total and soluble effluent COD concentrations recorded were similar i.e. little particulate COD leaving the system. Mean particulate COD values for both bioreactors over the complete trials, were <40 mg l⁻¹ COD, representing <33% of total effluent COD. The design offered much improved performance from that observed in chapter 2, with much more stable effluent quality being consistently obtained.

The applied AF also played an important and effective role in the retention of bioreactor biomass. Lettinga et al. (2001) highlighted the importance of the retention of viable psychroactive biomass in low-
temperature systems due to the slow microbial growth rates. The filter offered
effective separation of biomass from the bioreactor effluent ensuring that
slow growing microorganisms were maintained within the system. Indeed the
overall success of the process can be attributed to the retention of active
biomass within the bioreactors and the passive filtration of the bioreactor
effluent by the AF as it exited the system; the only loss of biomass recorded
was attributed to temporal sampling. Lettinga et al., (2001) reported that the
success of high-rate anaerobic treatment can be attributed to the development
of bioreactor designs in which biomass retention and liquid retention are
uncoupled. The bioreactor configuration allowed for increased solids
retention times (SRTs), which is necessary within low-temperature
bioreactors for successful high-rate anaerobic digestion (Zeeman and
Lettinga, 1999). The hydrolysis of retained particulates is often considered
the rate limiting step in the anaerobic treatment of sewage (Miron et al.,
2000), therefore the SRT needs to be suitably protracted to allow for
hydrolysis and methanogenesis to proceed fully.

The overall bioreactor configuration employed enabled the
exploitation of the EGSB bioreactor’s applied high superficial velocity and
mixing properties, overcoming problems such as preferential flows, hydraulic
short cuts and dead zones that might occur in the UASB bioreactors (Kato et
al., 2003), while also eliminating the issue in certain instances of biomass
washout under low-temperature conditions (Dries et al., 1998; McKeown et
al., 2009). The filter section, in addition to its physical role for biomass
retention and removal of suspended and colloidal COD, allowed for some
biological activity contributing to COD reduction in a zone which is lacking
biomass in the classical UASB/EGSB bioreactor designs.

3.4.4 Molecular Microbial Ecology
The feasibility of direct LtAD of raw domestic sewage has been demonstrated
as a feasible wastewater treatment option under low-temperature conditions
(Chapter 2 and current study). Despite the widespread harnessing of AD in
engineered bioreactors, there is a lack of systemic information regarding the
nature and role of anaerobic consortia responsible for this biological
wastewater treatment, which impacts on the design and operational stability
of these systems (O’Flaherty et al., 2006). In light of this, a polyphasic molecular approach was used to identify the composition and dynamics of the bacterial and archaeal communities present in the sewage-degrading consortia, in order to better understand and allow for optimisation of this low-temperature process. The reproducibility of anaerobic bioreactor trials with respect to microbial community dynamics was demonstrated with the relative abundance of archaeal and bacterial phylogenetic groups being similar for both bioreactors R4 and R5. Overall, the data indicated that a well-balanced, psychroactive microbial consortium composed of key hydrolytic, fermentative and methanogenic functional groups had developed during the LtAD of synthetic sewage.

3.4.4.1 Archaeal Community Structure

Studies of archaeal communities in natural, and engineered, cold environments have indicated an abundance of Methanosetaeaceae, Methanocorpusculaceae, Methanosarcinaceae, Methanobacteriaceae, as well as non-methanogenic groups, such as the Crenarchaeota (Chin et al., 1999; McHugh et al., 2003; Kotsyurbenko et al., 2004; Collins et al., 2005; Høj et al., 2005; Metje and Frenzel, 2007; Høj et al., 2008; Syutsubo et al., 2008). In this study, sequences affiliated within the phylogenetic divisions of the Methanosarcinales (Methanosetaeaceae and Methanosarcinaceae), Methanobacteriales (Methanobacteriaceae and Methanosphaera), and Crenarchaeota were obtained. 16S rRNA gene clone libraries of biomass from the seed inoculum and bioreactors biomass on day 282 established that Methanosaeta-like organisms were the predominant methanogens in this study, followed by members of the Methanobacteriales (Figs. 3.11). Quantitative analysis of 16S rRNA gene concentrations of methanogenic groups present was generally in agreement with semi-quantitative data from clone libraries (Fig. 3.14) and also demonstrated the dominance of the Methanosetaeaceae. This corresponds to the previously reported high abundance of Methanosetaeaceae-related species in stable granular anaerobic bioreactors (Diaz et al., 2006; Satoh et al., 2007; Fernandez et al., 2008). Methanosaeta are believed to be competitive in established methanogenic communities (Jetten et al., 1990; McMahon et al., 2001; McHugh et al., 2003;
Indeed, the predominance of methanogens closely related to *M. concilii* in anaerobic sludges, has been widely documented as important for the formation, functioning and maintenance of granular sludge (Griffin *et al*., 1998; MacLeod *et al*., 1990; Merkel *et al*., 1999). Deterioration in granule integrity has been attributed to decrease in the abundance of Methanosaeta. Gomec *et al.* (2008) observed granule deterioration in a laboratory-scale upflow anaerobic sludge bed (UASB) bioreactor treating raw sewage at 13°C; when numbers of filamentous Methanosaeta-like cells; detected by FISH appeared to alter to shorter forms and their overall cell densities decreased. However, this was not observed in the EGSB-AF bioreactors utilised in the current study, where robust granule structure was maintained in R4 and R5, and where stable temporal abundance of *Methanosaetaceae* 16S rRNA gene copies was observed.

Clone library analysis displayed an increase in *Methanobacteriales* by day 282 in both bioreactors, as eluded to earlier this may be as a response to the environmental stress of subjecting the mesophilic community of the seed inoculum to low-temperature conditions. Hydrogenothrophic methanogens are less sensitive to low-temperature conditions and the thermodynamics of hydrogen-mediated methanogenesis is greatly improved at reduced temperatures as a result of increased gas solubility (Lettinga *et al*., 2001). While the acetoclastic community adapted to the low-temperature conditions, there may have been a predilection towards hydrogenotrophic methanogenesis. An earlier sampling day for both SMA testing and qPCR analysis may have helped to elucidate and reinforce this assertion. The emergence of *Methanosphaera spp.* which had not been detected in the seed inoculum clone library was observed in the bioreactor biomass on day 282. This hydrogen/methanol-utilizing species was also observed in a similar study by Gao *et al*., (2010) using a mesophilic seed inoculum where it was reported that this species only began to emerge at 15 °C.

Sequences not affiliated with known methanogenic Archaea clustered within the Group 1.3b Crenarchaeota (Ochsenreiter *et al*., 2003). The presence and abundance of non-thermophilic Crenarchaeota in anaerobic wastewater treatment systems is well reported (e.g. Godon *et al*., 1997; Leclerc *et al*., 2001; Collins *et al*., 2005; Levén *et al*., 2007). While the
relative abundance of *Crenarchaeota*-affiliated sequences retrieved on conclusion of the trial (day 282) was reduced from that recorded for the day 0 clone library, the persistence of this group suggests a putative role of these non-methanogens in low-temperature anaerobic sewage treatment. However, the function of crenarchaeal organisms in AD and their influence on community ecology is still unknown, partly because culturing has largely been unsuccessful (Könneke et al., 2005). Collins et al. (2005) used a combination of FISH coupled with β-micro imaging to show the occurrence of these organisms in close proximity to acetate-utilising *Methanosaeta* in anaerobic granules, suggesting a putative symbiotic association. Recent findings have suggested their role in ammonia oxidation in the environment (Nicol and Schleper, 2006; Treusch et al., 2005) However, ammonia oxidation is unlikely the function of the *Crenarchaeota* present in the bioreactor biomass of this study due to the lack of oxygen and the persistence of high ammonia levels in the bioreactors (data not shown), more efforts are needed to understand their functions in anaerobic environments.

### 3.4.4.2 Bacterial Community Structure

Bacterial clone library analysis of the seed inoculum illustrated the predominance of *Bacteroidetes, Chloroflexi, Firmicutes, Actinobacteria* and *Proteobacteria* as the major phylogenetic groups present. Members of these phyla are widely distributed throughout anoxic engineered environments, where they are widely responsible for hydrolytic and fermentative reactions on a broad range of organic materials (Delbês et al., 2000; Godon et al., 1997; Levèn et al., 2007; Liu et al., 2002; Yamada et al., 2005). In addition, these groups are frequently recovered from both mesophilic (Godon et al., 1997; Hernon et al., 2006; Liu et al., 2002) and low-temperature, engineered systems (Collins et al., 2003; McKeown et al., 2009). The relative abundance of particular groups present in the seed inoculum was dramatically different to those observed on day 282 for bioreactors R4 and R5.

The dominance of *Bacteroidetes* in the seed inoculum clone library and the subsequent libraries on day 282 indicates the important role this branch plays in the conversion of organic matter both at mesophilic and low-temperature conditions. *Bacteroidetes* are known to be proteolytic bacteria
They mediate the degradation of proteins and are able to ferment amino acids to acetate. Microorganisms within the *Bacteroidetes* and *Firmicutes* are widely distributed throughout various anaerobic habitats, both natural and bioengineered, and have been demonstrated to have the ability to degrade a wide range of complex substrates and organic macromolecules, including proteins, starch and carbohydrates (Danon *et al.*, 2008; Levèn *et al.*, 2007).

The phylum *Chloroflexi* have long being recognized as an evolutionary and environmentally significant group of bacteria (Hugenholtz *et al.*, 1998; Sekiguchi *et al.*, 2001, 2003), associated with diverse habitats, including wastewater treatment systems (Kragelund *et al.*, 2007; Sekiguchi *et al.*, 2001). *Chloroflexi* formally known as the *Green non-sulfur* bacteria, are reported to be important community members within mesophilic and thermophilic wastewater treatment systems, representing up to 20% of the total microbiota in some instances (Björnsson *et al.*, 2002; Chouari *et al.*, 2005; Godon *et al.*, 1997; Levèn *et al.*, 2007; Rossetti *et al.*, 2003). This is in agreement with the relative abundance recorded for the mesophilic seed inoculum (18%, Fig. 3.13a). However, there importance in low-temperature environments appears much less, the relative abundance of this group within the bacterial consortia of both bioreactors was greatly reduced following 282 days of low-temperature cultivation. It has been suggested that members of this phylum may play key roles in the primary degradation of carbohydrates and cellular materials (such as amino acids) during AD (Nahiro & Sekiguchi, 2007). Few extensive reports exist which describe their importance within low-temperature environments, indeed a long term LtAD trial reported by McKeown *et al.*, (2009) showed that the group was functionally redundant under low-temperature conditions or lacked the ability to adapt to the psychrophilic conditions.

Greater diversity of the bacterial communities in the bioreactors was evident on day 282, as a result of the emergence of several groups, coupled with the loss of and or shifts in relative abundances of groups observed within the seed inoculum. The novel candidate division *TM7* emerged as a key group within the low-temperature sewage degrading bacterial communities. To date, there are no known cultivated members of this group and they have been
identified solely from environmental 16S rRNA sequence data (Hugenholtz et al., 2001). Previous research has found TM7 members in various habitats such as peat bog (Rheims et al., 1996), mature forest soils (Borneman and Triplett, 1997), sequencing batch reactors (Bond et al., 1995) and full-scale wastewater treatment plant sludges (Hugenholtz et al., 2001). More recent studies have focused on attempting to elucidate the ecological function of this group. Thomsen et al., (2002) confirmed that the filamentous bacteria TM7 can uptake carbon substrates under aerobic and anaerobic conditions. Bertin et al., (2006) through the use of in-situ techniques revealed that members of the TM7 division are capable of surviving and growing under a wide range of conditions, while Ariesyady et al., (2007) confirmed through a MAR-FISH approach that TM7 members were positive for glucose mineralization. An extensive study of the microbial communities involved in WWTP incorporating enhanced biological phosphorus removal carried out recently by Nielsen et al., (2010) revealed that the phylum TM7 were important protein-hydrolyzing organisms. It is anticipated that this is their major function in the EGSB-AF bioreactors employed in the current study, where in the absence of oxygen they use NO\textsuperscript{3-} and NO\textsuperscript{2-} as electron acceptors. Interestingly molecular analysis carried out on biomass samples from bioreactors R1-R3 of chapter 2 by Kelly et al., (2010) did not identify the TM7 as a key bacterial phylogenetic group. It is proposed that the recycling of entrapped complex particulate and polymeric compounds from the novel AF back to the sludge bed may have resulted in selective pressure preceding the development of these specialised hydrolyzing organisms, essentially enriching the biomass to deal with the trial influent. Other groups to emerge in both bioreactors included Termite Group 1 (TG-1)-affiliated clones, TG-1 species are abundant in bacterial clone libraries from the hindguts of termites (Herlemann et al., 2007; Ohkuma & Kudo, 1996).

