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INVESTIGATING THE TRUE LIMITS OF ANAEROBIC TREATMENT OF WASTEWATER AT LOW TEMPERATURE USING A COLD-ADAPTED INOCULUM

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September 2015

INVESTIGATING THE TRUE LIMITS OF ANAEROBIC TREATMENT OF WASTEWATER AT LOW TEMPERATURE USING A COLD-ADAPTED INOCULUM

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A Thesis Submitted for the Degree of Doctor of Philosophy

At Newcastle University

School of Civil Engineering and Geosciences

Newcastle upon Tyne

September 2015

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Abstract

Anaerobic batch reactors were inoculated with cold-adapted biomass (seed) to treat the organic material (COD) of domestic wastewater at 4, 8 & 15°C. The substrate was pre-UV sterilized to preclude competition between the cells thriving in the seed and the autochthonous, originated from wastewater cells. The performance in terms of organic removal showed that the specific cold-adapted inoculum efficiently treats anaerobically raw domestic wastewater at all temperatures based on the UWWTD (Urban Waste Water Treatment Directive) (91/271/EEC). The observed methanogenic taxa were *Methanomicrobiales*, *Methanosaetaceae*, and *Methanosarcina* during the whole experimentation. *Methanomicrobiales* were predominant at lower temperatures (4, 8°C) followed by *Methanosaetaceae*; at 15°C there was no distinct difference amongst them. Longer enrichment showed that further investigation may be required to clearly point the predominance between methanogens. Specific cellular activity was calculated (via qPCR, FISH) to enable scale-up & design simulation. The specific methanogenesis values showed that the activities at low temperatures are at least similar to those of typical mesophiles using a conservative cellular weighing reference to convert the cells to VSS. Higher specific activities were observed after acclimation of the cells at 4°C compared to 15°C regardless of the operational temperature (4 or 15°C). Acclimation at 4°C also resulted in a formation of a community that can be hardly disturbed from the competition of the wastewater cells when the seed:substrate ratio is low. This was not evident after acclimation at 15°C and it manifests that anaerobic treatment start-up at 4°C results in a sturdy and highly active methanogenic community. The COD_{RAW}:CH_4 conversion at 4°C was approximately 50% and reached up to 80% of the theoretically expected for sterile and non-sterile wastewater feed respectively. It is likely that the conversion was boosted from the synergy of the indigenous bacterial communities from wastewater and the cells originated from the seed. Enzymes (lipases) assays showed that the wastewater-originated group of cells (bacteria) contributed to the hydrolysis of insoluble organic material (lipids) and led to richer formation of intermediates that were subsequently utilized by the methanogenic populations of the seed. Limited lipid hydrolysis accounted for the organic material that remained insoluble. The lipases assays demonstrated that on equal temperatures (37°C) the specific activity of the enzymes secreted from the cells at low temperature (4°C) is higher than those secreted from cells at 15°C. This proves that the formation of a sturdier and of higher wastewater treatment performance community is likely when this is developed at low temperatures. The assay also demonstrated that a 4-degree temperature increase (from 4-8°C) is adequate to trigger the lipid:CH_4 bio-conversion. Thus, for a complete anaerobic wastewater treatment using the specific inoculum, the temperature limit lies in-between 4°C and 8°C. A scale up designation based on the differentiation of the specific methanogenic activity according to temperature shouted that this limit lies at 5°C. For operation at lower temperature (<5°C), the required vessel volume and the hydraulic retention time (HRT) become extremely high and consequently financially unattainable. The results suggest that inoculating digesters for low temperature operation with cold-adapted communities is a promising way to treat wastewater and an appropriate solution for the investigation of the process limits. Hence, my recommendation for successful low temperature carbon neutral wastewater treatment is the inoculation of anaerobic reactors with cold adapted or psychrophilic biomass strategy, acclimation at low temperature and operation at a temperature >5°C.

I n v e s t i g a t i n g t h e t r u e l i m i t s o f a n a e r o b i c t r e a t m e n t o f w a s t e w a t e r a t
l o w t e m p e r a t u r e u s i n g a c o l d a d a p t e d i n o c u l u m
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*"The impediment to action advances action.
What stands in the way becomes the way."*
— Marcus Aurelius ,121-180 A.D.

Contributions belong to the Thesis entitled: INVESTIGATING THE TRUE LIMITS OF ANAEROBIC TREATMENT OF WATEWATER AT LOW TEMPERATURE USING A COLD-ADAPTED INOCULUM

Evangelos Petropoulos

Newcastle upon Tyne (UK), September 2015

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l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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Contributions

1. The selected cold adapted biomass can, in principle, cope with extreme temperature conditions and render a wastewater effluent able to meet the UWWTD (91/271/EEC) COD standards for discharge on water surface at low temperatures (4, 8 and 15°C).

2. A safety factor for design purposes was estimated from the discrepancy between $COD_{removed}$ and COD_{CH4}. This disagreement suggests that the digester works partially as a clarifier the lower the temperature gets.

3. Specific rates for hydrolysis and methanogenesis of wastewater at 4, 8 and 15°C were calculated so they can be further used as fundamental parameters for applied engineering purposes.

4. The start-up of bio-reactors that operate at ambient or low temperature conditions needs to take place during cold periods as:

   - Acclimatization at 4°C results in a stable, sturdy community where hydrolysis limitation can be rapidly overcome during seasonal variation.
   - The biomass acclimatized to low temperatures produces more active enzymes than those secreted from the cells acclimatized to higher ones, when temperature increases.

5. The hydrolysis of lipids appears to be more temperature sensitive than the hydrolysis of proteins and carbohydrates. The inefficiency of lipids to be hydrolyzed could be a key factor that describes the sensitivity of methane production rates to temperature.

6. A 4-degree temperature increase (from 4-8°C) is adequate to trigger the COD_{lipid}:CH_4 conversion.

7. The anaerobic lipolytic activity in a bio-reactor operating at low temperatures is increasing by the addition of indigenous from raw wastewater communities.

8. Specific cellular activity as a function of temperature can assist in the estimation of the HRT and subsequently the volume of an anaerobic treatment tank. The relationship between HRT_t and HRT_{t-1} based on T_t, T_{t-1} respectively may nullify potential errors from a mistakenly selected HRT_t.

9. With regards to applicability, operation at 5°C is the lowest temperature limit of anaerobic wastewater treatment

Acknowledgements

I would like to express my gratitude to my supervisor, Professor Thomas P. Curtis, for the constant support during my studies. His ability to convey his enthusiasm and passion for research kept me motivated during the hard times. I would also like to thank Dr Jan Dolfing, Dr Russell J. Davenport and Dr Emma J. Bowen for the productive discussions and the assistance they offered me. Special thanks go to Dr Ziauddin Shaikh for the additional guidance at the beginning of my studies and the economist Mr Thomas Markopoulos who assisted and guided me with the regression analysis. I gratefully acknowledge the encouragement of my family and the patience of my friends throughout this journey. In addition I would also like to acknowledge the hospitality of the following cities during the writing of this thesis: Alfaro (Spain), Edessa (Greece) and Newcastle upon Tyne (UK). This research project was funded by the EPSRC (Reference No: EP/G032033/1).

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Nomenclature

General Terminology	
AD	Anaerobic Digestion
AF	Anaerobic Filter
AH	Anaerobic Hybrid
AnMBR	Anaerobic Membrane Bio Reactor
ASBR	Anaerobic Sequenced Batch Reactor
BOD	Biochemical Oxygen Demand
BW	Black Water
BWKW	Black Water Kitchen Waste
CLSM	Confocal Laser Scanning Microscopy
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
DGGE	Denaturating Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DPWW	Dairy Parlour Waste Water
EGSB	Expanded Granular Sludge Bed
F:M	Food to Microorganisms
FISH	Fluorescent In Situ Hybridization
FOV	Fields of View
G-AnMBR	Granular Anaerobic Membrane Bio Reactor
HRT	Hydraulic Retention Time
HUSB	Hydrolytic up flow Sludge Bed
Kcal	Kilo calories
kDa	Kilo Dalton
kJ	Kilo joules
LCFA	Long Chain Fatty Acids
LTAD	Low Temperature Anaerobic Digestion
MMB	*Methanomicrobiales*
MSA	Methanosulfonic Acid
MSH	Mineral media
MSC	*Methanosarcina*
MST	*Methanosaeta*
NA	Nutrient Agar
Nel	Number of electrons
OLR	Organic Loading Rate
PCR	Polymerase Chain Reaction
PGL	Polygalactorunase
pH	Power of Hydrogen
p-NPP	Para-nitrophenyl palmitate
qPCR	Quantitative Polymerase Chain Reaction
R2A	Agar Type
RNA	Ribonucleic Acid
RPF	Rigid Polyurethane Foam

S	Organic Material
SRB	Sulphate Reducing Bacteria
SRT	Sludge Retention Time
SVI	Sludge Volume Index
TOC	Total Organic Carbon
TSS	Total Suspended Solids
UASB	Up flow Anaerobic Sludge Bed
UV	Ultra Violet
UWWTD	Urban Waste Water Treatment Directive
VFA	Volatile fatty Acids
VSS	Volatile Suspended Solids
WW	Waste water
WWTP	Waste Water Treatment Plant
Xv	Bacterial Mass
Y	Yield

Chemical Compounds	
$C_4H_{6.1}O_{1.2}N)_x$	BSA protein
$C_6H_{12}O_6$	Glucose
$C_8H_{18}O$	Octanol
Ca	Calcium
$CH_3CH_2CH_2COOH$	Butyrate
CH_3CH_2COOH	Propionate
CH_3COOH	Acetate
CH_4	Methane
Co	Cobalt
CO_2	Carbon dioxide
Fe	Iron
FeS	Iron Sulphide
H^+	Proton
H_2	Hydrogen
H_2O	Water
H_2S	Hydrogen Sulphide
HCl	Hydrochloric Acid
HCO_3	Bicarbonate
KCl	Potassium Chloride
Mn	Manganese
N	Nitrogen
Na	Sodium
Ni	Nickel
P	Phosphorus
Pb	Lead

Contents

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
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Chapter 1: *Introduction – AD at low temperature, why?*
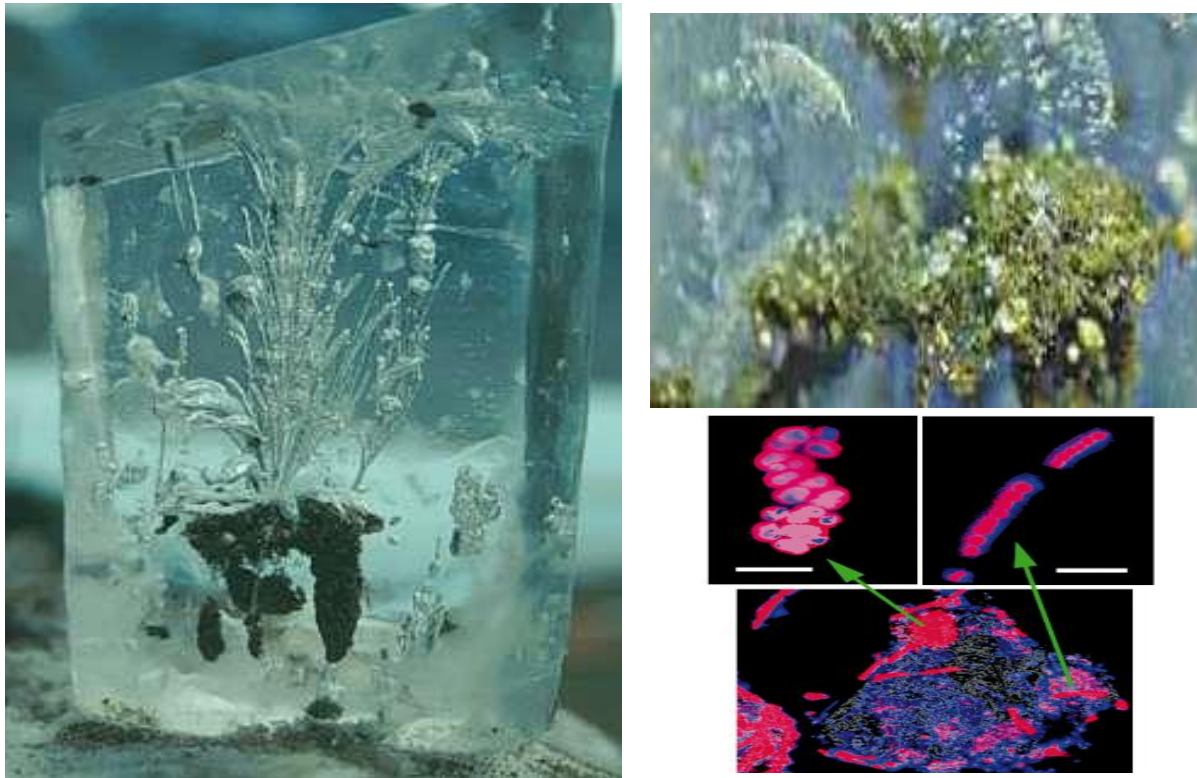
Figure 1.1 – left: Colony of Bacteria thriving a block of ice from Antarctica (Lake Bonney); right-up: same image, detail after zooming; right-bottom: Confocal laser photomicrographs from the same sample, showing microorganisms associated with a sediment particle, with enlarged views of two species of cyanobacteria (blue, DAPI-stained bacteria; red, chlorophyll auto fluorescence; gray, sediment particle); scale bars, 10mm (Priscu et al 1998).