Spirochaetes also emerged as an important group within the bioreactors. This group are typically found in organic-rich, oxygen poor environments, where they may demonstrate both fermentative and acetogenic metabolism, and may be significant bacterial community members within mesophilic granular biofilms, representing up to 25% of total bacterial community (Hernon et al., 2006). However, this group has not been reported
to play a significant role in low-temperature consortia, and while they appear
to be numerically important in our psychroactive consortium, their function
remains unclear.

While in relative terms the composition of the bacterial communities
in bioreactors R4 and R5 were similar, the diversity of R5 was greater than
R4. Clones from a number of bacterial groups were retrieved from the R5
biomass, which were not from R4. Groups to emerge in R5 included
\textit{Acidobacteria} 1\%, \textit{Planctomycetes} 3\% and \textit{Synergistetes} 1\% and the
candidate division \textit{OP11} 3\%. \textit{Acidobacteria}, which as by their name implies
are acidophiles, may have emerged in R5 as a response to the transient spikes
in acetic acid levels observed during Phases 3 and 4. The widespread
occurrence of environmental sequences belonging to the \textit{Acidobacteria}
division suggests that members of this group are ecologically significant
constituents of many ecosystems, particularly soil communities (Hugenholtz
\textit{et al.}, 1998). Recent research has demonstrated that genera of the phylum
\textit{Planctomycetes} play a central role in anaerobic ammonium oxidation
(Innerebner \textit{et al.}, 2007). Anaerobic ammonium oxidation (anammox)
bacteria, which were discovered in waste-water sludge in the early 1990s,
have the unique metabolic ability to combine ammonium and nitrite or nitrate
to form nitrogen gas (Kuenen, 2008). Effluent mean NH$_4^+$ concentrations were
6\% lower in R5, suggesting that the \textit{Planctomycetes} members identified only
in the R5 biomass may be playing a role in anaerobic ammonium oxidation
(anammox). \textit{Synergistetes} can use amino acids and in turn provide short-chain
fatty acids and sulphate for terminal degraders such as the methanogens and
sulphate-reducing bacteria (Vartoukian \textit{et al.}, 2007). The candidate group
\textit{OP11} have no cultivated members, so their physiological properties remain
unknown, however in general, representatives of the group are found in
reduced environments with a conspicuous presence of sulphur compounds
(Harris \textit{et al.}, 2004).

The molecular diversity of bacterial phyla in these low-temperature
ecosystems (R4 and R5 biomass) may be explained by the input of complex
organic substrates present in sewage and the ability of this anaerobic
microflora to degrade a wide range of macromolecules such as pectin,
cellulose, proteins and carbohydrates (Chouari \textit{et al.}, 2005). It is likely that
the aforementioned groups, along with the other diverse organisms present, contributed to the successful treatment of sewage through hydrolytic, proteolytic and fermentative degradation of the protein and carbohydrate constituents in the influent wastewater. Consequently, the necessary intermediate substrates for methanogenesis to occur were provided. Generally, a complex substrate composition allows for the development of a microbial community with a higher metabolic diversity compared to a substrate composed of only a few components (McHugh et al., 2003, 2004). Therefore, the diversity of bacterial phyla present in the sewage-degrading biomass is most likely a reflection of the complex nature of the sewage-based wastewater.

### 3.5 Conclusions

This chapter presents the results of a trial to investigate the use of an alternative fixed-film matrix material, granulated pumice stone, to enhance the efficiency and stability of LtAD of sewage. The novel EGSB-AF bioreactor design offered much improved performance from that observed in chapter 2.

This study also demonstrated that:

- Stable and efficient LtAD of synthetic sewage wastewater was successfully achieved at OLRs of up to 6 kg COD m\(^{-3}\) day\(^{-1}\) and HRTs of as low as 2 h;
- The addition of the modified AF section described in this chapter resulted in enhanced total COD removal efficiencies averaging > 75% and very stable performance across the range of loading rates applied. Effluent total COD values achieved throughout the trial were regularly at or below the discharge standard, with concentrations of \(\leq 125 \text{ mg l}^{-1}\) (Irish, Urban Waste Water Treatment Regulations, [S.I. No. 419/1994, 254/2001, 440/2004]);
- Reproducible wastewater treatment performance was observed, the data obtained suggests that the replication of anaerobic bioreactors, in terms of chemical oxygen demand removal, volatile fatty acid
degradation and accumulation, development of methanogenic activity and population dynamics, with respect to the operational pressures applied, is achievable once low-temperature adaptation has occurred, and was evident by the completion of the current trial;

- This packing material greatly increased the surface area of biofilm per unit of bioreactor volume and also due to its very high porosity, aided in the entrapment of suspended solids and colloidal material prior to biodegradation;
- The modified AF played a significant role in biomass retention;
- A high level of bacterial biodiversity was recorded; metabolically diverse fermentative and hydrolytic bacterial populations were evident within the R4 and R5 biomass, demonstrating rapid adaptability, with the necessary populations coming to the fore;
- Methanogenic communities exhibited robustness in response to changing environmental conditions, suggesting that the long-term operation of a low-temperature anaerobic bioreactor is feasible from a microbiological point of view, a suggestion strongly supported by the SMA profiles taken during the bioreactor trials;
- High levels of phosphate attenuation were again noted during the trials (averaging 50% and up to 80% P removal). The data from this trial suggests a biological, rather than a purely physical basis for phosphate attenuation in the system;
- Quantitative PCR offered a rapid snapshot of the methanogenic community of bioreactor temporal samples, helping to further elucidate the dynamics of the methanogenic communities at work while also demonstrating the benefit of using a polyphasic approach to microbial ecology studies.
3.6. References


ANAEROBIC TREATMENT OF RAW AND SETTLED SEWAGE AT 12°C IN EGSB-AF HYBRID BIOREACTORS: BIOPROCESS PERFORMANCE PHYSIOLOGICAL AND MOLECULAR CHARACTERISTICS

Bioprocess data from this chapter was presented at:
The 12th World Congress on Anaerobic Digestion, Guadalajara, Mexico
Abstract

The feasibility of direct anaerobic treatment of raw and settled sewage under temperate climatic conditions (12°C) was investigated. Two expanded granular sludge bed-anaerobic filter hybrid bioreactors, R6 and R7, were employed to treat raw sewage and settled sewage, respectively, sourced from the city of Galway, Ireland. The bioreactors were operated at hydraulic retention times (HRTs) of 3-24 h in a trial of >140 days. Successful treatment of both influent types was achieved with consistent effluent quality of <125mg/l chemical oxygen demand (COD) being obtained at organic loading rates of 0.2-2.4 kg COD m⁻³ d⁻¹, volumetric loading rate of 1-4 m³ wastewater m⁻³ bioreactor d⁻¹ and HRTs of 3-6 h, in addition significant phosphorus removal was recorded with both influent types. The successful use of membrane coupling was also demonstrated supplementing the COD removal of bioreactor R6. The ability of the bioreactors to quickly adapt to varying influent strength and changes in HRT suggest a direct low-temperature anaerobic digestion approach to sewage treatment is indeed feasible. Physiological characterisation of the sewage degrading biomass was carried out through specific methanogenic activity assays charting the effect of influent and temperature on bioreactor biomass. Values recorded indicated the successful adaptation of a mesophilic seed inoculum to low-temperature operation. Bacterial communities as deduced from clone library analyses at the conclusion of the trial were phylogenetically diverse, similar to those observed in chapter 3, consisting of key metabolically diverse hydrolytic and fermentative bacterial populations. Archaeal clone library analysis indicated that acetoclastic methanogenesis was dominant in both R6 and R7 by the completion of the trial.
Chapter 4

4.1 Introduction

The current approach to municipal wastewater treatment in the majority of developed countries involves the use of aerobic processes. The consequences of utilising aerobic systems such as activated sludge plants are large capital expenditure initially during installation, and subsequently through operation by the use of large amounts of fossil fuels. This approach makes the wastewater industry one of the most energy intensive sectors, indeed the energy requirements to treat the over 10 billion litres of sewage produced in the UK each day accounts for approximately 2-3% of net UK electricity, releasing approximately four million tonnes of greenhouse gas emissions (carbon dioxide equivalent) every year (Water UK Report, 2006; Postnote, 2007). In addition, aerobic wastewater treatment results in the generation of large quantities of secondary sludge requiring treatment before re-use/cycle. Increasing energy costs and a greater understanding of environmental issues indicate the need to secure a paradigm shift towards a more environmentally sustainable wastewater treatment system, an approach which produces renewable energy and or reduces energy consumption during treatment. This model could be realised through a shift from aerobic to anaerobic microbiology as the core technology for the treatment of wastewater. Anaerobic wastewater treatment is now an established wastewater treatment approach for a wide variety of wastewaters (Macarie, 2000; Elmitwalli et al., 2001; Bouallagui et al., 2005; Rincon et al., 2006), both industrial and municipal and offers the benefits of energy generation through biogas, reduced operational energy requirements and reduced secondary sludge production when compared with aerobic systems. The direct anaerobic treatment of municipal wastewaters such as sewage is well-established in tropical countries such as Brazil and India, where ambient temperatures (20-30°C) allow for the application of mesophilic anaerobic digestion (AD) in upflow anaerobic sludge bed (UASB) bioreactors (von Sperling and Chernicharo, 2005; Aiyuk et al., 2006). A challenge remains, however in countries with lower ambient temperatures such as Ireland and the UK, whereby a significant energy input to heat bioreactors would be required for treatment to proceed within a mesophilic temperature range, therefore
negating the cost saving associated with direct anaerobic treatment. However, recent advances and developments in anaerobic systems most notably advances in bioreactor design and the use of hybrid designs have overcome the limitations associated with applying AD at reduced temperatures (low-temperature anaerobic digestion [LtAD]). The treatment of low-strength wastewaters at low-temperature has benefited from the use of the expanded granular sludge bed (EGSB) bioreactor (Rebac et al., 1995, 1999; van Lier et al., 1997; Seghezzo et al., 1998; Kato et al., 1999, Chu et al., 2005), the design of which offers the benefit of greater mixing intensities than alternative bioreactor designs resulting in greater contact between the wastewater and biomass through increased height to diameter ratio and effluent recirculation (Puyol et al., 2009). Such approaches incorporating the EGSB bioreactor design have led to the successful LtAD of a wide variety of wastewaters as outlined by McKeown et al. (2009). Anaerobic treatment of low-strength wastewaters such as municipal wastewater at temperatures representative of those found in cooler and more temperate climates has been demonstrated, highlighting the potential for the direct anaerobic treatment of municipal wastewaters and those of varying organic load under low-temperature conditions (Uemura and Harada, 2000; Elmitwalli et al., 2001; Mahmoud et al., 2004; Gomec et al., 2004; Jamal and Mahmoud, 2009; Martín et al., 2010). The harnessing of a cost-effective low-temperature anaerobic sewage treatment approach offering efficient, stable and long-term operation would represent a significant breakthrough in the wastewater industry. The successful application of low-temperature anaerobic systems for the treatment of low-strength and complex wastewaters such as sewage requires a suitable bioreactor design. Seghezzo et al., (1998) reported that UASB and EGSB bioreactor designs are the most powerful anaerobic treatment systems for low-strength wastewaters such as sewage. Furthermore, Elmitwalli et al. (1999, 2002) demonstrated the importance of filter media in the anaerobic treatment of complex waste streams, such as domestic sewage, with respect to the removal of the various COD fractions and suggested that a hybrid granular sludge and anaerobic filter (AF) system would be advantageous for such applications.
The data obtained from the trials described in Chapters 2 and 3 suggested that the anaerobic treatment of sewage may be feasible under Irish climatic conditions. However, the use of a synthetic wastewater for those trials, although convenient and safe for laboratory applications, required that the results be verified with a non-synthetic sewage influent, which would be subject to the normal fluctuations in composition and strength associated with this wastewater type. This chapter, therefore, presents the results of an investigation as to the feasibility for direct anaerobic treatment of municipal wastewater using the EGSB-AF hybrid bioreactors described in Chapter 3. Both raw sewage and settled sewage obtained from the Mutton Island Wastewater Treatment Plant at Galway City were treated during a trial of c.140 days. Bioreactor performance and the physiological characteristics of the bioreactor biomass were monitored. Molecular ecology techniques were also applied through the use of clone library analysis of bacterial and archaeal 16S rRNA genes in order to monitor the development of the archaeal and bacterial populations of both systems. The hypothesis being tested was that long term, low-temperature (12°C), anaerobic treatment of sewage could be successfully achieved, with operational performance comparable to that of the synthetic sewage trials described in Chapters 2 and 3. In addition, the potential of an additional membrane-based module for further enhancement of effluent quality was investigated by the coupling of a tangential flow filtration system with the EGSB-AF bioreactor (R6).

4.2 Materials and Methods

4.2.1 Source of Biomass
A mesophilic, anaerobic granular biomass was obtained from a full-scale (1500 m³), internal circulation (IC) bioreactor at Carbery Milk Products (Ballineen, Co. Cork, Ireland) used to treat ethanol production wastewater. The volatile suspended solids (VSS) concentration of the granules was 94 g VSS l⁻¹. The granules were of a regular, spherical shape and a grey-green colour.
4.2.2 Source of Influent

The influent for the trial (raw sewage and settled sewage) was procured weekly from Mutton Island Wastewater Treatment Works (WWTW) Galway, Ireland. The Mutton Island site is located 1 km offshore of South Park in Galway City and was officially opened in May 2004. The treatment plant employs the conventional activated sludge (CAS) system including primary settlement, aeration and final settlement. Briefly, the wastewater arrives at the inlet to the treatment works via a number of trunk mains from the east and west sides of Galway City. The wastewater passes through coarse screens (100mm) to remove large objects followed by fine screens (6mm) to remove paper, plastics and large solids. Grit washed in from the streets by surface water is removed in two circular grit traps. Four primary settlement tanks allow up to 60% of the pollutants (primary sludge) in the wastewater to settle. Fats, oils and grease are removed from the surface of these tanks. The settled wastewater (primary effluent) is then pumped to four aeration lanes where air is introduced via three air blowers. The system was designed to operate as a plug flow diffused air system proceeded by an anoxic zone to provide for nitrification and partial denitrification. Aerobic microorganisms developed in the process are used to breakdown organic matter from the wastewater along with ammonia and nitrogen. The organic matter produced in the secondary treatment process is allowed to settle in the final settlement tanks, as secondary sludge. The treated effluent is discharged to Galway Bay via a sea outfall, where discharge occurs 24 hours per day under all tidal conditions. The secondary sludge is thickened and pasteurised to 60 degrees for 1 hour to kill pathogens and digested to reduce the volatile solids contents. The biogas produced during the sludge digestion process is in turn used to maintain the heat balance in the pasteurisation/digestion process. The final product, i.e. the dried sludge, is dewatered and transported off-site where it is spread on land as an organic fertiliser. The process flow employed at the Mutton Island WWTW is outlined in Figure 4.1.
Figure 4.1 Process flow diagram of the Mutton Island WWTW with sources of influent for R6 and R7 bioreactors indicated.

4.2.3 Bioreactor Design and Operation
Two identical 3.5 l glass laboratory-scale expanded granular sludge bed-anerobic filter (EGSB-AF) hybrid bioreactors (active liquid volume, 2.9 l) similar to that previously described by McHugh et al. (2004), were used. The upper ‘fixed-film’ anaerobic filter (AF) section consisted of granulated pumice stone (Ø c. 1-4 mm) tightly packed in a low density polyethylene (LDPE) cylinder. The lower ‘sludge bed’ section of the bioreactors (R6 and R7) was inoculated with 20g VSS l⁻¹ (58 g VSS; 0.62 l) of the granular seed biomass. The bioreactors were employed to treat: 1) raw sewage over four operational phases (R6); and 2) settled sewage over five operational phases (R7) (Fig. 4.2). Phases were characterised by changes applied to hydraulic retention time (HRT) and are outlined in detail in Table 4.1. The respective bioreactor influents were maintained at 4°C and continuously mixed with the aid of magnetic stirrers. A superficial up-flow velocity of 2.5 m h⁻¹ was
applied to the bioreactors for the duration of the trial, by continuous recirculation of effluent through the bioreactors.

**Table 4.1 Bioreactors R6 and R7 phases; operational conditions.**

<table>
<thead>
<tr>
<th>Bioreactor</th>
<th>R6 (Raw Sewage)</th>
<th>R7 (Settled Sewage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Days</td>
<td>0-37</td>
<td>38-70</td>
</tr>
<tr>
<td><strong>T</strong> a</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><strong>I COD&lt;sub&gt;tot&lt;/sub&gt; b</strong></td>
<td>512.8</td>
<td>291.4</td>
</tr>
<tr>
<td>±207.2</td>
<td>±86.0</td>
<td>±74.6</td>
</tr>
<tr>
<td><strong>I COD&lt;sub&gt;sol&lt;/sub&gt; c</strong></td>
<td>179.1</td>
<td>136.9</td>
</tr>
<tr>
<td>±50.1</td>
<td>±34.4</td>
<td>±17.7</td>
</tr>
<tr>
<td><strong>HRT</strong> d</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>±0.21</td>
<td>±0.18</td>
<td>±0.21</td>
</tr>
<tr>
<td><strong>OLR</strong> e</td>
<td>0.51</td>
<td>0.58</td>
</tr>
<tr>
<td>±0.21</td>
<td>±0.18</td>
<td>±0.21</td>
</tr>
<tr>
<td><strong>VLR</strong> f</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>±0.01</td>
<td>±0.01</td>
<td>±0.01</td>
</tr>
<tr>
<td><strong>SLR</strong> g</td>
<td>0.026</td>
<td>0.031</td>
</tr>
<tr>
<td>±0.01</td>
<td>±0.01</td>
<td>±0.01</td>
</tr>
<tr>
<td><strong>SLR</strong> h</td>
<td>0.05</td>
<td>0.13</td>
</tr>
<tr>
<td>±0.01</td>
<td>±0.01</td>
<td>±0.01</td>
</tr>
<tr>
<td><strong>UV</strong> i</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>±0.21</td>
<td>±0.21</td>
<td>±0.21</td>
</tr>
</tbody>
</table>

* Temperature (°C); b Influent chemical oxygen demand, total (mg l⁻¹); c Influent chemical oxygen demand, soluble (mg l⁻¹); d Hydraulic retention time (hrs); e Organic loading rate (kg COD m⁻³ d⁻¹); f Volumetric loading rate (m³ wastewater m⁻³ Reactor d⁻¹); g Sludge loading rate (kg COD kg [VSS]⁻¹ d⁻¹); h Sludge loading rate (m³ Wastewater kg [VSS]⁻¹ d⁻¹); i Up-flow velocity (m h⁻¹); Values for b and c are the phase mean ± phase standard deviation; Values for e and g are calculated on the mean influent COD<sub>tot</sub>.

**Figure 4.2 Bioreactor influent R6 (left) and R7 (right).**
4.2.4 Bioreactor Effluent and Biogas Analyses

Samples of bioreactor influent, effluent and biogas were routinely sampled for determination of total COD (COD\textsubscript{tot}), soluble COD (COD\textsubscript{sol}), total suspended solids (TSS), pH, and biogas analysis according to Standard Methods (American Public Health Association (APHA), 1998). Volatile fatty acid (VFA) concentrations were determined by GC-MS (Varian, USA). Phosphorus (PO\textsubscript{4}\textsuperscript{3-}) analyses of influent and bioreactor effluent was detected using a colorimetric spectrophotometer (HACH DR4000), according to the HACH molybdovanadate method with acid persulfate digestion. All samples were filtered through 0.45µm filter prior to PO\textsubscript{4}\textsuperscript{3-} analysis.

4.2.5 Tangential Flow Filtration

Secondary treatment of the bioreactor effluent of R6 was carried out during Phase 4 (days 124-140). A tangential flow filtration (TFF) system (Pall, Life Sciences: Fig. 4.3) incorporating a suspended screen cassette filter of 0.2µm pore size was employed for this purpose. The cassette filter had an area of 0.093 m\textsuperscript{2}. The system was designed to operate 4 times d\textsuperscript{-1} coinciding with the bioreactor HRT (6 h), i.e. processing the bioreactor volume 4 times d\textsuperscript{-1}, with an initial permeate flux rate of 65 l m\textsuperscript{-2} h\textsuperscript{-1}. 
4.2.6 Specific Methanogenic Activity Assays

The specific methanogenic activity (SMA) of the seed biomass (day 0), bioreactor biomass (R6 and R7) on day 86 and at the conclusion of each trial (R6 day 141; R7 day 149), were obtained according to Colleran et al. (1992) and Coates et al. (1996). SMA values were determined against the direct methanogenic substrates acetate and H$_2$/CO$_2$, and the indirect substrates propionate, butyrate and ethanol, as previously described by Collins et al. (2003). Briefly, biomass samples were washed in 1X PBS and transferred to sterile 20-ml (soluble substrates) or 60-ml (gaseous substrates) hypovials to achieve a final biomass concentration of 2-5 g VSS l$^{-1}$ in a total volume of 10 ml anaerobic activity test medium. Vials without substrate, or with only pressurised N$_2$/CO$_2$ headspace, were used as controls. Assays were carried out at 12°C and 37 °C.

4.2.7 DAPI staining of Polyphosphate Accumulation

Previous studies (Tijssen et al., 1982; Kjeldstad et al., 1991; Kawaharasaki et al., 1999; Liu et al., 2001) have indicated that 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) can bind to DNA and polyphosphates, and correctly differentiate between these two binding states by the use of epifluorescence microscopy, in which DNA-bound DAPI appears pale blue and polyphosphate bound DAPI appears strongly yellow when excited with light.
at a wavelength of 330-385 nm. Briefly, samples were washed with 5 ml Mill-Q water to remove any remaining substrate. Liquid nitrogen was used to embed the granules in OCT freezing medium prior to sectioning. Serial cryosections of 5-8 µm thickness (Ø, 0.5-2 mm) each were prepared, as described previously by Sekiguchi et al. (1999), and were immobilised on acid-washed glass slides. 10-20 µl of DAPI (50 µg ml⁻¹) was applied to the sections and allowed to dry for 0.5 h in the dark. The anti-bleaching agent VECTASHIELD® (5µl drop) and a glass cover-slip were then applied. Individual cells were also observed and obtained by physical disruption of the anaerobic granules. Slide preparations were viewed by epifluorescence microscopy with a specific DAPI light filter (365 nm).

4.2.8 DNA Extraction
Biomass total genomic DNA was obtained using the MoBio Soil DNA Extraction kit (Cambio, Cambridge, UK), according to the manufacturer’s instructions. All granular sludge samples for molecular analysis were first mechanically crushed by grinding using a pestle and mortar and 0.5g of sludge granules was used for extractions. DNA was extracted in triplicate and pooled for each sample prior to analysis. Extracted DNA and downstream PCR reactions were electrophoresed on 1% (w/v) agarose gels in 1X TAE, containing 1 µg ethidium bromide ml⁻¹ (Maniatis et al., 1989), with visualization by UV excitation to determine DNA integrity or to check PCR amplicon size.

4.2.9 Generation of Clone Libraries, Amplified rDNA Restriction Analysis (ARDRA) and Phylogenetic Classification
Archaeal and bacterial clone libraries were generated through PCR amplification of 16S rRNA genes from total genomic DNA using the bacterial primers 27F (5′-GAGGTGATCCAGGTAG-3′) DeLong 1992) and 1392R (5′-ACGGCCTGATCCCTGAC-3′) Lane et al., 1985) and the archaeal primers 21F (5′-TTCGGATCCCTGACGAG-3′) Stackebrandt and Goodfellow, 1991) and 958R (5′-YCCGGCCTGAMTCCATT-3′; DeLong, 1992). Amplicons were ligated into plasmid vector pCR 2.1-TOPO® XL (Invitrogen) and used to transform chemically competent Escherichia coli
Chapter 4

cells as described by McHugh et al. (2003). Amplified rDNA restriction analysis (ARDRA) was performed, whereby clones were categorized into distinct operational taxonomic units (OTUs) based on restriction with the tetrameric restriction enzyme Hae III (McHugh et al., 2004). Unique clonal restriction patterns were identified, and representatives of these sequenced. The retrieved sequences were compared to previously identified sequences using the web-based Basic Local Alignment Search Tool, (BLASTn) (www.ncbi.nlm.nih.gov/BLAST) and the Ribosomal Database Project (RDP) (Maidak et al., 2000) and aligned using the ARB database software programme’s (Ludwig et al., 2004) Fast Aligner Tool. Phylogenetic trees were constructed using the neighbor joining (Jukes–Cantor correction) (Saitou & Nei, 1987) algorithms implemented in ARB.