1.1. General Introduction

2.5 billion people lack of sanitation (WHO & UNICEF 2012) and clean water is becoming the ultimate limiting resource (AWA/Deloitte 2011). As urban populations increase, water scarcity (WWAP 2012) becomes more widespread and prices inevitably increase (Figure 1.2) (UN Population Division 2008) (Figure 1.4.a, b) (WWAP 2012) (Figure 1.3), subsequently the need for water reuse, recycling and reclamation becomes more pressing. Wastewater, after treatment, is a source of water and can be returned to the natural environment ensuring no disturbance of the ecosystem (Water UK report – Wastewater Treatment and Recycling 2006).

Most wastewater treatment technologies are energy intensive and are becoming increasingly expensive as fuels and electricity prices increase (Figure 1.2) (Haarmayer, 2011). Energy neutral or positive wastewater treatment plants can, in principle, be developed as the energy in wastewater is far greater than the energy required to treat it (Heidrich et al 2011). Hence, the question is how we can re-use this energy rather than let it be lost. A convenient way is the bioconversion of the organic material in wastewater to biogas via anaerobic digestion.

Anaerobic treatment of domestic wastewater is an established technology (or suite of technologies) in countries with warm climates (Haandel and Lettinga 1994). At lower ambient temperatures (<20ºC) these systems are less effective. The rates of substrate utilization, growth, methane production and wastewater hydrolysis all decline with temperature drop and previous attempts to treat domestic wastewater at less than 8ºC have resulted in treatment process failure (Alonzo et al 1969; Kettunen and Rintala, 1997; Bowen et al 2014). However, most attempts to date were focused on acclimatizing mesophilic biomass to low temperature.

I have sought to determine if inoculation of a reactor with psychrophilic/cold-adapted biomass would be a more effective way to investigate the performance and limits of the process at low temperatures.

A positive answer would pave a new path in **conservation of our environment, passing it to our children in as good or better condition than it was passed to us**.
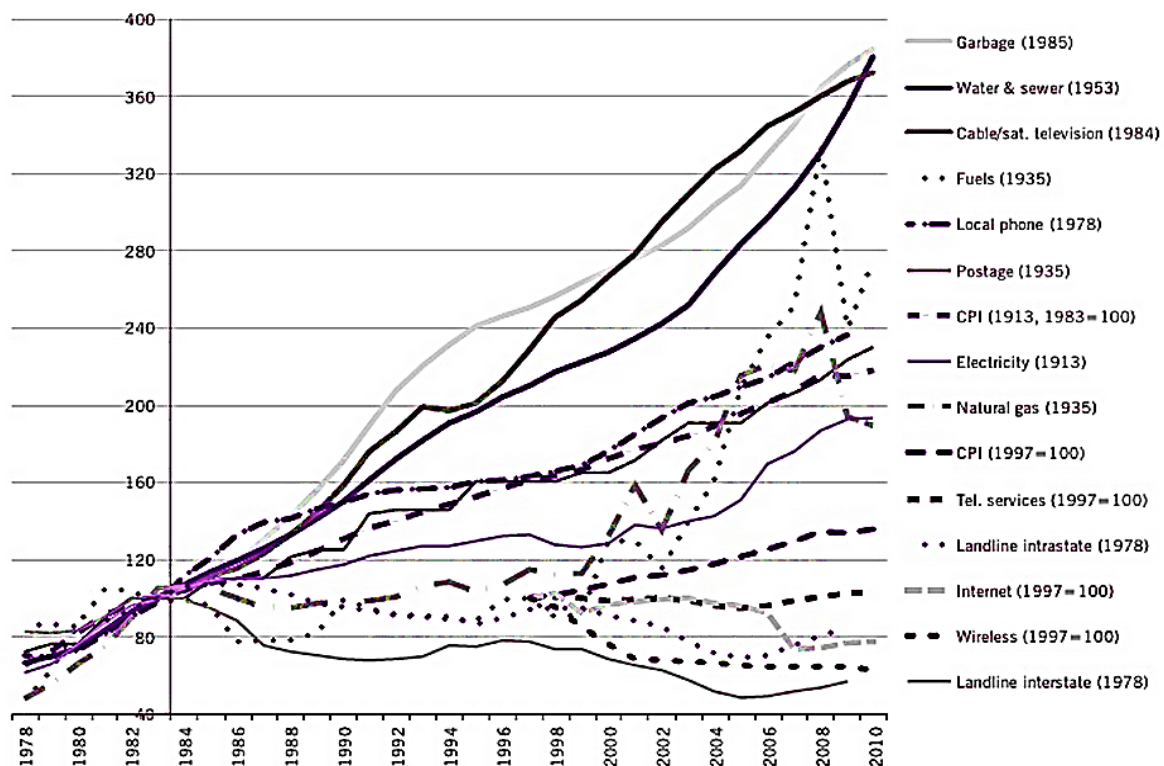
Figure 1.2 – Trends in consumer prices for utilities index (CPI) 2010, for the last 31 years (Haarmayer, 2011); the index is set to 100 for 1982-1984 except for telephone, wireless and internet services where the index is set to 100 at 1997.
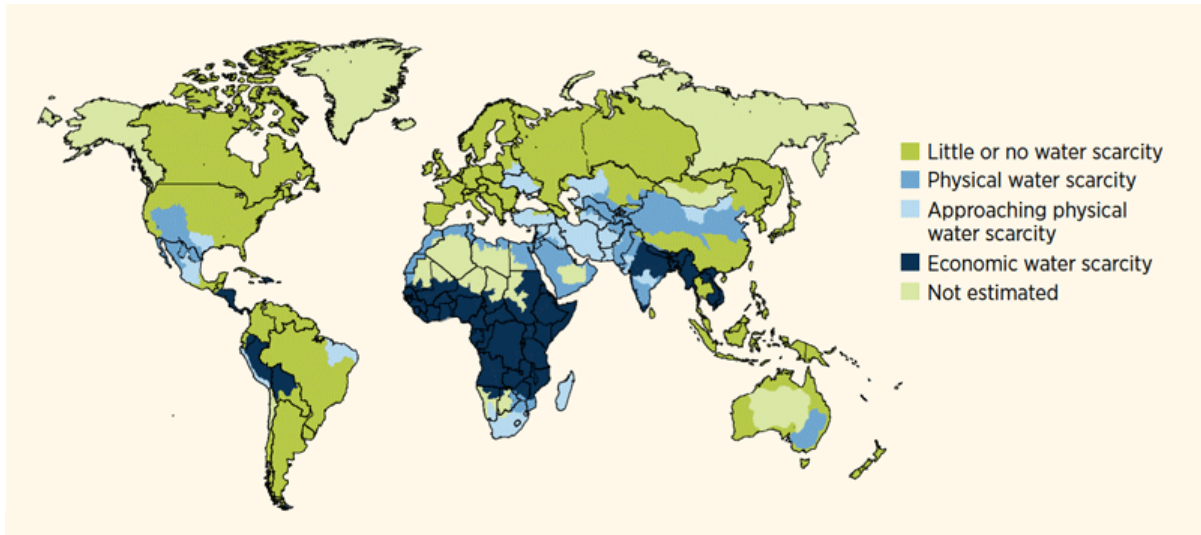
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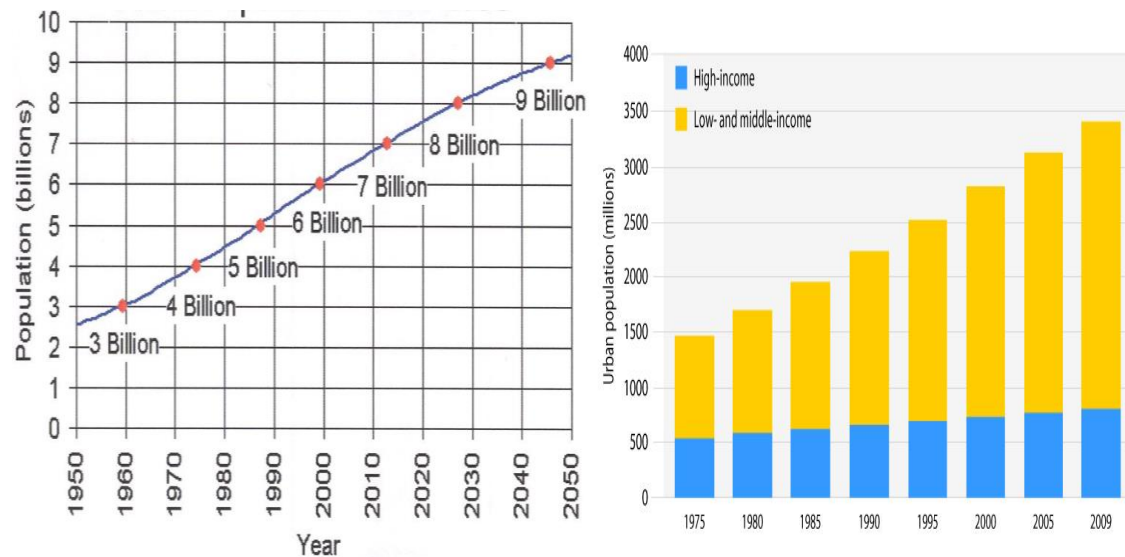
Figure 1.4 - a) World Population status since 1950 (U.S. Census Bureau, International Database 2011); b) Urban population in low and middle-income countries, 1975-2009 (UN Population division 2008).

Chapter 2: *Literature Review on low temperature AD*
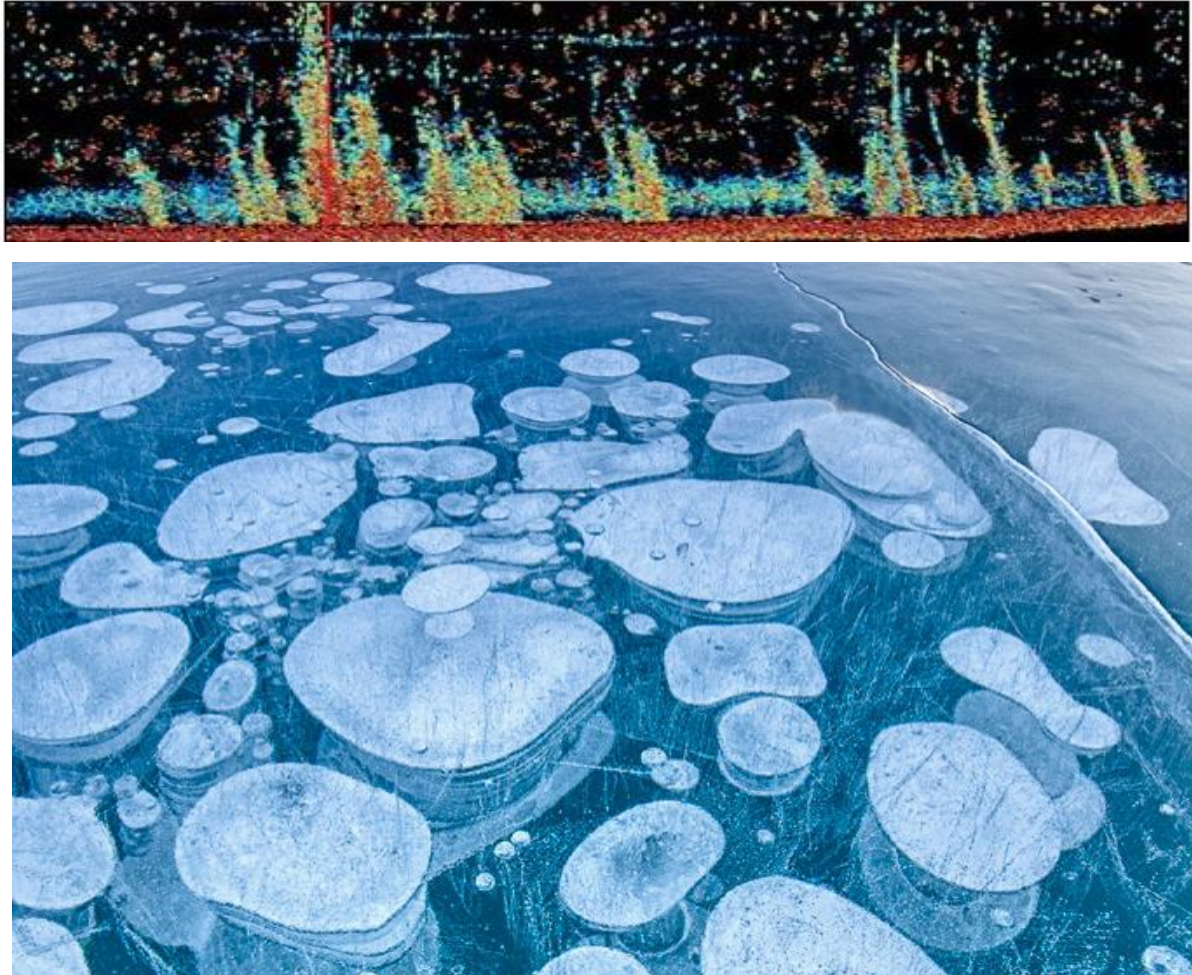
Figure 2.1 – Methane bubbles observed by sonar, escape from sea-bed as temperature rise (BBC News 19 Aug. 2009); Methane bubbles formed in the bottom of the Arctic sea covered in ice trying to escape the atmosphere penetrating the ice (Alaska Dispatch 30 April 2012).

2.1. Wastewater Treatment and Systems

Wastewater etymology & description: Wastewater (also written as waste water) is any water that has been adversely affected in quality by anthropogenic influence. Municipal wastewater is usually conveyed in a combined sewer or sanitary sewer, and treated at a wastewater treatment plant or septic tank. Treated wastewater is discharged into receiving water via an effluent sewer (Directive 91/271/EEC).

2.1.1. General View

The main aim of sewage treatment is to obtain an effluent that complies with the relevant discharge standards. In Europe this will typically mean meeting with the standards of the Urban Waste Water Treatment Directive (UWWTD 91/271/EEC) to protect the environment from the adverse effects of urban waste water discharges and discharges from certain industrial sectors. Parameters that might be taken into consideration include organic-biodegradable material (BOD, biodegradable COD), nutrients (P, N), total suspended solids (TSS) and pathogens (Metcalf and Eddy 2002). In certain circumstances other pollutants/parameters such as heavy metals, pesticides sulphur containing compounds and inorganic elements, turbidity and color might also be relevant.

Wastewater engineering includes both physical and biological processes. In the latter, organic matter is degraded by the microorganisms present in the wastewater or the inoculum. Broadly speaking there are two types of biological reaction: aerobic and anaerobic. In the anaerobic reactors, mineralization takes place following a fermentative path where biogas (CH_4, CO_2) is typically the final product (Schrurer and Jarvis 2009). Nutrient removal mainly occurs via de-nitrification in specific reactor setups to achieve nitrification followed by nitrate removal (>90% in a 2 phased submerged filter - UASB); phosphorus removal takes place after its conversion to struvite (80% removal at 9.0pH and Mg:P of 1.6:1) (Sousa et al 2008, Jordaan et al 2010). Nitrogen can also be sufficiently removed with minimum amounts of O_2 via anaerobic ammonia oxidation (Ahn et al 2004; Hu et al 2013, etc.). Finally if pathogens are present a 3rd step of disinfection/sterilization is added (usually chlorination, UV irradiation or Ozone) (Metcalf and Eddy 2002).

2.1.2. Wastewater Characteristics

Engineering a reactor requires an understanding of parameters that are often site specific as socio-economic and climatic factors change the nature of the waste (Hussain et al 2001) (e.g. water content and organic material concentration).

Solids: are classified on the basis of size into to dissolved, colloidal solids and particulate matter (the solids and particulates are known as suspended solids (TSS); TSS containing organic carbon are referred to as volatile suspended solids (VSS) (Hammer and Hammer 2002).

Organic material: is the source of energy for a wide range of heterotrophic cells participating in biological reactions. Utilization of organic compounds by microorganisms is known as "metabolism" and can be divided into two classes of processes. Consumption for energy production is called "catabolism", whereas use for multiplication is "anabolism" (Malina and Pohland 1992).

2.1.2.1 Chemical Oxygen Demand

Chemical oxygen demand or COD is a key parameter in wastewater treatment (Ritmann and McCarty 2001, Henze et al. 2008). It is used to estimate the concentration of organic compounds and define effluent quality (for example UWWTD 91/271/EEC). Usually expressed as mg/L (or g/m^3 or ppm) COD indicates the oxygen required for the total oxidation of the organic material in the waste (Metcalf and Eddy 2002). COD can give an indication of the potential amount of CH_4 that could be derived from the organic fraction of wastewater (Heidrich et al 2011).

2.1.3 Chemical Energy from Organic Material

The mineralization of organic material releases energy which is then available for metabolic processes (Malina and Pohland 1992). Though some of energy is dissipated or lost as heat to the environment (Haandel and Lettinga 1994). Due to the complex composition of most wastewaters it is not possible to define a standard energy for all types of wastewater. A rational approach for the estimation of the free energy is by the thermodynamic calculation

upon oxidation of common wastewater compounds. As the values vary significantly when expressed in kJ/mol a more appropriate way is to state them as kJ/gCOD (Table 2.1).

This can be deduced by assuming that a hypothetical wastewater compound $C_xH_yO_z$ is being oxidized:

$$CxHyOz + (2x - z)H_2O \rightarrow xCO_2 + (4x + y -2z)H^+ + (4x + y + 2z)e^- \ (Eq.1)$$

or

$$(1/(4x+y- 2z))CxHyOz + ((2x–z)/(4x+y+2z))H_2O \rightarrow (x/(4x+y–2z))CO_2 + H^+ + e^- \ (Eq.2)$$
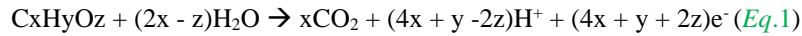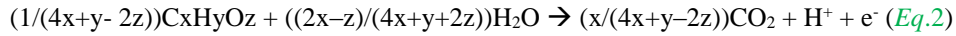
The *Eq.* 1 shows that the oxidation of 1 mol of organic compound transfers of $(4x + y + z)$ electrons. In other words for every C atom the number of electrons being transferred is:

$$Nel \text{ (number of electrons transferred per C atom)} = (4x + y -2z)/x = 4 + (y − 2z)/x \ (Eq. 3)$$

For domestic wastewater, the electrons transferred per C atom ranges from four (for carbohydrates and most of the proteins) to six (Fats) (Table 2.1).

The Nel describes the oxidative state of every compound. The higher the Nel per C atom the more reduced the compound and the less free energy released from oxidation (Figure 2.2). For most of the common compounds with Nel >3el/C the energy is roughly 14.0 Kj gCOD^{-1} (approx. 3.3Kcal mol^{-1}).

Table 2.1 - Values of free released energy & electron transfer on oxidation of organic compounds (Haandel and Lettinga 1994)

	Compound	Kcal/mol	Kj/gCOD	Kcal/g COD	Kcal/g TOC	Nel (el/C-atom)
1	Oxalic Acid	82	21.47	5.13	3.44	1
2	Formic Acid	68	17.96	4.29	5.71	2
3	Citric Acid	916	14.99	3.58	7.16	3
4	Glucose	686	14.94	3.57	9.53	4
5	Lactic Acid	326	14.19	3.39	9.05	4
6	Acetic Acid	207	13.52	3.23	8.62	4
7	Glycerine	387	14.44	3.45	9.39	4.67
8	Phenol	723	13.48	3.22	10.01	4.67
9	Ethylene Glycol	281	14.69	3.51	11.69	5
10	Benzene	761	13.27	3.17	10.55	5
11	Acetone	410	13.39	3.2	12.18	5.33
12	Palmitic Acid	2338	13.31	3.18	12.18	5.75
13	Cyclohexane	901	13.06	3.12	12.48	6
14	Ethylene	314	13.69	3.27	13.08	6
15	Ethanol	312	13.60	3.25	13	6
16	Methanol	165	14.40	3.44	13.76	6
17	Ethane	344	12.85	3.07	14.33	7
18	Methane	191	12.47	2.98	15.88	8
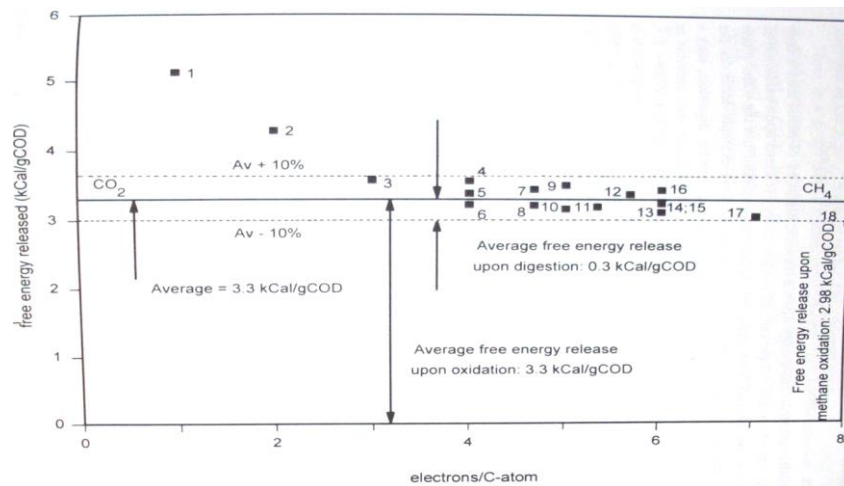
Figure 2.2 - Free energy released from the oxidation of organic compounds per gram of COD as a function of the N-el. Transferred per carbon atom (Haandel and Lettinga 1994); data points refer to the free energy released from Table 2.1.

2.1.4. Bacterial Metabolism

Biological wastewater treatment is the removal of organic material based on bacterial metabolism (anabolism - catabolism) (Malina and Pohland 1992).

2.1.4.1. Fermentative Catabolism

The catabolic fermentation in anaerobic digestion results in methane formation (Malina and Pohland 1992). Methane is the most reduced in Nel/C atom^{-1} organic compound (Nel 8). Thus AD is the quintessential fermentative process as all the organic material from various compounds is transformed to the least oxidized product, methane (Eq.4). The methane retains the chemical energy from the reduced compounds allowing only a minor fraction to be released in the environment. In aerobic processes by contrast, the energy is lost as CO_2 is the final product and the anabolic fraction is much higher as described below.

$$CxHyOz + (4x-y-2z)/4H_2O \rightarrow (4x – y + 2z)/8CO_2 + (4x+y-2z)/8CH_4 \ (Eq.4)$$
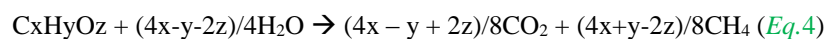
CO_2 is also formed in anaerobic digestion and the gases (CO_2 and CH_4) escape from the liquid phase forming biogas.