4.2.10 Accession Numbers

The partial 16S rRNA gene sequences determined in this study through clone library analysis were deposited in the GenBank database and assigned the following accession numbers: Bacteria - GQ423770-GQ423785 (seed inoculum); HQ008146-HQ008179 (R6, day 141); HQ008180-HQ008216 (R7, day 149); Archaea - FJ005019-FJ005025 (seed inoculum); GU982662-GU982667 (R6, day 141); GU982668-GU982677 (R7, day 149).

4.3 Results

4.3.1 Bioreactor Performance

Bioreactor performance during the low-temperature anaerobic treatment of raw sewage (R6) and settled sewage (R7) is summarised in Table 4.2. Influent and effluent COD_{tot} and COD_{sol} concentrations and total and soluble COD removal efficiencies (CODRE_{tot+sol}), biogas quality (% methane) and phosphorus concentrations are presented in Figures 4.4-4.7. Operational phase conditions and parameters are previously outlined in Table 4.1.
Table 4.2 Performance characteristics of bioreactors R6 and R7 during operation.

<table>
<thead>
<tr>
<th>Bioreactor</th>
<th>R6 (Raw Sewage)</th>
<th>R7 (Settled Sewage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase Days</td>
<td>1  2  3  4</td>
<td>1  2  3  4  5</td>
</tr>
<tr>
<td>I COD&lt;sub&gt;tot&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>512.8 ±207.2</td>
<td>194.1 ±55.9</td>
</tr>
<tr>
<td>E COD&lt;sub&gt;tot&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>175.7 ±60.2</td>
<td>114.1 ±40</td>
</tr>
<tr>
<td>CODRE&lt;sub&gt;tot&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>57.2 ±5.2</td>
<td>39.6 ±40</td>
</tr>
<tr>
<td>I COD&lt;sub&gt;sol&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</td>
<td>179.1 ±50.1</td>
<td>141.3 ±38.2</td>
</tr>
<tr>
<td>E COD&lt;sub&gt;sol&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>101.7 ±25.2</td>
<td>94.5 ±40</td>
</tr>
<tr>
<td>CODRE&lt;sub&gt;sol&lt;/sub&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
<td>40 ±5.2</td>
<td>33.7 ±3.7</td>
</tr>
<tr>
<td>I P&lt;sup&gt;g&lt;/sup&gt;</td>
<td>18 ±3.9</td>
<td>5.2 ±1.4</td>
</tr>
<tr>
<td>E P&lt;sup&gt;h&lt;/sup&gt;</td>
<td>4.1 ±0.9</td>
<td>3.6 ±2.1</td>
</tr>
<tr>
<td>PRE&lt;sup&gt;i&lt;/sup&gt;</td>
<td>77.6 ±5.6</td>
<td>30.5 ±2.1</td>
</tr>
<tr>
<td>VFA&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.62 ±0.54</td>
<td>0.53 ±0.65</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;k&lt;/sup&gt;</td>
<td>16.7 ±10.5</td>
<td>3.5 ±0.5</td>
</tr>
<tr>
<td>Biogas&lt;sup&gt;l&lt;/sup&gt;</td>
<td>129.9 ±8.2</td>
<td>18.6 ±8.2</td>
</tr>
</tbody>
</table>

*<sup>a</sup> Influent chemical oxygen demand, total (mg l<sup>-1</sup>);<sup>b</sup> Effluent chemical oxygen demand, total (mg l<sup>-1</sup>);<sup>c</sup> Total COD removal efficiency (%);<sup>d</sup> Influent chemical oxygen demand, soluble (mg l<sup>-1</sup>);<sup>e</sup> Effluent chemical oxygen demand, soluble (mg l<sup>-1</sup>);<sup>f</sup> Soluble COD removal efficiency (%);<sup>g</sup> Influent phosphorus (mg l<sup>-1</sup> PO<sub>4</sub><sup>3-</sup>);<sup>h</sup> Effluent phosphorus (mg l<sup>-1</sup> PO<sub>4</sub><sup>3-</sup>);<sup>i</sup> Phosphorus removal efficiency;<sup>j</sup> VFA effluent (mg l<sup>-1</sup> acetic acid);<sup>k</sup> Methane in biogas (%);<sup>l</sup> Biogas production (ml d<sup>-1</sup>). All values are the phase mean ± phase standard deviation.

**R6 Process Performance**

The feasibility of direct unheated anaerobic treatment of raw sewage was investigated in bioreactor R6 over 4 operational phases (Phase 1-4) as outlined in Table 4.1.

During the initial phase of operation the bioreactor adapted quickly to the influent with COD removal being recorded within 5 days (Fig. 4.4). Phase 1 was characterised by fluctuations in influent strength as a result of unsettled weather conditions experienced locally, this resulted in similar responses in effluent quality. However this trend ceased from day 16, with greater
consistency in effluent quality being observed. The remainder of Phase 1 recorded average COD\textsubscript{tot} concentration of 135 mg l\textsuperscript{-1}, with COD\textsubscript{sol} concentrations exhibiting similarly low values, averaging marginally above 100 mg l\textsuperscript{-1} for the phase. Biogas methane content improved incrementally as the phase progressed (Fig. 4.6).

The introduction of Phase 2 which involved a decrease of HRT to 12 h resulted in an instant decrease in effluent quality with a COD\textsubscript{tot} concentration of 245 mg l\textsuperscript{-1} being recorded on day 38; this effect appeared only transitory as effluent quality returned rapidly to the level recorded prior to Phase 2 introduction. Fluctuations in influent strength were observed during Phase 2 however despite these continuing fluctuations in influent COD, effluent quality remained consistent. The improved performance observed as Phase 2 progressed resulted in a high quality effluent being obtained during the final 15 days of phase operation; mean COD\textsubscript{tot} and COD\textsubscript{sol} of 81 mg l\textsuperscript{-1} and 52 mg l\textsuperscript{-1} respectively.

The consistent performance which was a feature of Phase 2 continued in Phase 3 with the decrease in HRT to 8 h displaying no adverse effect on removal efficiencies. Stable influent strength was a feature of Phase 3 and while it may be considered low; the high quality effluent obtained during Phase 2 persisted; with <100 mg l\textsuperscript{-1} COD\textsubscript{tot} representing >60% COD\textsubscript{RE}\textsubscript{tot} being recorded. Biogas quality was directly related to influent COD and the fluctuations observed in influent strength were correspondingly translated in the recorded % methane in the biogas. The mean effluent COD\textsubscript{tot} concentration for Phases 2 and 3 was 111 mg l\textsuperscript{-1}.

The final operational change of the trial was the introduction of Phase 4 which involved a decrease in HRT to 6 h, the increase in influent strength observed towards the conclusion of Phase 3 continued during Phase 4, with a mean influent COD\textsubscript{tot} of >550 mg l\textsuperscript{-1}. The increased influent strength resulted in increased biogas quality and yield. The combination of increased influent strength and decreased HRT attributed to a decrease in effluent quality. Mean effluent quality was 200 mg l\textsuperscript{-1} COD\textsubscript{tot}, representing >63% COD\textsubscript{RE}\textsubscript{tot}. 
Figure 4.4 R6 Phase 1-4 chemical oxygen demand concentration; (a) total and (b) soluble; influent (▲), effluent (□); COD removal efficiency (—×—).
In summary, the influent concentration fluctuated greatly, in the range of 163-897 mg l\(^{-1}\) for COD\(_{\text{tot}}\) (mean: 436 mg l\(^{-1}\), devst: 179). Influent COD\(_{\text{sol}}\) ranged from 78-340 mg l\(^{-1}\) (mean: 159 mg l\(^{-1}\), devst: 56). Thus, particulate COD comprised approximately 64% of the total COD. The effluent COD\(_{\text{tot}}\) was in the range 62-280 mg l\(^{-1}\) (mean: 159 mg l\(^{-1}\), devst: 61), effluent COD\(_{\text{sol}}\) ranged 50-204 mg l\(^{-1}\) (mean: 104 mg l\(^{-1}\), devst: 45). Effluent quality was stable throughout the trial and in particular during Phases 2 and 3. Mean COD\(_{\text{tot}}\) and COD\(_{\text{sol}}\) removal was 60% and 32%, respectively. Effluent VFA concentrations were negligible with only acetic acid being occasionally detected above the detectable limit of analysis (1 mg l\(^{-1}\), Table 4.2). Biogas methane content was greatly affected by influent COD strength and averaged 24% (range: 1-45%).

**R7 Process Performance**

R7 was employed to treat settled sewage over 5 operational phases (Phase 1-5), as outlined in Table 4.1. This is the liquid fraction following settlement of primary sludge from raw sewage. The characteristics of this wastewater were considerably different to the R6 influent (raw sewage). The settling out of the majority of solids resulted in a reduced influent polluting potential (COD) and phosphorus concentration.

A successful start-up was achieved for R7 at the applied HRT of 24 h. Within 16 days of operation effluent was of a high quality and remained so for the rest of the phase with mean effluent COD\(_{\text{tot}}\) and COD\(_{\text{sol}}\) <100 mg l\(^{-1}\) (Fig. 4.5). An immediate deterioration in effluent quality was observed upon the decrease in HRT (Phase 2), however this drop in performance was temporary, as stable performance was observed for the remainder of Phase 2.

The decrease in HRT on initiation of Phases 3 had little effect on system performance, with effluent quality remaining high, mean COD\(_{\text{tot}}\) concentration over Phases 2 and 3 of <78 mg l\(^{-1}\). The decrease in HRT to 6 h (Phase 4) had no immediate effect on effluent quality with mean effluent COD\(_{\text{tot}}\) concentration remaining low for the initial 10 days of Phase 4 (59 mg l\(^{-1}\)). The increasing influent strength in terms of COD concentration which was observed with the raw sewage trial (R6) from day 90 onwards was also observed with the settled sewage trial. This resulted in decreased effluent...
quality for the latter half of Phase 4 (COD$_{\text{tot}}$ 194 mg l$^{-1}$). However, this decline ceased towards the conclusion of the Phase 4.

Phase 5 involved a further drop in HRT to 3 h, influent COD concentration remained consistent from that recorded towards the end of the previous phase as did effluent quality, as the final phase progressed effluent quality improved returning a mean effluent COD$_{\text{tot}}$ concentration of 146 mg l$^{-1}$, with COD$_{\text{sol}}$ also improving as the phase concluded (105 mg l$^{-1}$).

To summarise, as with R6, the influent concentration fluctuated greatly, in the range of 62-360 mg l$^{-1}$ for COD$_{\text{tot}}$ (mean: 190 mg l$^{-1}$, dev$_{\text{st}}$: 75). Influent COD$_{\text{sol}}$ ranged from 50-240 mg l$^{-1}$ (mean: 134 mg l$^{-1}$, dev$_{\text{st}}$: 52). Thus, particulate COD comprised approximately 29% of the total COD. The effluent COD$_{\text{tot}}$ was in the range 50-208 mg l$^{-1}$ (mean: 109 mg l$^{-1}$, dev$_{\text{st}}$: 48), effluent COD$_{\text{sol}}$ ranged 50-200 mg l$^{-1}$ (mean: 85 mg l$^{-1}$, dev$_{\text{st}}$: 39). Effluent quality was relatively stable throughout the trial and in particular during Phases 1-4. Mean COD$_{\text{tot}}$ and COD$_{\text{sol}}$ removal was 41% and 34% respectively. As with the raw sewage trial effluent, VFA concentrations were negligible, with only acetic acid being detected above the detectable limit of analysis (1 mg l$^{-1}$, Table 4.2) during Phase 5. As a result of the low influent COD concentration biogas methane content was low throughout the trial, with significant values being recorded only during Phases 1 and 5 (Fig. 4.6). Mean biogas methane concentration was 2% (range, 0.1- 10%).
Figure 4.5 R7 Phase 1-4 chemical oxygen demand concentration (a) total and (b) soluble; influent (♦), effluent (○); COD removal efficiency (---×---).
Figure 4.6 Biogas methane concentration; R6 (■) and R7 (○). Dashed line indicates Phase 5 of the settled sewage trial (R7).

4.3.2 Phosphorus Analysis

Phosphorus (PO$_4^{3-}$) measurements of both influent and bioreactor effluent were carried out and suggested the occurrence of anaerobic phosphorus uptake/accumulation by the bioreactor biomass. R6 influent phosphorus concentration ranged 6-29 mg l$^{-1}$ (mean: 17 mg l$^{-1}$, devst 6), while effluent concentration ranged 1-10 mg l$^{-1}$ (mean: 4 mg l$^{-1}$, devst 2) representing a mean influent phosphorus attenuation of >76% (Table 1; Fig. 4.7a). The influent phosphorus load of the settled sewage trial (R7) was much reduced from that of the raw sewage influent (R6), as a result of the settling out of solids and possible use of a coagulant at the Mutton Island WWTW. However, phosphorus attenuation during the five phases of operation was similarly observed (Fig. 4.7b). R7 influent phosphorus concentration ranged 1-16 mg l$^{-1}$ (mean: 6 mg l$^{-1}$, devst 4), while effluent concentration ranged 0-9 mg l$^{-1}$ (mean: 3 mg l$^{-1}$, devst 2), representing a mean reduction of 50%. Fluorescent microscopy of DAPI-stained bioreactor granules, following conclusion of the trial, indicated polyphosphate accumulations on the surface of the granular biomass of R6 and R7 (Figs. 4.8b and c respectively). Staining of individual cells showed no indication of intracellular polyphosphate inclusions (Fig.
4.8a) which appear as bright yellow globules known as volutin or metacromatic granules within the cell (Tijssen et al., 1982; Kjeldstad et al., 1991; Kawaharasaki et al., 1999; Liu et al., 2001).

Figure 4.7 Phosphorus concentration (a) R6; influent (▲), effluent (□) and (b) R7; influent (♦) effluent (○).
4.3.3 Secondary Effluent Treatment

Processing of R6 bioreactor effluent was carried out towards the completion of Phase 4 of the raw sewage trial (day 124-140). As the HRT was reduced to 6 h, bioreactor effluent quality decreased and hence effluent filtration was investigated by tangential flow filtration in order to recover an effluent of discharge quality in terms of COD concentration (125 mg l⁻¹, [S.I. No. 419/1994, 254/2001, 440/2004]), as was previously achieved by the bioreactor during Phases 2 and 3. The CODₜₒᵗ concentrations of the raw sewage influent, bioreactor effluent and TFF permeate are presented in Figure 4.9a, COD removal efficiency of both bioreactor and overall, i.e. bioreactor plus TFF system are presented in Figure 4.9b. While the majority of CODₜₒᵗ was removed by the bioreactor, the TFF system supplemented removal efficiency, with mean removal of CODₜₒᵗ from the bioreactor effluent of >42%. TFF processing increased overall CODₜₒᵗ by a mean of >16%, representing a mean overall COD removal efficiency of 78% (range: 58-89%) (Fig. 4.9b). The permeate CODₜₒᵗ from the TFF system ranged from 82-204 mg l⁻¹, with a mean of 122 mg l⁻¹ (Fig. 4.9a). In general, fluctuations in TFF permeate displayed a similar trend to the bioreactor effluent, however, after the initial 5 days of membrane processing, the TFF permeate displayed stable CODₜₒᵗ concentrations, 82-122 mg l⁻¹. The greatest stability observed by the TFF system was following the initial exposure of the filter cassette to the bioreactor effluent (day 5 onward), this may be attributed to increased
bioreactor effluent quality, however on days 136-138 bioreactor effluent was similar to concentrations observed during the first 5 days of processing, despite this TFF permeate remained stable at 122 mg l\(^{-1}\) COD\(_{\text{tot}}\).

**Figure 4.9** R6 membrane processing of bioreactor effluent (a) total chemical oxygen demand concentrations; influent (▲), bioreactor effluent (□), TFF permeate (■) and (b) chemical oxygen demand removal efficiency; bioreactor (○), bioreactor + TFF (●).
The initial membrane processing runs of bioreactor effluent resulted in a sharp drop in permeation flux; within 48 hr operation (8 processing runs) membrane flux had decreased by 50%. Following this dramatic decrease, flux rates stabilised somewhat for the following 5 days of operation (20 processing runs). This coincided with the observed greater stability of bioreactor effluent and TFF permeate COD\textsubscript{tot} concentrations. From day 131 to the completion of the trial the permeate flux rate decreased gradually, this decrease was also translated through increased transmembrane pressure. The membrane was operated without cleaning for 17 days, at which point permeate flux had reached $<10 \text{ l m}^{-2} \text{ h}^{-1}$ (Fig. 4.10).

![Figure 4.10](image_url)

**Figure 4.10** Membrane permeate flux and transmembrane pressure (TMP) rates.
4.3.4 Physiological Characterisation of Biomass

The values obtained from specific methanogenic activity testing of the seed inoculum (day 0), and bioreactors biomass on days 86, 141 (R6) and 149 (R7) at 12°C and 37°C are outlined in Table 4.3

Table 4.3 Specific methanogenic activity (ml CH₄ g [VSS]⁻¹ d⁻¹) of seed inoculum and bioreactor biomass from R6 and R7.

<table>
<thead>
<tr>
<th>Day</th>
<th>Biomass</th>
<th>Test temp. (°C)</th>
<th>Ethanol</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate</th>
<th>H₂/CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12°C</td>
<td>37°C</td>
<td>12°C</td>
<td>37°C</td>
<td>12°C</td>
<td>37°C</td>
</tr>
<tr>
<td>Seed</td>
<td>GSB</td>
<td>1.7 ±0.2</td>
<td>2 ±0.3</td>
<td>1.3 ±0.1</td>
<td>1.8 ±0.2</td>
<td>4.6 ±0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.8 ±1.3</td>
<td>19.7 ±3.1</td>
<td>1.9 ±0.4</td>
<td>2.5 ±0.1</td>
<td>11.4 ±0.3</td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>R6 GSB</td>
<td>5.1 ±0.8</td>
<td>5.4 ±0.7</td>
<td>1.1 ±0.2</td>
<td>1.8 ±0.1</td>
<td>23.9 ±0.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.9 ±0.4</td>
<td>56.8 ±3.9</td>
<td>1.5 ±0.1</td>
<td>2.5 ±0.5</td>
<td>85.9 ±5.8</td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>R7 GSB</td>
<td>3.6 ±0.1</td>
<td>3.3 ±0.1</td>
<td>1 ±0.1</td>
<td>1.7 ±0.1</td>
<td>7.7 ±0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.1 ±0.3</td>
<td>30.1 ±0.1</td>
<td>1.5 ±0.1</td>
<td>3.8 ±0.2</td>
<td>74.2 ±3.1</td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>R6 GSB</td>
<td>7.7 ±0.5</td>
<td>8.3 ±0.4</td>
<td>1.6 ±0.3</td>
<td>2.9 ±0.6</td>
<td>7.1 ±0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.8 ±2.0</td>
<td>49.6 ±1.9</td>
<td>1.5 ±0.1</td>
<td>4.5 ±0.1</td>
<td>53.1 ±0.1</td>
<td></td>
</tr>
<tr>
<td>149</td>
<td>R7 GSB</td>
<td>4.3 ±0.2</td>
<td>4.2 ±0.3</td>
<td>1.2 ±0.1</td>
<td>1.9 ±0.1</td>
<td>6.3 ±0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.1 ±0.8</td>
<td>30.3 ±0.7</td>
<td>2.6 ±0.1</td>
<td>3.9 ±0.1</td>
<td>69.9 ±5.2</td>
<td></td>
</tr>
</tbody>
</table>

GSB – granular sludge bed biomass; All values are the mean of triplicates ± standard deviation.

4.3.4.1 Seed Inoculum Methanogenic Activity (day 0)

SMA values obtained from the mesophilic seed biomass suggested reduced activity against all substrates when tested at 12°C than those recorded at 37°C (Table 4.3). Decreases in methanogenic activity against propionate and butyrate at 12°C were less pronounced than for the other substrates tested. Values obtained against the direct methanogenic substrates acetate and H₂/CO₂ at 37°C suggested the dominance of aceticlastic methanogens, this was in contrast with the values obtained at 12°C which returned greater activity against H₂/CO₂.

4.3.4.2 R6 and R7 Methanogenic Activity (day 86)

SMA assays carried out on day 86 assessed the adaptation of the seed inoculum to the influent applied, bioreactor operating temperature and the operational changes associated with Phases 1 and 2. For both the R6 and R7 biomass, tested at the bioreactor operational temperature of 12°C, increases in potential activity were observed for all substrates with the exception of
propionate and butyrate, where no significant change was recorded suggesting the development of a psychrotolerant biomass. Increases in SMA were more evident for the R6 biomass; the more concentrated influent (raw sewage) presumably offering higher substrate concentration abundant for the various trophic groups involved in the AD process. Assays against the direct methanogenic substrates at 12°C indicated that a predominantly hydrogenotrophic methanogenic community was active in both bioreactors R6 and R7. A >4:1 and >2:1 hydrogenotrophic:acetoclastic methanogenic activity was recorded for R6 and R7 respectively. The increased hydrogenotrophic methanogenic activity was particularly evident for R6 where a 5-fold increase in activity from the seed inoculum against H2/CO2 was observed. Biomass from both bioreactors tested at 37°C returned greater values than those recorded at 12°C with similar relative values against test substrates being observed between the two test temperatures.

4.3.4.3 R6 and R7 Methanogenic Activity (day 141 and 149 respectively)
SMA testing of bioreactor biomass samples at their operational temperature (12°C) on completion of the trial (R6 day 141, R7 day 149) displayed increased activity against the indirect methanogenic substrates from those observed on the previous test day suggesting both bioreactors contained biomass which is adapted to their respective influents and also to the low-temperature conditions applied. The increased influent strength observed for both bioreactors during Phase 4 (R6 and R7) and Phase 5 (R7) may have also contributed to the improved activity of the various trophic groups, through greater substrate availability. The apparent shift to more prevalent hydrogenotrophic methanogenic activity which was observed on day 86 appeared reduced on testing of the takedown biomass with decreases in H2/CO2 mediated methanogenic activity being recorded, this was coupled with increased activity against acetate suggesting the emergence of a more balanced methanogenic community structure; this was particularly evident in R6. Indeed values obtained suggest a slightly greater affinity for acetoclastic methanogenesis in the R6 biomass tested at 12°C. On day 141 the potential SMA against propionate and butyrate of the R6 biomass at 12°C was greater than that observed with the seed biomass further confirming the development
of the psychroactivity observed on day 86. Once again SMA was greatly increased when the biomass was tested at 37°C for both R6 and R7.

4.3.5 Biomass Properties

Volatile suspended solids analysis was carried out periodically throughout the bioreactor trials. The VSS and structural integrity of the granular biomass was maintained throughout the trial. The VSS concentration of the bioreactor biomass of R6 and R7 decrease on takedown was 5% and 7% respectively (Table 4.4). No operational loss of biomass was recorded during both bioreactor trials, with temporal sampling the only source of biomass diminishment. The total volume of VSS recorded within each bioreactor throughout the trials showed only marginal decreases.

<table>
<thead>
<tr>
<th>R6 Phase</th>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g VSS $\text{GSM}^1$</td>
<td>94</td>
<td>83.9</td>
<td>86.4</td>
<td>84.7</td>
</tr>
<tr>
<td></td>
<td>g VSS $\text{Reactor}^{-1}$</td>
<td>58</td>
<td>52.0</td>
<td>53.6</td>
<td>52.5</td>
</tr>
<tr>
<td></td>
<td>g VSS $\text{Reactor}^{-1}$</td>
<td>20</td>
<td>17.9</td>
<td>18.5</td>
<td>18.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R7 Phase</th>
<th>Day</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g VSS $\text{GSM}^1$</td>
<td>94</td>
<td>85.3</td>
<td>82.5</td>
<td>85.1</td>
<td>80.4</td>
</tr>
<tr>
<td></td>
<td>g VSS $\text{Reactor}^{-1}$</td>
<td>58.0</td>
<td>52.9</td>
<td>51.2</td>
<td>52.8</td>
<td>49.8</td>
</tr>
<tr>
<td></td>
<td>g VSS $\text{Reactor}^{-1}$</td>
<td>20.0</td>
<td>18.2</td>
<td>17.6</td>
<td>18.2</td>
<td>17.2</td>
</tr>
</tbody>
</table>

GSB - granular sludge bed biomass.