2.1.4.2. Anabolism

Anabolism and catabolism can be quantified. Catabolism can be estimated from the quantity of methane produced and anabolism can be quantified from the increase in the amount of biomass.

At the same time catabolism and anabolism (metabolism) can be described from the decrease in organic matter. Figure 2.2 briefly explains how substrate is being utilized through cellular metabolism.
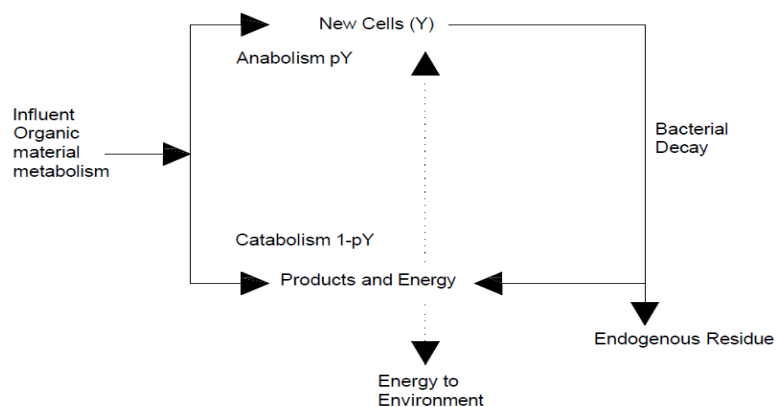
Figure 2.2 - Representation of microbial metabolism and decay (Haandel and Lettinga 1994)

*Eq.*5 describes the relationship between the microbial mass produced and the metabolized substrate mass:

$$Y = - (\Delta X_v / \Delta S)_m \ (Eq.5)$$

Where Y is the Yield coefficient; X_v the bacterial mass (volatile solids); S the organic material or substrate being utilized; m indicates that the changes between Xv and S occurred from the metabolic activity (Haandel and Lettinga 1994) and that no external factors affected the process (e.g. removal from chemical or physical processes).

For aerobic reactions Y yield can reach up to 0.45g VSS/g COD (Hammer and Hammer 2002). For anaerobic digestion at mesophilic temperature conditions the Y is lower reaching the amount of 0.02-0.03 mgVSS/gCOD$_{removed}$ (McCarty 1990, Stronach et al 1986). Thus in aerobic processes the production of biomass is higher leading to increased sludge handling costs.

In aerobic systems approximately the 1/3 of the metabolized COD mass is converted to CO_2 whereas the 2/3 is anabolized and becomes biomass. Under anaerobic conditions the low yield results in a significant conversion of the organic material into methane (approximately 97% of COD) (Marais and Ekama 1976). In anaerobic treatment the number of cells does not necessarily increase; as all living organisms decay they themselves become the organic substrate for other organisms' metabolic processes.

Marais and Ekama (1976) assert that during periods of starvation the biomass produced from aerobic metabolism has a higher decay rate than the biomass generated through anaerobic metabolism. This indicates that anaerobic biomass is more robust than the aerobic one and gives an extra advantage to the anaerobes at low temperatures as metabolic rate is generally slow, and 'food' is limited. Such limitations are mainly caused by the changes that occur to the substrate structure at low temperatures (Neidleman 1987), resulting to lower amounts of bioavailable substrate for the cells to uptake, grow and thrive.

2.2. Anaerobic Digestion of domestic wastewater

The conversion of sewage to biogas is a complex procedure; however it can be separated in four phases, hydrolysis/fermentation, acidogenesis, acetogenesis and methanogenesis (Figure 2.3.a, b). Different microbial populations are thought to participate in each interlinked process (Angelidaki et al 1999, Haandel and Lettinga 1994, Malina and Pohland 1994).
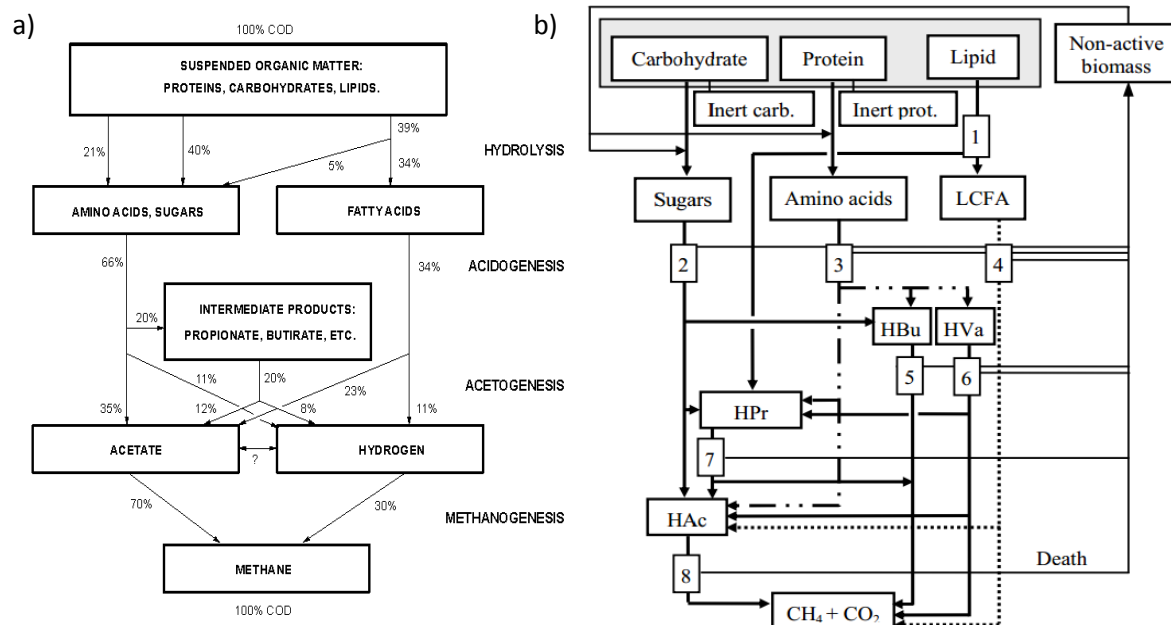
Figure 2.3 – a) Reaction sequence for the anaerobic digestion of complex organic substrate; all values expressed in %; b) Anaerobic degradation steps: 1) acidogenesis from glycerol combined with lipid (triglyceride) hydrolysis; 2) acidogenesis from sugars (glucose); 3) acidogenesis from amino acids; 4) acetogenesis from long chain fatty acids (LCFA); 5) acetogenesis from butyrate (HBu); 6) acetogenesis from valerate (HVa); 7) acetogenesis from propionate (HPr); 8) acetoclastic methanogenesis (Angelidaki et al 1999).

Hydrolysis; complex organic material (proteins, carbohydrates and lipids) is dissolved into smaller molecules (glycerin, fatty acids, amino acids and sugars). Hydrolysis is not necessarily a strictly anaerobic process as studies showed that micro-oxygenation enhances the physiological metabolism of the facultative hydrolytic bacteria assisting substrate hydrolysis (Qi et al 2005, Chu et al 2012).

Acidogenesis; material produced by hydrolysis is utilized by fermentative bacteria and simpler compounds (LCFAs & VFAs, alcohols, CO_2, ammonia and H_2S) are released. This step is carried out by diverse consortia of organisms (Haandel and Lettinga 1994). As in hydrolysis, facultative acidogenic microorganisms can also metabolize organic material through micro-aerobic processes (Haandel and Lettinga 1994, Qi et al 2005, Chu et al 2012).

Acetogenesis; the organic compounds released from the cells in the previous phase are converted to CH_3COOH and H_2/CO_2 as shown on Figure 2.3.a. As indicated on the same figure a fraction of 70% of the COD is converted to acetic acid where only 30% turns into hydrogen. Hydrogen may also be released from acetate formation depending on the oxidative state of the original organic compound (Nel <4 gives acetate and CO_2, Nel >4 gives acetate and H_2) (Haandel and Lettinga 1994). As wastewater nature is multi-dimensional usually both H_2, CO_2 are produced.

Acetate may also be formed from hydrogen through homoacetogenesis and vice versa through acetate oxidation when specific microbial communities are present (acetate oxidizing bacteria and homoacetogens respectively) (Batstone et al 2002). Homoacetogenesis usually requires a high H_2 partial pressure (1-10Pa) to occur over methanogenesis (Kotsyurbenko et al 2001). At lower temperatures though acclimation of the cells seems to be a key factor in the competition for H_2 between methanogens and homoacetogens (Kotsyurbenko et al 2001).

Methanogenesis; is the process whereby methane is produced from acetate and H_2/CO_2. For methane formation the presence of acetotrophic and hydrogenotrophic methanogens is essential (Thauer et al 1993); the reactions taking place are shown below (*Eq.*6, 7):

$$CH_3COOH \rightarrow CH_4 + CO_2 - \text{Acetotrophic Methanogenesis } (Eq.\ 6)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O - \text{Hydrogenotrophic Methanogenesis } (Eq.\ 7)$$

Commonly, under mesophilic conditions hydrogenotrophic methanogenesis is thermodynamically favored compared to acetoclastic methanogenesis. As 70% of the methane comes from acetate, methanogenesis tends to be the rate limiting step at mesophilic conditions (Henze and Herramoes 1983); however it is not certain if this is the case at low temperatures.

Methanogenesis may also be derived from propionate and butyrate which can be converted to acetate and thus methane (*Eq.*8, 9). These reactions are thought to be less common (Gerardi 2003).

$$4CH_3CH_2COOH + 2H_2O \rightarrow 4CH_3COOH + CO_2 + 3CH_4 \ (Eq.8) \text{ followed by } Eq.6$$

$$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 4CH_3COOH + CO_2 + 3CH_4 \ (Eq.9) \text{ followed by } Eq.6$$

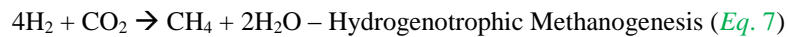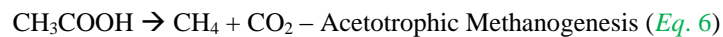Methane can be also formed from hydrogenotrophic reduction of CO_2 (*Eq.*10) under special conditions (e.g. specific H_2 partial pressure to promote syntrophic interactions between microbial communities) (He et al 2006).

$$HCO_3^- + H_2 + H^+ \rightarrow CH_4 + 3H_2O \ (Eq.10)$$
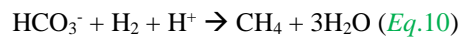
2.2.1. Environmental Factors

The performance and feasibility of anaerobic digestion is dictated by environmental factors such as: pH, temperature, nutrients and presence of toxic compounds (Malina and Pohland 1992, Haandel and Lettinga 1994). In domestic wastewater the most important factor is arguably temperature as it cannot be controlled. Domestic wastewater is usually buffered by the carbonate-bicarbonate buffering system (Malina and Pohland 1992, Haandel and Lettinga 1994); however, pH control may be required at high VFA or ammonia concentrations to avoid inhibition of methanogenesis. Domestic wastewater as substrate also provides with adequate amounts of nutrients (micro-macro nutrients and phosphorous) and rarely contains toxic compounds.

2.2.1.1. Temperature

Anaerobic wastewater treatment is significantly affected by temperature. Theoretically there are two optimum temperatures, near 35°C and near 55-60°C for mesophilic and thermophilic consortia respectively. At temperature more than 70°C rates decrease even in the presence of excess substrate (Zinder et al 1984). On the other hand methanogenesis is feasible at temperatures lower than 20°C, though slower growth and reaction rates imply the need for larger reactors and longer residence times (Mackie and Bryant 1981, Chapter 2.2.4). **Which begs the question what if the population was adapted to low temperatures**?

Microorganisms are classified according to optimum growth and metabolism temperature into psychrophilic, mesophilic and thermophilic (Figure 2.4); the boundaries among them are not clear (Lettinga et al 2001).
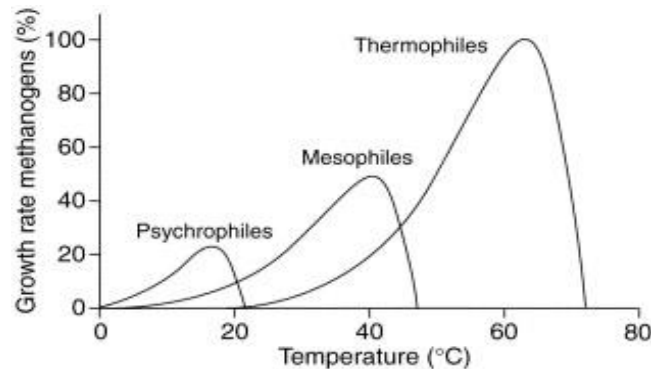
Figure 2.4 - Relative growth rates of methanogens according to their optimal temperature conditions (Lettinga et al 2001)

2.2.1.2. Other factors

Reactor pH; the operating pH needs to be stable in the neutral range (6.8-7.2); change in pH may affect the archaeal population and thus methanogenesis (Liu et al 2008). Acidogenic populations compete with methanogens for specific substrates (e.g. propionate) (Hwang et al 2001) and may be less affected by lower pH values resulting to limited methane production rates. However, a suboptimal pH does not necessarily stop methanogenesis. The process has been observed at pH values low as 5.0 by *Methanosarcina* b*arkeri* and *Methanosarcina vacuolata*, (Maestrojuan and Boone 1991) as well as at a pH > 7.2 (Liu et al 1991, Boone et al 1986, Mathrani et al 1986).

Nutrients; the C:N ratio (C:N as COD:Nitrogen) in anaerobic reactors usually vary from 400:7 to 1000:7 depending on the substrate (Henze and Harremoes 1983). A typical ratio in wastewater is 1000:18:5 (Spencer 2005). The N:P also varies but usually a good working ratio is 7:1 (Malina and Pohland 1992). Other, less common C:N:P ratios have also been studied and found workable (Stronach et al 1986). Certain micronutrients are also required at low concentration (Fe, B, Zn, Cu, Mn, Mo, Al, Co, Ni, Se). Studies showed that their use as trace element solution promotes biomass growth and subsequently methanogenesis stability (Zhang et al 2012, Jansen et al 2007).

Toxic compounds; there are various toxic compounds that can adversely affect methanogenesis. Accumulations of fatty acid, ammonia and hydrogen sulfide, excess of heavy metals (Ni, Pb, Co, etc.) can cause inhibition or failure of the process. In small amounts though these elements can be useful (e.g. Zhang et al 2012 enhanced anaerobic digestion of food waste using 0.05ml of a trace element solution containing 563, 8.9, 23.9, 14.2, 138.9, 27.2, 10.1, 495.8, 35.2, 56.1 mg.L^{-1} of Fe, B, Zn, Cu, Mn, Mo, Al, Co, Ni, Se respectively).

2.2.2. Need of carbon neutral/positive wastewater treatment

The advantage of the anaerobic treatment of domestic wastewater at low temperatures is that wastewater can be treated under ambient conditions with no need to expend energy to maintain a specific temperature. Any biogas generated can be used on the site or sold.

Three quarter of our biosphere is situated at low temperatures in hypoxic and anoxic environments (i.e. deep oceans and lakes) (Metje and Frenzel 2007). Biomass from these environments might be appropriate for the investigation of the true temperature limits for the anaerobic treatment of domestic wastewater.

There is methanogenic capacity at low temperature. 30-40 Tg/year methane is emitted from the arctic wetland (approx. 6% of humanity's annual needs (Reeburgh and Whalen 1992) or approximately 8% of the gases causing global warming per year (Cao et al 2012).

However, lower temperatures can lead to lower growth and substrate utilization rates. This in turn may drive to slow degradation and increase in biomass yield due to accumulation of the un-hydrolyzed organic material (0.03-0.18 gVSS/gCOD; Stronach et al 1986). Accumulation of un-hydrolyzed material may also lead to changes in the psysico-chemical properties of the substrate. Other challenges that may occur due to low temperatures are high gaseous solubility, high liquid viscosity, and lower diffusion of soluble compounds (Lettinga et al 2001).

2.2.3. Why low temperature

As metabolic rates are related to temperature, treating wastes anaerobically at low temperatures is challenging. The approach becomes more difficult when the substrate is low strength domestic wastewater. Low levels of substrate, poor substrate-biomass contact, low gas production and the need for excellent biomass retention (Lettinga et al 2001) are the main barriers to the anaerobic treatment of wastewater at ambient temperature in temperate climates. For me, the answer why I do it, as J.F. Kennedy said when he was asked why investing time and money to moon expedition, saying: *'we do it not because it is easy but because it is hard'*.

2.2.4. Metabolism at low temperature

Studies of cells in cold environments (permafrost ice, snow, clouds) showed that their metabolic rates are similar to those of normal cells living in water and soil (Price and Sowers 2004). These rates fall into 3 sub-groups: the rate $_{ug}$(T) for growth, $_{um}$(T) for sufficient maintenance and $_{us}$(T) for survival when the cells are imprisoned (e.g. trapped in ice) and micro-molecular damage repairs (especially when cells are dormant). The activation energy required for the three metabolic processes was estimated at 110kJ/mol and the energy distribution among them follows the ratio of $10^6:10^3:1$ respectively (Price and Sowers 2004).

2.2.5. Mesophilic biomass as seed

Thus far, attempts to treat wastewater anaerobically at ambient temperatures in temperate countries have sought to acclimatize mesophilic sludge to low temperature, typically by using either 1 or 2 phase reactor configurations. Under limited mesophilic temperature conditions methane to COD conversion can reach the 0.34LCH$_4$/gCOD (Borja et al 2002) at 15-19°C (close to the theoretically expected, 0.35gCOD) at a prolonged HRT of up to 20 days. The COD:CH$_4$ conversion and the methanogenic activity is lower though between 5-15°C (Figure 2.5.a).
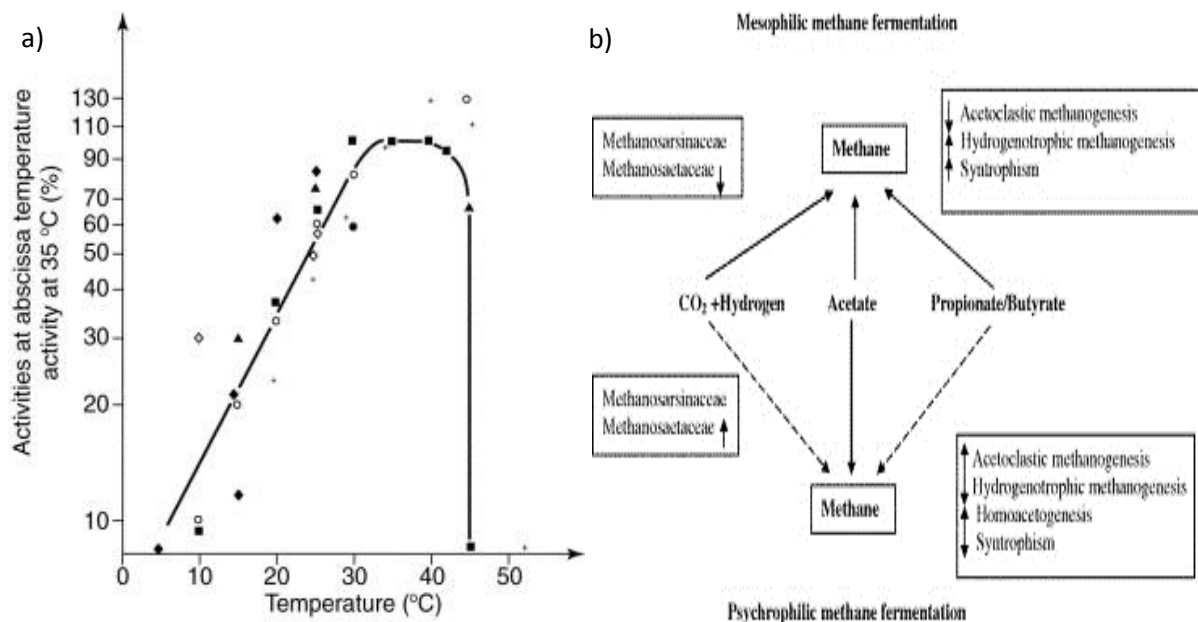

Figure 2.5 – a) Temperature dependency of methane production using mesophilic biomass as seed (reproduced from Lettinga et al 2001); b) Influence of temperature on methane production when temperature decreases or increases (Dhaked et al 2010).

Slow performance might be attributed to limited acclimation or less favorable thermodynamics. Lettinga et al (2001) showed that at >17ºC the substrate utilization activity follows a linear trend reaching the 35ºC that appears to be the optimum temperature. Thus lowering operational temperature of AD with mesophilic biomass causes COD:CH$_4$ conversion decline and potentially change in the metabolic CH$_4$ pathways (Dhaked et al 2010) (Figure 2.5.b).

2.2.5.1. 1 step configuration

Engineering studies typically focus on treating wastewater using a single reactor.

Uemera and Harada (2000) tested the feasibility of low strength domestic sewage digestion at 25 to 13ºC using a UASB reactor seeded with mesophilic granular sludge rich in *Methanothrix*-like archaea at an HRT of 4.7 hours. Average total COD removal reached 69.4% with poor gaseous COD:CH$_4$ conversion (74% and 45% for 25 and 13ºC respectively); large amounts of methane were detected in the effluent. After 6 months operation the biomass properties changed; MLVSS acidification and deterioration (based on SVI) resulting in limited settleability increasing the likelihood of washout. Similarly F$_{420}$ co-enzyme associated with hydrogenotrophic methanogenesis (Dolfing and Mulder 1985) was reduced, implying a reduced capacity for methane production from hydrogen or formate. This suggests that there were changes in the structure of hydrogenotrophic community and in the overall reduced biomass. The hypothesis was supported by scanning electron microscopy which showed that granules had the tendency to break or collapse and the *Methanothrix*-like communities had disappeared The ATP concentration was also reduced throughout the reactor apart from the lowest level of 10cm. The qualitative and quantitative changes in biomass were attributed to starvation and limited hydrolysis at low temperatures.

Biomass deterioration and washout were also observed by Xing et al (2009) in a study of low strength wastewater treatment at 20 and 15ºC. Lowering the temperature from 20 to 15ºC resulted in a limited performance and a decrease in the proportion of *Methanosaeta* sp.; however other genera seemed to increase (notably *Methanospirillum*); no effect on the bacteria was observed (the predominant eubacteria being *Firmicutes*).

Maharah and Elefsiniotis (2001) set up CSTR reactors to treat municipal wastewater and a mix of municipal-industrial (starch rich) wastewater using a seed:substrate ratio of 1:1. The aim was to promote acidogenesis for biological nitrogen and phosphorus removal. The experiment started at 25ºC and the temperature was gradually reduced to 8ºC. An HRT of 30 hours was chosen based on the optimum hydrolysis rate achieved in a similar study at 25ºC by Benerjee et al (1999). Switching of HRT to 48 and 60 hours led to limited substrate after a certain period, followed by bacterial decay (as VSS) and resulted in low VFA production. Lowering the temperature to 8ºC also reduced the VFA production, presumably due to a reduction in the activity, but not the abundance of the acidogenic bacteria as the VSS concentration remained stable. By contrast Alvarez et al 2008 found that biomass (VSS) increased when the operational temperature was reduced from 21 to 14ºC in a 2-phased (hydrolytic - methanogenic) reactor treating raw domestic wastewater. The increase was attributed to limited hydrolysis as the dilute nature of the wastewater (COD<250mg L^{-1}) led to poor contact between cells and substrate.