4.3.6 Clone Library Analysis

4.3.6.1 Archaeal 16S rRNA Clone Library Analysis

ARDRA identified 23 different operational taxonomic units (OTUs) from archaeal clone libraries; 7 OTUs from the seed inoculum, 6 OTUs from bioreactor R6 on day 141 and 10 OTUs from bioreactor R7 on day 149 (Figs. 4.11 and 4.12).

The archaeal community of the seed inoculum could be divided into two distinct clusters, namely the *Euryarchaeota* and *Crenarchaeota*.
Euryarchaeotal clones consisted of two orders *Methanosarcinales* (family; *Methanosetaecae*) and *Methanobacteriales*. *Methanosaeta*-like organisms appeared to dominate the archaeal community of the seed inoculum (72% of clones), this also appeared to be the case for R6 and R7 bioreactor biomass on completion of the trial (92% and 84% respectively), with the acetoclastic methanogen; *Methanosaeta* like clones displaying an increase in relative abundance in both bioreactors. In contrast, clones with strong sequence-similarity to *Methanobacteriales*-like organisms (hydrogenotrophic methanogens) represented 12% of the seed inoculum clone library; however a significant decrease was observed in relative abundance in the bioreactor biomass of R6 and R7 at 3% and 2% respectively.

*Crenarchaeota*-like clones identified were highly similar to members of the Group 1.3 division of the *Crenarchaeota*, or Group 1.3b as proposed by Ochsenreiter *et al.* (2001). *Crenarchaeota* comprised one OTU in the seed sludge (clone FJ005022), representing 16%. Clonal analysis of the R6 biomass indicated a dramatic decrease in the relative abundance of this non-methanogenic *Archaea*, with *Crenarcheota* like clones representing just 3% of the library, while the R7 biomass displayed only a slight decrease at 12%.
Figure 4.11 Phylogeny of archaeal 16S rRNA gene sequences obtained from the seed inoculum (Seed) and bioreactor (R6 and R7) clone libraries calculated using the Maximum Likelihood algorithm (Felsenstein, 1981). GenBank accession numbers of the reference and clonal sequences from this study are indicated and the relative abundance (%) of each OTU is shown in parentheses.
FJ005025, 9%
FJ005023, 18%
FJ005021, 11%
FJ005020, 13%
FJ005019, 21%
FJ005022, 16%
FJ005024
Methanobacteriales
GU982664, 3%
GU982665, 3%
GU982662, 33%
GU982663, 57%
GU982666, 2%
GU982667, 2%
Methanosarcinales
72%
Methanosarcinaceae
GU982667, 2%
Methanosarcinales
94%
Methanaesetaceae
GU982666, 2%
Methanosarcinales
72%
Methanosarcinales
94%
Methanosarcinales
72%
Figure 4.12 Comparative distribution of archaeal OTUs retrieved from (a) seed inoculum (day 0); (b) bioreactor biomass R6 (day 141) and (c) bioreactor biomass R7 (day 149).

4.3.6.2 Bacterial 16S rRNA Clone Library Analysis
ARDRA identified 87 different operational taxonomic units (OTUs) from bacterial clone libraries; 16 OTUs from the seed inoculum, 34 OTUs from bioreactor R6 on day 141 and 37 OTUs from bioreactor R7 on day 149 (Figs. 4.13 and 4.14).

Seed Inoculum
The seed inoculum clone library consisted of sequences related to the Bacteroidetes (33%), Firmicutes (27%), Chloroflexi (18%), Actinobacteria (11%), δ-proteobacteria (8%) and Spirochaetes (3%) groups (Figs. 4.13 and 4.14a). The dominant group consisting of Bacteroidetes-like clones was divided into two family subgroups, viz. Porphyromonadaceae and Bacteroidetes. The phylum Firmicutes was represented by sequences related to the obligate anaerobes Clostridia, order Clostridiales.

R6 Biomass
On day 141, the bacterial clone library of R6 appeared more diverse to that observed in the seed inoculum however was similarly dominated by sequences related to the Bacteroidetes (36%) and Firmicutes (30%) (Figs. 4.13 and 4.14b). All of the groups identified in the seed inoculum were
present with the exception of the *Actinobacteria*, with none of the OTUs retrieved on day 141 representing this group. Clonal sequences affiliated within the *Proteobacteria* observed in the seed inoculum were located in the δ-subclass, however by the completion of the trial the group appeared to diversify with representatives of the α-, β- and δ-proteobacterial divisions present on day 141. During the trial, the relative abundance of the *Chloroflexi* decreased to 5%, with only the family *Anaerolineaceae* being identified. OTUs representing the candidate division *TM7* emerged and appeared to supersede the *Actinobacteria* by the completion of the trial, representing 9% of the clone library. Other groups to emerge included the *Planctomycetes* (1%), *Synergistetes* (2%) and *Thermotogae* (1%).

**R7 Biomass**

The bacterial clone library of R7 on day 149 was similar to the R6 biomass in that sequences related to the *Bacteriodetes* and *Firmicutes* dominated the community, however *Firmicutes* had a greater relative abundance in the R7 biomass (Figs. 4.13 and 4.14c). The emergence of the *TM7* group and the representatives of the *Planctomycetes*, *Synergistetes* and *Thermotogae* which emerged in R6 was not a feature of the R7 biomass. The *Actinobacterial* clones which were present in the seed inoculum but which were not retrieved in the R6 biomass were maintained in R7 albeit at a decreased relative abundance at 5%. The diversification of the *Proteobacteria* observed in R6 was similarly observed in R7. A *WS6*-affiliated clone, isolated previously from a contaminated aquifer and anaerobic sediment environments (Dojka et al., 2000), was also detected. *Spirochaete*-like OTUs had a greater relative abundance in R7 than that observed in either the seed inoculum or R6 biomass.

Overall, the majority of sequences retrieved from clone library analyses of the seed sludge and R6-R7 bioreactors were related to uncultured bacteria from diverse anaerobic habitats.
Figure 4.13 (a) Phylogeny of bacterial 16S rRNA gene sequences obtained from the seed inoculum (Seed) and bioreactor (R6) clone libraries calculated using the Maximum Likelihood algorithm (Felsenstein, 1981). *GenBank* accession numbers of the reference and clonal sequences from this study are indicated and the relative abundance (%) of each OTU is shown in parentheses.
Figure 4.13 (b) Phylogeny of bacterial 16S rRNA gene sequences obtained from the seed inoculum (Seed) and bioreactor (R7) clone libraries calculated using the Maximum Likelihood algorithm (Felsenstein, 1981). GenBank accession numbers of the reference and clonal sequences from this study are indicated and the relative abundance (%) of each OTU is shown in parentheses.
Figure 4.14 Comparative distribution of bacterial OTUs retrieved from (a) seed sludge (day 0); (b) bioreactor R6 biomass (day 141) and (c) R7 bioreactor biomass (day 149).

4.4 Discussion

4.4.1 Bioprocess Performance

The current study demonstrated the successful direct anaerobic treatment of raw sewage (R6) and settled sewage (R7) in EGSB-AF hybrid bioreactors operated at 12°C. A rapid start-up was observed for both bioreactors indicating the successful adaptation of the mesophilic seed inoculum to low-temperature conditions and the supplied influent. It should be noted, that when treating a wastewater with such a variable and fluctuating strength, % removal can sometimes be misleading indicator of performance; a set effluent quality (COD\textsubscript{tot} concentration) as a performance goal is a more relevant parameter to report. Both bioreactors achieved effluent of discharge quality in terms of COD\textsubscript{tot}.
No. 419/1994, 254/2001, 440/2004])}, with concentrations of ≤125 mg l⁻¹ (Figs. 4.4a and 4.5a). R6 achieved this standard at HRTs of 6-24 h and OLRs of 0.3-2.2 kg COD m⁻³ d⁻¹, and R7 at HRTs of 3-24 h and OLRs of 0.2-2.4 kg COD m⁻³ d⁻¹ with concurrent methane production. Despite the fluctuations observed in influent CODₜot, following a short start-up period (15-16 days) effluent CODₜot was very consistent. The bioreactors demonstrated rapid adaptability to changes in HRT and influent strength, thereby indicating robust anaerobic systems capable of withstanding the perturbations associated with municipal wastewater treatment systems; as a result of the cyclical nature of human activities and seasonal variations. The majority of research into direct anaerobic treatment of sewage at low-temperatures has concentrated on the UASB bioreactor design, or indeed two stage approaches, where the primary stage is aimed at removal and partial hydrolysis of suspended COD and the second stage mainly for conversion of dissolved COD to methane (Wang, 1994; Sayed and Fergala, 1995; Zeeman et al., 1997; Elmitwalli et al., 2002; Mahmoud et al., 2004). However this two-stage approach encounters challenges at high loading rates, namely the accumulation of suspended solids in the first stage, while the removal of colloidal COD is also limited (Elmitwalli et al., 2002; Mahmoud et al., 2004). The results presented in the current study represent an efficient one-stage approach for the anaerobic pre-treatment of sewage at 12°C. The performance in terms of COD₅₉₈₉°₅tot and effluent CODₜot concentrations achieved by the EGSB-AF hybrid system treating raw sewage (R6) compare favourably with results from previous one-stage LtAD (<15°C) sewage applications with the UASB bioreactor design (de Man et al., 1986; Maaskant et al., 1991; Bodik et al., 2000; Mahmoud et al., 2004; Gomec, 2005; Mahmoud, 2008; Gomec et al., 2008) and also with two-stage applications (Wang, 1994; Zeeman et al., 1997; Elmitwalli et al., 2002; Mahmoud et al., 2004). The results obtained at 13°C by Uemura and Harada, (2000) and Chunjuan et al. (2009) in UASB and EGSB bioreactors respectively, marginally exceed the results presented here; however those trials involved protracted staged decreases in operating temperature from 25°C. Elmitwalli et al. (2002) achieved 34% COD₅₉₉₉°₅tot employing a hybrid UASB bioreactor for sewage treatment at 13°C, demonstrating the effectiveness of the bioreactor design employed in the
current study. While successful treatment of settled sewage in (R7) was achieved, the strength (COD) of influent being treated was low (often below discharge standard), therefore offering little recompense in terms of bioenergy (methane) produced. Some previous studies have demonstrated the successful treatment of pre-settled sewage (van der Last and Lettinga, 1992; Elmitwalli et al., 1999), however the influent concentrations being treated were much greater for those trials than observed in the current study.

It is important to state that the application of LtAD for sewage treatment will not be a single stage full treatment process. Rather, LtAD can act as an energy-producing core-technology in a de novo municipal wastewater treatment plant configuration, which will remove the great majority of organic pollutants, pathogens and oxygen demand from the wastewater. Foresti (2002) reported that effluents from anaerobic bioreactors treating domestic and municipal sewage rarely comply with discharge standards. While biodegradable organic compounds are effectively removed in anaerobic treatment, mineralized compounds like NH₄⁺, PO₄³⁻, and S²⁻ are left in the solution necessitating an additional post-treatment (Lettinga, 1996). Post-treatment involves the removal of pathogens and remaining organic matter as well as removal or recovery of nutrients, depending on local requirements and reuse potential (Kujawa-Roeleveld and Zeeman, 2006).

4.4.2 Secondary Effluent Treatment
The use of a microfiltration (MF) membrane (0.2µm) for secondary treatment of bioreactor (R6) effluent of LtAD of sewage was investigated, and proved successful in recovering an effluent of discharge quality in terms of CODₜₗ. MF achieved CODₜₗ removal in the range 40-160 mg l⁻¹ (mean: 90 mg l⁻¹), thereby increasing overall CODREₜₗ by a mean of >16% (Fig. 4.9), which compares favourably with a similar membrane coupled study under low-temperature conditions (Chu et al., 2005). While the membrane trial was relatively short the results obtained in the current study show the potential for the use of a TFF system for membrane processing of bioreactor effluent from the LtAD of sewage. The unique AH bioreactor utilised in this study and in particular the pumice stone filter section’s passive filtration properties helped to alleviate the problem of membrane fouling, which is regarded as the main
challenge for anaerobic membrane bioreactors (Choo and Lee, 1996; Elmaleh and Abdelmoumni, 1997; Choo et al., 2000; He et al., 2005). The adhesion of biomass and colloidal organic material to the membrane surface has been identified as one of the major obstacles for MBR (Choo and Lee, 1996); however, the retention of particulate matter, both biomass and particulate COD within the bioreactor minimised this issue. In addition, the design of the TFF system allowed for high flux rates to be applied, in turn facilitating high fluid flow velocities at the surface of the membrane; this high shear feature helps to counteract membrane fouling (Stephenson et al., 2000). The performance achieved by the anaerobic membrane bioreactor; up to 89% (mean: >78% COD\text{tot} removal) is in agreement with similar low-temperature applications (Wen et al., 1999; Chu et al., 2005; Ho and Sung, 2010; Smith et al., 2013).