Dague et al (1998) tested the treatability of synthetic low strength dairy wastewater (600mgCOD/L) using a continuous ASBR inoculated with mesophilic granular sludge. Starting at 25ºC the temperature was reduced to 5ºC. The experiment was aiming to convert an ASBR to a high rate reactor by lowering the HRT from 24 to 6 hours and achieve a higher operational OLR. Lowering the temperature reduced the COD removal efficiency from >90% at 25ºC to 60-70% at 5ºC at all HRT. The temperature decrease limited methane production but reduced HRT increased the gas production showing successful conversion of a conventional ASBR to a high rate reactor. Additionally lowering the HRT resulted in a higher than theoretically expected methane production. This is attributable as the lower HRT allowed un-degraded material to accumulate in the reactor that could be utilized in the subsequent batch feed leading to the putative excess of methane. This suggests that intermediates accumulated at lower temperature may be subsequently degraded causing an increased F:M ratio and a higher microbial growth rate.

Akila and Chandra (2007) tried to treat synthetic glucose-based wastewater at 15ºC achieving an 83±6% and 90±4% COD removal for low (950 mgCOD/L) and high (8000 mgCOD/L) strength wastewater with 70±2% and 78±3 COD:CH$_4$ efficiency respectively using a UASB reactor. The initial HRT of 7 days was reduced to 1 day for each substrate. Despite the HRT and OLR changes the removal efficiency was stable. The inoculum was cattle

manure, operated at 37°C and gradually reduced to 15°C; the adaptation lasted 21 months. The results showed that the higher the COD content of the influent (8000mg/L) the poorer the effluent quality; however the conversion of COD to methane was better at the higher COD values. The effluent contained non-degraded VFAs rendering it unsuitable for discharge without further treatment. Activity test showed that the predominant methane pathway was through acetate (>70 times higher than H_2).

Treatment of brewery wastewater (1000-6000 mgCOD/L) at 15°C was reported by Connaughton et al (2006). They use an EGSB-AF reactor in a comparative study with a control reactor at 37°C with a mesophilic inoculum. In the first 100 days performance was not stable, however the process eventually stabilized (in terms of COD removal and CH_4 production) at both temperatures. Some washout was inferred at 15°C but it was observed that a higher OLR could be tolerated. Significant effluent polishing was undertaken by an upper fixed film layer. Enright et al (2007) using toluene-containing wastewater in a similar setup to Connaughton et al 2006, at 15°C achieved high COD removal efficiencies (70-90%). Collins et al (2005) in the same group with a similar reactor setup using anaerobic granules from a full-scale citric acid production plant found that high rate anaerobic digestion is feasible and reproducible under favorable conditions (18-20°C) with low VFA accumulation (<1000 ppm). However, when temperature decreased to10°C a system shock was evident. This suggests that the granules cannot operate at a very low temperature level, possibly due to limited hydrolysis that limits the sCOD production which is necessary for granules preservation (Kalogo and Verstraete 2001). However the study showed presence of putative psychrophilic propionate and butyrate degraders with the former being the most sensitive to temperature perturbations (a finding repeated by Collins et al 2006).

Elmitwalli et al (2002) compared a hybrid anaerobic filter (AF) with anaerobic hybrid (AH) reactor at 13°C using low strength domestic wastewater at an HRT of 4 hours with a view to determining which configuration is more efficient for wastewater pre-treatment. The AF performed better than the AH in terms of COD removal with 70.7% and 58.9% for AF and AH respectively; SS removal was also higher for AF (82% over 53% for AH); similar amounts of methane were produced in both systems. Excess sludge was formed in both systems with more being found in the AF. The AH sludge quality was better than the sludge formed in AF in terms of settleability and filterability expressed as SVI index and VSS/TSS. This means that the AF sludge has higher organic content and further stabilization is required rendering a post-stabilization step necessary at a higher degree than for AH. Hence, although pre-treatment may promote the biodegradability of the wastewater substrate by removing the hard to degrade material, it may also result in increased amount of sludge that requires extensive post-treatment and subsequently increased handling costs.

2.2.5.2. 2 step configuration

Single stage AD reactors operating at low temperatures suffer from poor contact between the biomass and wastewater, accumulation of inert suspended solids and the consequent deterioration of treatment efficiency (Seghezzo et al 1998). Two stage reactors might therefore be worth considering.

Rebac et al (1999) carried out a comparative study between one and two-phased EGSB reactors at 10-15°C, treating malting and synthetic waste using a mesophilic inoculum. The single reactor removed 90% of COD at an HRT of 1.6 hours and an OLR of 12 gCOD/m^3/day, at a temperature of 10-12°C. The use of a second compartment allowed the application of a higher OLR (2.8 and 12.3 kgCOD/m^3/day for the two compartments respectively), at an HRT of 3.5 hours at 10-15°C, achieving 67-78% and 90-96% removal for soluble COD and VFAs respectively. The second phase mainly polished the effluent, utilizing the VFAs that were formed in the 1st one. The same study showed that after long acclimation of cells to low temperatures one compartment is adequate to achieve sufficient anaerobic low strength wastewater (mainly of VFAs) treatment at 3-12°C.

Alvarez et al (2008) treated low strength raw domestic wastewater (400- 118 mgCOD/L) at 21-14°C via a 2 stage hydrolytic upflow sludge blanket – upflow sludge blanket (HUSB-UASB) configuration at an overall HRT of 9.3-17.3 hours. The system managed to reduce the COD by 49-65% and TSS by 49-89% (for cold and warm periods respectively); importantly COD solubilization occurred in the HUSB indicating high hydrolytic capacity. Methane formation was modest: 36.1% of COD was converted to CH_4 probably due to low sCOD/TSS especially in the cold period. sCOD mainly generated in the HUSB (11-26% depending the period) suggesting that hydrolysis and acidification primarily takes place in that compartment, followed by sCOD uptake in the UASB (10-38% sCOD

removal for cold and warm period respectively). The low sCOD removal resulted in the accumulation of VFAs in the UASB that inhibited methanogenesis. This suggests that acidification was not complete in HUSB especially at cold periods where hydrolysis rates were limited. Thus, an increase in HRT is required in the first compartment. The VSS removal was 56.8 and 81.4% for HUSB and overall system respectively. Sludge SMA was stable throughout the experimental period and the hydrogenotrophic pathway predominated at both temperatures. No granulation occurred in the system, probably due to low sCOD generation from low strength wastewater in conjunction with the high VSS concentrations (sCOD/VSS >10 (Kalogo and Verstraete 2001) or 6.6 (Ligero and Soto 2002)). Granulation was achieved though by O'Reily et al (2010) who tested methane production and granulation feasibility with a synthetic glucose-based wastewater at 15°C. The study showed that carbohydrates degradation and presence of hydrogenotrophic methanogens (e.g. *Methanomicrobiales*) promoted granulation at low temperature. This is expected as carbohydrates are considered the easiest degraded compound (Eastmean and Ferguson 1981) in anaerobic treatment providing with high sCOD.

A 2-phased UASB-septic tank setup inoculated with mesophilic sludge was selected by Luostarinen and Rintala (2006) to treat black water (BW) and black water-kitchen waste (BWKW) (COD: 1g/L) at 20-10°C. The run was continuous and semi-continuous. The removal efficiency for BW at both temperatures and feed types remained the same for COD (90%) but significantly lower for dissolved COD_{dis} (20 & 70% for semi- and continuous BW feed respectively no matter the temperature). For BWKW the efficiency was similar however the effluent had higher total COD content rendering post-treatment necessary. A similar reactor configuration was used for synthetic BW and dairy parlour wastewater (DPWW) at 20-10°C by Luostarinen and Rintala (2005) achieving a 90 and 80% COD removal for each substrate. This suggests that a single phase is adequate for BW however a second step is required for DPWW. TSS and VSS were efficiently removed from the 1st step however the good performance for BW resulted in elevated sludge production, hence more frequent emptying. SMA tests showed that the sludge is not properly stabilized due to limited hydrolysis. Methane production was proportional to temperature; although the biomass had a mesophilic origin activity was detected even at 5°C in batch systems. In a similar study from Luostarinen and Rintala (2007) treating BW and BWKW it was found that lowering of temperature from 20 to 10°C affects the methane production rate (for both BW and BWKW). The treatment of BW was found once again to be easier than of BWKW suggesting that for the former a single phase is adequate. At lower temperature the sludge bed height increased suggesting limited hydrolysis. Low $COD:CH_4$ conversion resulted to poor sludge stability even after 198 days for both substrates.

The same UASB-septic tank setup was used as a BW pre-treatment step rather than polishing the effluent by both Bogte et al (1993) and Luostarinen et al (2007), inoculated with paper-mill sludge operating at ambient temperature acclimatized at the local seasonal variation (5-13°C and 14-17°C for cold and warm periods). The results from Bogte et al (1993) were promising as the cold period setup was more efficient than the warmer one in terms of COD_{ss} (particulate COD) removal but not for total COD, suggesting that the reactor was operating more like a settler during the cold period. For the first year the $COD_{removed}:CH_4$ conversion during cold could not exceed the 5% of the theoretically expected. The performance increased throughout the warmer period showing that seasonal variation can tackle the issue of poor methanogenesis with $COD_{removed}:CH_4$ conversion reaching close to 20% (2nd year, cold period). Further acclimation also promoted dissolved COD degradation, reducing the scum-forming potential. Luostarinen et al (2007) re ran this reactor after 13 years to treat BW and compared the findings with the study from Bogte et al (1993) for the warm period. The performance was similar in terms of biogas production regardless the 13 years´ adaptation. After 13 years, the start-up occurred faster compared to Bogte et al (1993) supporting higher microbial adaptation to low temperature (≤17°C). The COD removal never exceeded the 60% for both eras showing that treatment is not dependent on adaptation, or that further acclimation is required. Dissolved COD removal increased, reaching 53% from a negative reduction observed on the first operational year (Bogte et al 1993) supporting the hypothesis that adaptation of the methanogenic populations to low temperature is feasible. The effluent after 13 years could not meet the Class II Finnish and Dutch discharge standards as high accumulation of organic material rendered a polishing step mandatory.

In the same study Luostarinen et al (2007) set a UASB-septic tank reactor re-inoculated with the above 13 years old mesophilic sludge acclimated to low temperatures to treat BW at 15°C and compare the performance with a reactor that was remained unseeded operating at 25°C. The results at low temperature were similar with those obtained from the '13 years old' reactor operating at 14-17°C as expected. The performance of the control at 25°C

though was higher in terms of COD to methane conversion showing that the particular substrate is difficult to degrade at low temperature. Organic solid material (CODss) removal at 15 and 25ºC though was similar showing once again that temperature does not affect CODss removal even after long acclimation to low temperatures.

Elmitwalli et al (2003) also treated concentrated domestic wastewater (grey water 3600mg COD/L) at 13ºC and tried to address hydrolysis limitation by using a two-phased digester (2x AH$_{RPF}$-septic tank) achieving 94% COD removal at an HRT of 2.5 days for each digester. The experiment was based on Zeeman and Lettinga's (1999) suggestion that a 2-phased AD configuration would promote hydrolysis and prevent acidification. The AH reactor is an AF using RPF media (Huysman et al 1983). Poor biogas production was attributed to limited hydrolysis causing accumulation of insoluble matter in the first step. Thus, the COD removal was a mixture of physical separation (22%) and biological reaction and took place mainly in the first tank that filled with sludge after 4 months. The seed was mesophilic flocculent and granular sludge. From the data extracted and ADM1 modeling it was suggested that for a single reactor an HRT of 5.5-7.5 days is required to achieve similar results. Such a long HRT is perhaps too long to be practicable; thus, the use of mesophilic sludge may not be efficient enough for successful single compartment anaerobic treatment at low temperature.