4.4.3 Microbial Physiology
The SMA values obtained from the seed inoculum confirmed the mesophilic nature of the seed inoculum, with the reduced temperature of 12°C having an effect on the biomass with all substrates displaying a decrease in activity (Table 4.3). This was to be expected as the seed biomass was sourced from a full-scale bioreactor operating in a mesophilic temperature range and was not of particular concern as the development of low-temperature activity following exposure to low-temperature conditions in bioreactors has been previously documented (Enright et al., 2005, McKeown et al., 2009). Recorded values for the direct methanogenic precursors acetate and H\textsubscript{2}/CO\textsubscript{2} at 37°C on the seed inoculum indicated the dominance of acetoclastic methanogens producing CH\textsubscript{4} through the decarboxylation of acetate, as the primary route of methane production in favour of the reductive action of hydrogenotrophic methanogens acting on CO\textsubscript{2} with hydrogen. This contrasted with the recorded values of the seed inoculum tested at 12°C, which suggested the slight dominance of a hydrogenotrophic methanogenic population, however this may be as a result of increased gas solubility at lower temperatures (Lettinga et al., 2001). By day 86, SMA assays at 12°C and 37°C (R6 and R7) indicated that maximum potential methanogenesis was channelled through hydrogen, rather than acetate. Under low-temperature
conditions, the thermodynamics of hydrogen-mediated methanogenesis are greatly improved, resulting in the observed predilection towards a hydrogenotrophic methanogenic community (Conrad and Wetter, 1990).

In addition, in both the natural environment and in engineered systems, the rate of acetoclastic methanogenesis is reported to be more strongly affected by decreasing temperature than their hydrogen-utilising methanogenic counterparts (Kotsyurbenko et al., 1996; Nozhevnikova et al., 1997; Lettinga et al., 1999). The dominance of hydrogenotrophic methanogens in LtAD is not uncommon or indeed is a shift towards hydrogenotrophic methane production under low-temperature conditions (Enright et al., 2005; Collins et al., 2005; Connaughton et al., 2006; Syutsubo et al., 2008; McKeown et al., 2009). In addition, McHugh et al. (2003, 2004) suggested that hydrogenotrophic methane production was a feature of bioreactors operating under stressed conditions. By the trial completion SMA testing of the R6 biomass demonstrated the recovery of acetoclastic methanogenic activity with values at this point suggesting the emergence of a more balanced methanogenic community structure. The importance of a stable acetoclastic methanogenic community to the successful operation of anaerobic digesters has been well documented in terms of both methane production and granule integrity (Lettinga 1995; O’Flaherty et al., 1997). It has been reported that two thirds of methane is produced through acetate under anaerobic conditions (Zinder, 1993; Conrad, 1999) and acetoclastic methanogenesis has been widely reported as the dominant methanogenic pathway in low-temperature natural environments (Schulz et al., 1997; Falz et al., 1999; Fey and Conrad, 2000; Conrad, 2002) and engineered systems (McHugh et al., 2003; Akila and Chandra, 2007). One hypothesis put forward for this occurrence in low-temperature environments is increased production of acetate through enhanced homoacetogenic activity (Schulz and Conrad, 1996).

The enrichment of psychroactive acetogenic and methanogenic populations during low-temperature cultivation has been well documented (Collins et al., 2006; McKeown et al., 2009) and was evident in the current study by day 86 for both bioreactors when values recorded at 12°C for the direct methanogenic substrates acetate and H₂/CO₂ and the carbon
intermediate ethanol returned greater values than those recorded on the seed inoculum (day 0) at 12°C, and similarly on bioreactor takedown. SMA assays carried out using bioreactor biomass generally did not indicate the development of truly low-temperature (psychrophilic) microbial communities but rather suggested the development of a low-temperature tolerant mesophilic biomass. Biomass tested from both bioreactors on day 86 and on takedown returned greater activity against all test substrates when tested at 37°C indicating that the seed inoculum had retained its mesophilic nature. Recently some progress has been made in isolating and characterising psychrotolerant and psychrophilic methanogens from cold, natural environments (Franzmann et al., 1992, 1997; Simankova et al., 2003; Zhang et al., 2008), however to date there has been no report of methanogenic psychrophiles being isolated from engineered anaerobic environments such as anaerobic bioreactors. Rebac et al. (1995), have suggested that while psychrophiles are likely to be present in anaerobic granular sludge cultivated under low-temperature conditions, SMA testing is unlikely to elucidate them due to the greater numbers of mesophiles. In this context, it is suggested that longer trials under low-temperature conditions would be required to promote the selective enrichment, and ultimate emergence, of dominant psychrophilic populations (McKeown et al., 2009).

4.4.4 Phosphorus Removal
Phosphorus attenuation was recorded throughout both bioreactor trials, with >75% removal being recorded for R6 (Fig. 4.7a). Effluent total phosphorus values recorded during Phase 3-4 (R6 and R7) were within discharge standards to sensitive areas (Irish, S.I. No. 419/1994, 254/2001, 440/2004). Excess phosphate presenting in wastewater is one of the main causes of eutrophication (McGrath et al., 2001), the main removal techniques from wastewaters include physical, chemical, and biological methods. Analysis of bioreactor biomass indicated the accumulation of polyphosphate (polyP); DAPI staining displayed polyP deposits on the surface of the granular biomass. It has been shown in previous studies (Fuhs and Chen, 1975; Buchan, 1983; Lotter 1985; Mino et al., 1984, 1985) that polyphosphate-accumulating organisms (PAO) play an essential role for enhanced biological
phosphorus removal (EBPR) in the anaerobic-aerobic process. EBPR involves alternating anaerobic and aerobic periods, the anaerobic phase is characterised by PAO assimilating short chain fatty acid fermentation products such as acetate, producing intracellular polyhydroxybutyrate (PHB) with associated release of phosphorous from stored polyphosphates (poly-P), followed by an aerobic phase where stored PHB is metabolized, providing energy for new cell growth of PAO, resulting in intracellular poly-P uptake (Brett et al., 1997; van Loosdreacht et al., 1997; Mino et al., 1998). However, biological polyP uptake has not been previously reported under anaerobic conditions, moreover no intracellular polyP was identified by DAPI staining. This indicates that the polyP accumulation observed was not biological but merely due to physical adherence, and may be the result of the formation of metal polyP complexes (Wild et al., 1997) such as struvite (Marti et al., 2008). Batch assays to quantify these polyP deposits may help to elucidate this occurrence and the mechanism by which it occurs; determining if the adherence is physical, directly biological, or indirectly biological as a result of the biomass response to low-temperature conditions, operational conditions or substrate.

Interestingly greater phosphorus reduction was observed as the trial progressed and decreased HRTs were applied (Fig. 4.7). While polyP deposits were observed on the granular biomass, the greatest source of phosphorus removal is likely to be as a result of absorption to the pumice in the AF. Pumice has been found to be effective for the removal of phosphate ions from water (Onar et al., 1993, 1996), with compounds present in actual wastewater having no significant effect on the removal of phosphate. In recent years, considerable attention has been paid to low-cost adsorbents for adsorption of phosphate from wastewater (Liu et al., 2007) and the use of pumice as an adsorbent to remove metals and dyes from wastewater at low cost is a well-established process (Catalfamo et al., 2006). The structure of pumice stone contains open channels that allow water to move into and out of the crystal structure, the skeletal structure allows ions and molecules to reside and move within the overall framework (Yavuz et al., 2008). Adsorption is a technique which would be comparatively useful and economical for phosphate removal from sewage, offering advantages over the processes currently employed in
conventional activated sludge plants; EBPR, despite the high removal efficiencies which can be achieved the process is highly variable due to operational difficulties (Clark et al., 1997), while chemical precipitation incurs large costs and results in increased sludge volumes (Mullan et al., 2002). The phosphate load which the pumice can absorb is yet to be determined, however, the advantage of the modular filter unit employed in this study is that removal and recovery of valuable phosphate is possible with relative ease.

4.4.5 Molecular Microbial Ecology
4.4.5.1 Archaeal Community Structure
Archaeal clone library analysis carried out on completion of the bioreactor trials displayed a dominance of acetoclastic Methanosaeta-like ribotypes for both R6 and R7 (Figs. 4.13b and c) becoming even more dominant than that observed in the seed biomass. Due to their low acetate thresholds, Methanosaeta are typically associated with stable anaerobic systems and are believed to play an important role in the formation and maintenance of granular sludge in anaerobic bioreactors (Grotenhuis et al., 1991; Uemura and Harada, 1995; Griffin et al., 1998; McHugh et al., 2005). A Methanosarcina clone was identified in both the R6 and R7 biomass, however these represented just 2% of all archaeal clones. Methanosarcina have been frequently described in the methanogenic community of cold-terrestrial environments (Kotsyurbenko et al., 2004) and low-temperature anaerobic bioreactors (Collins et al., 2003; McHugh et al., 2004; Connaughton et al., 2006; Enright et al., 2007). Methanosaeta have a higher substrate affinity for acetate than Methanosarcina; VFA analysis of bioreactor effluent demonstrated that acetate concentrations remained low throughout the trial. Low acetate concentrations and low-temperature conditions may have offered a competitive advantage for Methanosaeta over Methanosarcina and other acetoclastic groups, resulting in their high relative abundance in both R6 and R7 (Griffin et al., 1998; Fey and Conrad. 2000; McMahon et al., 2004). Methanobacteriales appeared to become less important in R6 and R7 as both systems revealed a significant decrease in relative abundance from that observed in the seed biomass. Crenarcheota-affiliated sequences which had
a significant relative abundance in the seed inoculum appeared to have less importance in R6 accounting for just 3% of the clone library, however they prevailed to a greater extent in R7. *Crenarcheota* has been well documented in both mesophilic (Chouari et al., 2005; Levèn et al., 2007) and low-temperature anaerobic systems (Collins et al., 2005; McHugh et al., 2005), however the exact functional role of these organisms has yet to be fully elucidated. Collins et al. (2005) discovered a close association to *Methanoseata* spp. suggesting they may play a homoacetogenic type syntrophic role.

### 4.4.5.2 Bacterial Community Structure

Nelson et al. (2011) showed that the majority of the bacterial sequences in methanogenic bioreactors belonged to four “major” phyla, i.e. *Bacteroidetes, Chloroflexi, Firmicutes* and *Proteobacteria*. This was indeed the case for the seed inoculum with these phyla accounting for 86% of sequences retrieved (Fig. 4.14a). Clone libraries of R6 and R7 biomass on completion of the trials demonstrated a significant increase in diversity of bacterial phyla. (Figs. 4.14a and 4.14c). However core phyla (*Bacteroidetes, Firmicutes* and *Proteobacteria*) identified by many researchers in anaerobic mesophilic (Godon et al., 1997; Hernon et al., 2006; Liu et al., 2002) and low-temperature engineered systems (Collins et al., 2003; McKeown et al., 2009) remained dominant in both bioreactors.

The identification of phyla responsible for hydrolytic and fermentative conversions on a broad range macromolecules indicate a biomass which has adapted well to the subjected substrate. In particular the maintenance of a substantial *Bacteroidetes* population is important for the LtAD of sewage as they are involved in the hydrolytic and acidogenic step of anaerobic digestion (Délbes et al., 2000). Regueiro et al., (2012) showed that the higher the *Bacteroidetes* percentage (as semi-quantified by FISH) the higher the hydrolytic activity of the biomass.

*Chloroflexi* are in general well-represented in anaerobic engineered systems (Godon et al. 1997; Chouari et al., 2005) where they have been suggested to play an architectural role, promoting and maintaining granule integrity (Sekiguchi et al., 1999). They are also recognised for their role in
carbohydrate degradation (Sekiguchi et al., 2001). However the relative abundance of Chloroflexi decreased in both R6 and R7 from that of the seed inoculum. This is a similar finding to that observed in R4 and R5 (chapter 3), suggesting that their role is greatly diminished in low-temperature environments.

The phylum Firmicutes was well represented in both R6 and R7, with this phylum dominating bacterial sequences retrieved from R7 on day 149. Members of the class Clostridia are known organic acid oxidizers (Bunge et al., 2008) and may be involved in ethanol fermentation, as well as homoacetogenesis (Lee et al., 2008). Homoacetogenesis may indeed be the role of the Firmicutes in this current study. Archaeal clone library analysis suggested the almost complete dominance of acetoclastic methanogens in both R6 and R7 biomass, however SMA data on trial completion demonstrated that methanogenic activity was balanced between hydrogenotrophic and acetoclastic methanogenesis for R6, and SMA testing of R7 biomass on day 149 indicated that the maximum potential methanogenesis was channelled through hydrogen, rather than acetate.

The diversity of the phylum Proteobacteria was much greater in the R6 and R7 biomass than that observed in the seed biomass. OTUs affiliated with Syntrophobacter, which are known syntrophic propionate-oxidisers, were obtained from the seed, R6 and R7 biomass. Syntrophobacter and other Proteobacteria are important fatty acid oxidizers (Sousa et al., 2007) which are frequently observed in bacterial communities within anaerobic bioreactors (Chouari et al., 2005; Bafana et al., 2008; Siggins et al., 2012).

In addition to the relatively well-known taxa, phylotypes belonging to uncultured candidate phyla were detected. The emergence of the candidate division TM7 in R6 biomass is of particular interest. The novel candidate division TM7 has no cultivated representatives and has been predominantly characterized by environmental sequence data. This division takes its name from the German peat bog from which the first sequence was obtained (Rheims et al., 1996). Additional TM7 sequences have been deposited by several other investigators, demonstrating that members of this division are present in extremely diverse environments, including soil, activated sludges and in human subgingival plaque samples (Borneman and Triplett, 1997;
Hugenholtz et al., 2001; Paster et al., 2001). In situ analysis revealed that members of the uncultivated TM7 division are capable of up taking carbon substrates under aerobic and anaerobic conditions and surviving in a wide range of environments (Thomsen et al., 2002; Bertin et al. 2006). Ariesyady et al. (2007) confirmed through a MAR-FISH approach that TM7 members were positive for glucose mineralization while Nielsen et al. (2010) revealed that the phylum TM7 were important protein-hydrolyzing organisms.

The emergence of this candidate phyla is of particular interest in the current study as it may play a key role in the hydrolysis of sewage solids. TM7 emerged as a key bacterial phylogenetic group in R4 and R5 biomass (chapter 3) while treating synthetic sewage. Moreover, TM7-like sequences were not identified in the biomass of R7 which was treating settled sewage. The removal of settled solids from this wastewater may account for their absence. Clone library analysis from chapter 3 and the current chapter would suggest that the candidate group TM7 may play a key role in the low-temperature anaerobic treatment of raw sewage.

Overall bacterial clone library analysis demonstrated diverse bacterial communities in both R6 and R7. The complexity and wide range of organic substrates present in the sewage influent likely led to the emergence and development of diverse bacterial phyla in these low-temperature systems and the presence of key groups enabled the hydrolytic, proteolytic and fermentative degradation of the protein and carbohydrate constituents of the influent wastewater.

### 4.5 Conclusions

The approach taken in the present study and the results obtained suggest the low-temperature (12°C) anaerobic digestion of raw sewage in an EGSB-AF hybrid bioreactor is feasible at HRTs of 6-24 h with effluent of discharge quality in terms of COD concentration being consistently achieved at HRTs of 8-24 h with concurrent methane production. Settled sewage can be treated effectively at HRTs of 3-24 h. This study also demonstrated that:
• Stable and efficient LtAD of raw sewage (R6) and settled sewage (R7) is feasible at applied OLRs of 0.3-2.2 kg COD m\(^{-3}\) d\(^{-1}\) and 0.2-2.4 kg COD m\(^{-3}\) d\(^{-1}\) respectively;
• a mesophilic seed inoculum can adapt quickly to low-temperature conditions and can be successfully used for the start-up of a low-temperature bioreactor, without the requirement for stepwise temperature decreases to enable biomass adaptation;
• phosphorus attenuation during LtAD of sewage can be achieved;
• hybrid bioreactors incorporating an effective anaerobic filter can offer much improved performance and treatment over single stage UASB and EGSB bioreactors in terms of biomass retention and effluent polishing through solids/particulates entrapment;
• secondary effluent processing through microfiltration membranes can supplement overall COD removal by >16%;
• granulated pumice stone is a suitable packing material for the filter section of AH bioreactors, aiding biomass retention, suspended solids removal, phosphate removal;
• methanogenic communities were dominated by acetoclastic Methanosaeta-like ribotypes for both R6 and R7;
• a diverse bacterial community developed, with key hydrolytic fermentative bacterial populations present in both bioreactors, with a possible hydrolytic role for the candidate group TM7 being hypothesised.
4.6 References


CONCLUDING REMARKS AND FUTURE RECOMMENDATIONS
5.1 Concluding Remarks

The results presented in this thesis demonstrate at lab-scale the feasibility of LtAD for the treatment of municipal wastewater under Irish conditions. The results obtained are encouraging for future developments in this area and compare favourably with previous experience of direct LtAD for municipal wastewater. Stable bioprocess performance was demonstrated during the various trials, to a greater extend when an innovative hybrid EGSB-AF was employed (chapters 3 and 4). The successful adaptation of a mesophilic inoculum operating at sub-optimal temperature conditions was also evident during all trials. It is thought that the use of a pre-acclimatised seed inocula may reduce start-up times and improve stability of LtAD, however the mesophilic unacclimatised seed inocula employed in the current study demonstrated metabolic versatility to adapt quickly to low-temperature conditions with rapid start-up achieved during all trials. Indeed, the results gathered implied that the sub-mesophilic anaerobic treatment of municipal wastewater was facilitated by the development of low-temperature/psychro-tolerance within the inoculum while low-temperature mediated successional development of particular microbial groups was also evident. The enrichment of syntrophic and methanogenic psychroactivity was evident during this study, however the emergence of truly psychrophilic populations was not demonstrated. It remains to be seen if long-term low-temperature cultivation (longer trial periods) would promote the emergence of psychrophilic populations in the bioreactor biomass.

Molecular clone library analysis identified the acetoclastic Methanosaeta as the most prominent methanogenic archaeal group in the seed inoculum and all bioreactor biomass (chapters 3 and 4). SMA data of bioreactor biomass tested at operational temperature during all trials suggested a shift to hydrogenotrophic methanogenesis in the start-up phase (~100 days) of all trials. Subsequent SMA testing demonstrated a re-balancing of methanogenic activity or greater affinity for acetoclastic methanogenesis. This may suggest that during an acclimation period to low-temperature conditions, H2-mediated methanogenesis is favourable, and once acclimation is achieved acetoclastic methanogenesis begins to re-establish.
However the action of homoacetogens may also account in part for the H₂/CO₂ conversion observed in SMA profiles. An assessment of the various methods which studied methanogenesis in this trial tentatively suggest homoacetogenic activity in these LtAD systems. Homoacetogens are capable of a diverse range of metabolic transformations; demonstrating the ability to grow autotrophically on H₂/CO₂ or heterotrophically on a wide range of sugars, methyloxylated aromatic and single carbon compounds, generating acetate as the sole end-product. A failing of the SMA protocol used in this trial is the disregard for the role of homoacteogenic bacteria. Homoacetogens should in theory be out-competed by hydrogenotrophic methanogens, which have a much lower Ks value for H₂, however they are widely reported to persist in both natural and bioengineered anaerobic environments where they compete for H₂. An adaptation of the SMA protocol employing methanogenic inhibitors and measurements of acetate formation may help to elucidate the presence/absence of homoacetogenic activity.

The data obtained demonstrate the importance of bioreactor design and configuration for this AD application. The EGSB-AF hybrid bioreactor configuration compensated for the negative effects of low operational temperature on metabolic activity and rates of microbial growth. By applying effluent recirculation increased mixing was achieved, providing enhanced substrate-biomass contact thereby increasing mass transference rates. The configuration also provided a support matrix for the retention of biomass through entrapment, and also the development of fixed film growth on the support matrix. The material (granulated pumice stone) used in chapter 3 and 4 provided more stable operational performance to that recorded during chapter 2. The structural properties of pumice stone, namely high specific surface area, porosity and surface roughness contributed to the retention of biomass and particulate COD and the development of fixed film growth. Preliminary investigation into the use of membrane coupling with the EGSB-AF design offered an increase in overall COD removal efficiency of >16%. More research is needed to fully assess the feasibility of this setup.

Clone library, gene sequencing and phylogenetic analysis provided detailed information on the microbial community structure of the seed inoculum and the bioreactor biomass on trial completion (chapter 3 and 4).
Clone library analysis revealed metabolically diverse fermentative and hydrolytic bacterial populations had developed in the bioreactor biomass, with key phylogenetic groups being identified. Monitoring of temporal samples from the bioreactors though the use of practical, high-throughput techniques such as terminal restriction fragment polymorphism (TRFLP) may have shed light on the development of the resulting diverse bacterial community.

While COD concentrations achieved during chapter 3 and 4 were routinely below local discharge limits, it is important to state that the application of LtAD for municipal wastewater treatment will not be a single stage full treatment process. The results obtained indicate that LtAD can act as an energy-producing core-technology in a de novo municipal wastewater treatment plant configuration, where it will remove the majority of organic pollutants, pathogens and oxygen demand from the wastewater. Remediation of effluent nutrients, such as $\text{NH}_4^+$, $\text{PO}_4^{3-}$ will be required in most cases to achieve a final effluent which conforms to discharge licence limits.

### 5.2 Future Perspectives and Recommendations

While the results presented in this thesis provide a basis for optimism with respect to the future application of LtAD of municipal wastewater under Irish conditions, it is apparent that much basic and applied research remains to be carried out in the field.

**Recommendations**

- Longer term (>1 yr) laboratory- and pilot-scale trials with actual sewage, rather than synthetic will need to be carried out in order to fully assess the effect on performance of seasonal variation in influent strength and variation in ambient temperatures experienced locally.
- A comparative study investigating the potential benefits of using acclimated seed inoculum versus unacclimated inoculum should be carried out to demonstrate any process advantages which may be gained.
• A full assessment of external membrane coupling with the current bioreactor design to yield a high quality effluent should be carried out. The demonstration of long term, low cost operation of such membranes; in terms of membrane fouling, cleaning regime, applied flux rates, membrane material and the overall economics of this process could prove very attractive to industry.

• A basis for the phosphorus removal/attenuation observed during the LtAD of municipal wastewater which was noted during this study should be established. The mechanism of this occurrence should be determined, to discover if the process is merely physical, directly biological, or indirectly biological as a result of the biomass response to low-temperature conditions, operational conditions or substrate.

• Investigate in greater depth the microbial consortia responsible for the LtAD of municipal wastewater through the use of higher throughput molecular analysis tools and approaches (e.g. next generation sequencing).

• The use of targeted techniques such as micro-autoradiography-fluorescence in situ hybridization (MAR-FISH) to study the physiological properties of the microorganisms involved in LtAD could help to distinguish the in-situ metabolic function of key bacterial organisms within the microbial community. In addition the use of new and emerging disciplines such as functional metagenomics, and targeted metabolomics may provide valuable insight into the complex interactions which occur within LtAD systems.

Scale up and economics

• The future development of low-temperature anaerobic digestion will be dependent on the amalgamation of laboratory- and pilot-scale bioprocess data, preferably in conjunction with mathematical modelling of the process to generate performance and operational guidelines. In order to progress towards implementation of LtAD of
municipal wastewater, the process must be demonstrated at a scale convincing to the water industry and policy makers.

- A full cost-benefit analysis of LtAD for municipal wastewaters versus the CAS systems or alternative treatment approaches such as physiochemical technologies should be carried out. In the case of the treatment approach proposed in this study, the cost implicated with any tertiary treatment that may be required should also be taken into account. In addition the particular benefits of coupling sustainable bioenergy production with pollutant removal and reduced secondary sludge production, and the absolute requirement for humanity to address the linked issues of global warming and dwindling fossil fuel reserves should also be taken into account.