Elmitwalli et al (2002) reported domestic sewage treatment using a combination of an AF followed by AH inoculated with mesophilic sludge at 13ºC. Various HRT were tested with an optimum of 4 and 8 hours for the AF and AH respectively. Hydrolysis took place mainly in the AF reaching the 81% CODss removal at an overall of 91%. 60-74% COD was converted to methane irrespective of the HRT from both steps. Most of the gas was produced in the second compartment though as the AF mainly hydrolyzed a high fraction of CODss that was converted to CH$_4$ at a lower level of 20-35%. Excess sludge produced mainly in the AF than at the AH (81 and 58% VSS/SS for AF and AH respectively). An OLR <0.38kgCOD$_{ss}$/m^3/day was suggested for stabilized AH sludge at an optimal HRT operation of 4-8 hours for the two steps (AF, AH). At lower HRT poor CODss removal in the AF lead to acidification, deterioration of the biomass and poor sludge settleability, washout and poor treatability.

McHugh et al (2006) also found that biomass can be affected by high VFA concentrations and that the associated disintegration of granular sludge affected the COD removal efficiency. The study employed whey-based wastewater in a double UASB reactor setup, operating at temperature that was reduced from 20 to 12ºC at an OLR of 0.5 and 13.3 kgCOD/m^3/day for the first and second compartment respectively. Lower temperatures caused process inhibition mainly on the second phase as a result of excess VFA and it was found that an OLR <6.6kgCOD/m^3/day was required to avoid inhibitory conditions, as methanogens could not utilize the amount of intermediates that were generated. The limited VFA degradation at low temperatures was supported from the activity tests that did not reveal presence of any putative psychrophilic organisms although the experiment ran at low temperature for 500 days.

A VFA based substrate was digested at low temperatures (8-3ºC) by Lettinga et al (1999) in a 2 phased EGSB setup inoculated with mesophilic granular sludge. The overall COD removal was adequate reaching 63-92% (around 80% for lower temperature) at an HRT of 2-5 hours. Although butyrate and acetate were easily degraded (100 & 90-100% respectively) in the first compartment, propionate was not; perhaps due to the large quantities of acetate from the evidently decelerated acetate utilization. Homoacetogenesis was less likely as the hydrogen concentration was low. Molecular analysis showed that the dominant methanogenic communities contained both acetotrophic and hydrogenotrophic (*Methanosaeta*-like and *Methanospirillum*-like) bacteria. Limited propionate oxidizers growth suggests that inoculation of enriched cultures is necessary; batch experiments showed that such cells can be cultivated using H$_2$ or formate as substrate at 10ºC.

2.2.5.3 Anaerobic-membrane configuration

Membrane reactors have also been used to promote low temperature anaerobic treatment of wastewater. It is thought that membranes can achieve a higher biomass-substrate ratio that could lead to better performance. Hu and Stuckey (2006) found that low strength domestic synthetic wastewater could be treated successfully (>90% sCOD removal) with no washout phenomena achieving a high COD:CH$_4$ conversion at mesophilic temperatures (close to 0.395m^3 CH$_4$/kgCOD). The same study showed that generation of a gel layer on the membrane boosts the process; however membrane fouling was observed and appeared to be due to particles of 0.15-0.14μm in

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
P a g e |15

diameter. Fouling was tackled by Smith et al (2013) who sparged and back flushed the membrane using the biogas formed in a study with actual and synthetic wastewater using a similar setup to that of Hu and Stuckey (2006) at 15ºC. The inoculum had a mesophilic origin and the efficiency reached the 69±10% and 92 ±5% COD removal for real and synthetic wastewater respectively. This study found that acetoclastic anaerobic digestion is predominant and *Methanosaeta* sp. was the key genus. The biomass yield was considered high (~0.10 gVSS/gCOD removed) and a substantial fraction of the methane produced (40-50%) was dissolved in the effluent. The increase in solids seemed to be beneficial to the performance as it increases adsorption of the soluble material. Contradictory results were reported by Krzeminski et al (2012) who showed that large molecules such as un-hydrolyzed proteins, fats and carbohydrates have a detrimental effect on sludge filterability and are likely to contribute in membrane fouling resulting to limited performance and subsequently poor treatability, especially at lower temperature where hydrolysis rates are slower. The colloidal and soluble COD fraction <1μm accounts for more than 63% of the COD in the system and plays an important role increasing the filterability deterioration. Additionally, failure seems likely when temperature decreases (experimental temperature: 10-18ºC) unless easily biodegradable material (e.g. VFAs) is present. Evident accumulation of non-degraded compounds was detected from Garcia et al (2013), who compared a granular and a suspended growth anaerobic membrane bio reactor (G-AnMBR, AnMBR) at moderate temperatures (25-10ºC), treating settled domestic wastewater. The results showed that there is no significant difference between granular or suspended biomass in COD and BOD removal at moderate temperatures. Remarkably though differences appeared between the fouling compounds on the membranes, with the G-AnMBR less clogged. This signifies that G-AnMBR is highly recommended to low temperature operation as cleansing is less energy intensive and less gas sparging is required. Yoo et al (2013) showed that the problem of membrane fouling can be overcome by the addition of activated carbon as a membrane scraper so the un-hydrolyzed material can be absorbed onto it. The advantages of this technique must be set against costs of membranes and activated carbon.

2.2.5.4. Summarizing

Thus far, attempts to operate a reactor seeded with mesophilic biomass at low temperatures have resulted in both limited hydrolysis and methanogenesis. Limitations have increased the risk of failure due to the physical deterioration of the biomass, reduced settleability and led to washout. No significant treatment has been observed with real wastewater at less than 8ºC and no recognizable psychrophilic community has been detected. As a result high bacterial performance at low temperatures remains problematic (according to Figure 2.4). In general adequate performance at low temperatures is favored by low organic loading rates, high retention times and a two stage process.

2.2.6. Psychrophilic and cold-adapted strategy

One solution would be inoculating digesters with cold-adapted inocula to create a psychrophilic reactor biomass. The choice of seed is of major importance (Collins et al 2006). As most cold-adapted cells are either stenopsychrophiles or eurypsychrophiles ($T_{opt.}$<15ºC but unable or able to tolerate T>15ºC respectively). According to Sheford's law of tolerance for successful growth (Sheford 1931) psychrophiles are more likely to thrive at low temperatures than at traditional mesophilic conditions. Moreover, psychrophilic seeds from low nutrient environments might have at an advantage in low strength wastewater (Cavicchioli 2006). Interestingly, Cavicchioli (2006) found that methanogens are the most abundant archaea in many samples from cold environments (Figure 2.6) suggesting that growth and multiplication of methanogenic organisms at low temperatures is common in Nature.

Figure 2.6 - 16S rRNA archaeal tree; in blue the isolates corresponding to archaea originated from cold climate environments (Cavicchioli 2006)

2.2.7. Adaptation to cold

Limitations that organisms come across when exposed to low temperature include decreased enzymatic rates, lower membrane fluidity (Ganzert et al 2007) and increased stability of nucleic acid structures (Thomas and Cavicchioli (1998).

Studies carried out by Russell (1999), Cavicchioli (2006) and Thomas and Cavicchioli (1998) found that psychrophilic microorganisms produce proteins that are not only active at low temperatures, but are more efficient and flexible than those of mesophilic and thermophilic cells. Other proteome adaptations to low temperature include "antifreeze proteins" (Feller and Gerday 2003), extracellular enzymes that prevents unwanted ice formation in their surrounding environments (for example the permafrost's 'veins', enhancing liquid transfer between cells and environment (Junge et al 2004), or enzymes adapted to substrates whose structure changes at low temperatures (Saunders et al 2003), promoting substrate utilization and uptake at such conditions.

The molecular basis for the cold adaptation in proteins includes a higher proportion of non-charged polar amino acids (glutamine, threonine) and hydrophobic residues for higher protein-substrate activity and thus higher catalytic efficiency (Saunders et al 2003).

Microbial life has developed a wide range of other strategies including: thickening of the cellular lipid bilayer at such conditions providing them insulation (Saunders et al 2003), "cannibalism" at long periods of starvation, development of ultra-microcells, dormancy, sporulation, cell size reduction, formation of capsular polysaccharide coats, adjusting the cellular water volume, use of permafrost/ice veins for energy extraction from trapped organic compounds (Price and Sowers 2004, Price 2007).

2.2.8. Psychrophilic and cold-adapted biomass as seed

Communities with these myriad adaptations to low temperature environments could be very useful seeds for anaerobic treatment reactors operating at low temperatures.

A wide variety of cold-adapted methanogens and other organisms have been described. Simankova et al (2003) revealed a psychrotolerant hydrogenotrophic ecotype of the genus *Methanocorpusculum* belonging to the order of *Methanomicrobiales* having a morphology of motile irregular cocci (Figure 2.7.d) from Baldegger lake (Switzerland). Interestingly this organism had more than one copy of the mcrA gene. The same study isolated a

strain of *Methanomethylovorans hollandica* (Figure 2.7.c) which was uniquely able to use methanol, methylamines, DMS and methanomethiol as substrates at temperatures of 4-5°C. *Sarcina*-like cells were also detected with no unique properties apart from psychro-tolerance and the formation of large agglomeration (up to 1mm) (Figure 2.7.a). Franzman et al (1997) isolated an obligate psychrophilic methanogen from Ace lake (Antarctica), *Methanogenium frigidum*, the first psychrophilic methanogen known to be able to catabolise H_2/CO_2, with a growth rate of 0.24 day^{-1} (doubling time 2.9 days). The effect of temperature on its growth has been established (Figure 2.7.e) and it can tolerate a pH range: 6.3-8.0. Nozhevnikova, et al (2003) isolated a psychrophilic methanogenic community after incubation experiments of sediments from Lake Baldeg and Lake Soppen (Switzerland). The samples were pre-incubated at 4 to 60°C and then transferred to 6°C. The methane production rates were higher for the bacteria pre-incubated at low temperatures. The acetate methanogenic pathway predominated and *Methanosaetaceae* was the most dominant methanogenic genus. A hydrogenotrophic psychrotolerant methanogen (*Methanosarcina lacustris*) was found to dominate in a study on Siberian permafrost, carried out by Metje and Frenzel (2007) trying to reduce iron (II), VFAs and ethanol. Methanogenesis follows the acetoclastic pathway at the optimum temperature (26-28°C) but H_2/CO_2 became more important at lower temperature (4°C). Butyrate was consumed during methanogenesis and accumulated when methanogenesis was inhibited (with BES and CH_3F), suggesting that butyrate serves as precursor of methane, presumably providing acetate and H_2 by syntrophic oxidation. A Ratkowsky plot (square-root of growth rate versus temperature plot (Ratkowsky et al 1983) suggested that participate in anaerobic treatment could survive and grow at temperatures as low as 7.15°C. Another example of syntrophy at low temperature anaerobic conditions is *Syntrophus aciditrophicus*, a psychrophilic/psychrotolerant syntrophic microorganism studied by McInerney et al (2007). This particular organism is able to metabolize fatty acids, benzoate, cyclohexane carboxylate and cyclohex-l-ene and crotonate if a CO_2 reducer methanogen or an SRB is present.
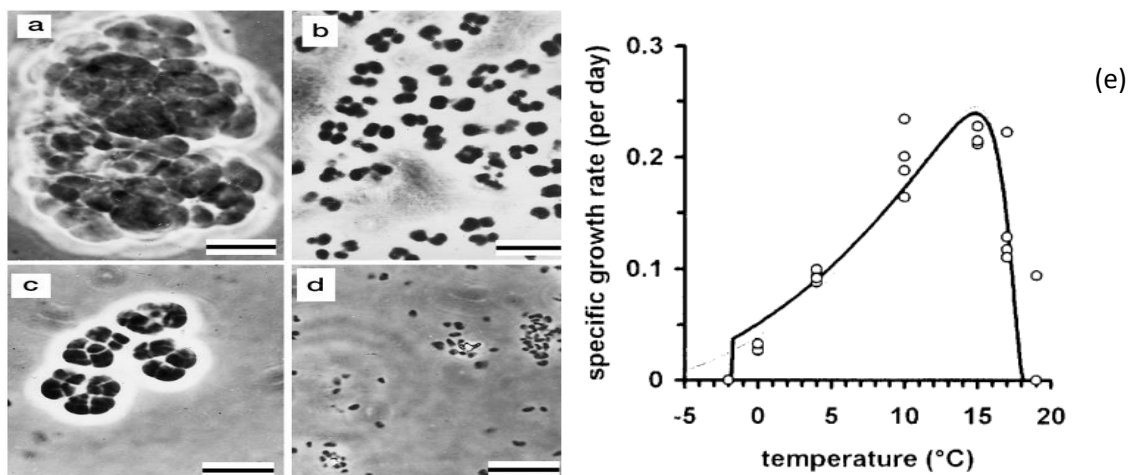
Figure 2.7 – (left) Micrographs of a) strain MT, b) strain MM (both Sarcina-like), c) strain ZB, d) strain MSP. (Simankova et al 2003); (2e) effect of temperature to growth of *Methanogenium frigidum*, data points represent values of μ during growth in MSH medium, dark line: best fit of the modified square root equation (Franzmann et al 1997).

Ya Lokshina and Vavilin (1999) compared the kinetics of microbial consortia from Syktyvar Forest, Polar Ural (Varkuta) and East Central Germany at low temperature (mean annual 4-6°C), and found little difference between growth rates at 6°C (0.011-0.022 day^{-1}). When temperature was increased the growth rate increased substantially.

Nozhevnikova et al (2007) using sediments from Lake Baldegg, Switzerland found that methanogenesis mainly followed the acetoclastic pathway (predominance of *Methanosaetaceae*); H_2/CO_2 methanogenesis was outcompeted by homoacetogenesis at 5-15°C. In this study the fraction of hydrogenotrophic methanogenesis was proportional to temperature attaining its maximum under thermophilic conditions (>50%, up to 70°C). Similar results in terms of homoacetogenesis at low temperatures were obtained by Kotsyurbenko et al (1993) using an inoculum from a pond in the Urals. Homoacetogens out-competed methanogens and subsequently methane formation rate was poor at low temperature and increased at higher (6 to 28°C). Kotsyurbenko et al (1993) also evaluated the effect of temperature on hydrolysis and acidogenesis using various organic substrates at 6, 15 and 28°C. They found that temperature affects the rate of cellulose hydrolysis, fermentation and

acidogenesis/acetogenesis. The last was found to be the rate limiting step and the most temperature sensitive as it required the longest start-up period (at 6°C) amongst the other processes mentioned.

The relative rates of homoacetogenesis and methanogenesis at low temperatures were also examined by Kotsyurbenko et al (2001). H_2 uptake of isolates of methanogens and homoacetogens were compared. Methanogens had an advantage at low H_2 pressure, however when H_2 is not limited homoacetogens did better than methanogens. The results showed that with excess H_2 homoacetogenesis leads to the re-distribution of the organic matter in the psychro-active communities. Thus, the presence of syntrophic methanogenic communities able to outcompete homoacetogens and uptake H_2 reducing partial pressure is of major importance (Kotsyurbenko 2005). Nozhevnikova et al (2001) in a study using inocula taken from 50cm sediment depth of a Swiss lake showed that temperature decrease adversely affects methane formation (tested from 25 to 5°C) and that methanotrophs seem to be more tolerant to temperature decrease than methanogens.

Hoj et al (2005) using soil from Spitsbergen wetlands showed that there is both acetotrophic and hydrogenotrophic methanogenesis at 10°C in communities with *Methanomicrobiales*, *Methanonobacteriaceae* and *Methanosaeta*. The relative importance of the H_2/CO_2 pathway to methane production at low temperature was not clear. The pathway appears to be favoured if polysaccharides are present as an important fraction of the organic material (e.g. rice paddy soils or lake sediments) or if the cells are exposed to nutrient-poor environments (Kotsyurbenko et al 2004).

Kotsyurbenko et al (2004) also stated that apart from temperature, sampling depth plays a vital role for methanogenesis indicating that the optimal depth for high methane production is 30-50cm below the ground surface. Yavitt et al (2006) also detected higher methanogenic activity from the samples that were taken slightly deeper than 20cm. Ganzert et al (2007) also mentioned that soils that were sampled from 20-35cm depth or close to the surface (<5cm) were the optimum in terms of methane production. This was supported by the in increased possibility of methanogenic substrate´s presence in the form of solubilized TOC and DOC (total and dissolved organic carbon) from decomposed vegetation at deep and shallow depths respectively.

Yavitt et al (2006) sampled 3 peat soils from the continental western Canada to test methanogenesis at low temperature and defined specific cellular activity. The maximum performance was 250nmol/g/day (or 250 femtomols/cell/day) at 25°C for un-amended incubation. The activity was enhanced by 600 times after addition of ethanol. After storage at 0°C for 18 months the experiment was repeated demonstrating decreased activity equal to 10-221 femtomols CH_4/cell/day; addition of ethanol only doubled the amount. Decay of active methanogens from starvation and low or no adaptation to 0°C possibly contributed to the lower activity. 16S rRNA sequence identified species affiliated with *Methanosaetaceae*, *Methanosarcinaceae*, *Methanomicrobiales* and *Methanobacteriales*; all psychrotolerant.

Psychrophilic cells had been found in ice core samples from Lake Vostok by Price (2000) at temperature below freezing point. The cells were able to survive and metabolize although the nutrients were dispersed in the solid ice; they could not multiply. Sulphate (SO_4^{-2}) and nitrate (NO_3^-) were found to be the main electron acceptors; methanosulfonic acid (MSA), formic and acetic acid are the main energy/carbon providers. This reveals potential for methanogenesis at low temperatures as formic and acetic acids are fundamental substrates to the methane pathway.

Although psychrophilic and cold-adapted inoculum had been tested for their ability to anaerobically degrade a variety of substrates (Alvarez et al 2008; Elmitwalli et al 2002; Luostarinen et al 2007; Rebac et al 1999; Sanz and Fernando-Polanco 1990), only a few of the systems could adequately operate at <13 °C with real wastewater as substrate, fact that has recently been attributed to failure in methanogenesis at temperatures below 8 °C (Bowen et al 2014).

Hydrolysis was the rate limiting step in most previous studies of the treatment of real wastewaters employing mesophilic or cold-adapted inocula. Hydrolysis is the step in which carbohydrates are converted to sugars and subsequently fermented to VFAs; proteins are hydrolyzed to amino acids and further degraded to VFAs through anaerobic oxidation linked to H_2 production or via fermentation and lipids, mainly triglycerides are hydrolyzed to LCFAs and further oxidized via b-oxidation to acetate or propionate.

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
P a g e | 19

2.2.9. Effect of temperature to hydrolysis

At low temperatures slow hydrolysis rates lead to the accumulation of suspended solids and consequently reduced efficiency. For hydrolysis optimization the control of HRT and pH (Hwang et al 2001) (Figure 2.8.a, b, c, d) in addition an appropriate SRT is required (Miron et al 2000). Miron et al (2000) also revealed that a CSTR treating domestic wastewater, operating at 25°C needs at least 8 days for complete hydrolysis and prevention of biomass acidification that might lead to washout and deterioration phenomena; in detail it also revealed potential implications that might occur at low temperature operation (see also Figure 2.9.a, b, c, d, e):
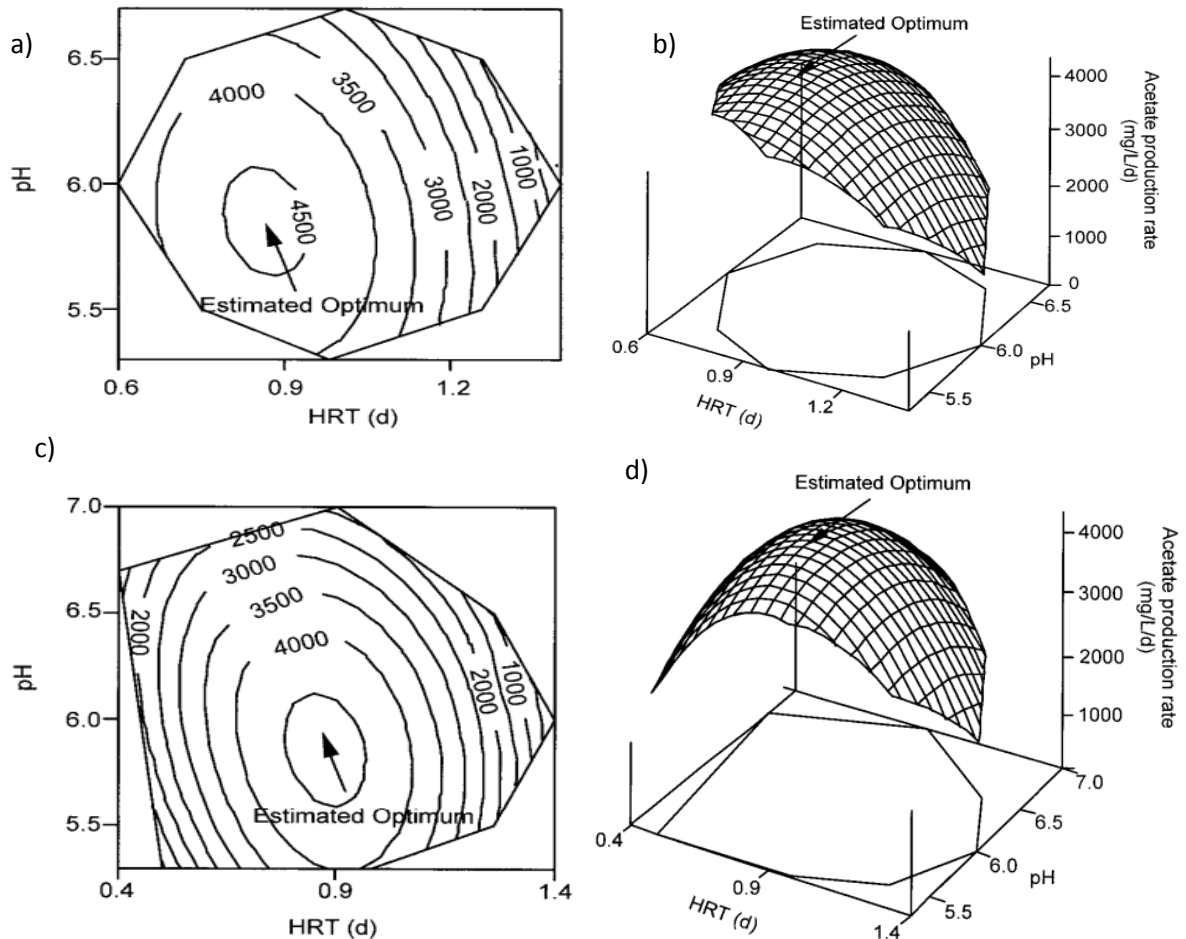
Figure 2.8 - a) two and b) three dimensional contour plots of a quadratic model predicting acetate production with respect to pH and HRT; c) two and d) three dimensional plots of a model model for the acetate production rate in the extended region for optimized butyrate production (Hwang et al 2001).

The study in hydrolysis of lipids by Miron et al (2000) showed that:

- 39% of the lipids are already converted to LCFA before entering the WWTP. At lower temperatures completed hydrolysis may require more time.
- Low SRT inhibits the b-oxidation of LCFA rendering degradation of lipids impossible, leading to accumulation of lipids.
- At an SRT >3 days, accumulation of LCFA occurs (Figure 2.9.c) leading to acidogenic conditions as b-oxidation of LCFA becomes the rate limiting step compared to hydrolysis; for SRT >15 days lipids hydrolysis becomes rate limiting.
- Lipid unsaturation accelerates degradation. LCFA such as C16:1 (palmitoleic), C18:1 (oleic), and C18:2 (linoleic) were found to be the easiest degradable acids under acidogenic conditions (removal by hydrogenation stimulated by the high H_2 pressure). At lower tempertures the ease in the degradation of the above compounds isn't certain and higher SRT may be required affecting lipid hydrolysis.

The hydrolysis of proteins was evaluated in the same study and Miron et al (2000) concluded that:

- Proteins can be identified by the amount of NH_4^+-N
- Most protein hydrolysis occurs in the sewer however at low temperatures this may not occur.
- An SRT of 8-10 days is enough to achieve high protein hydrolysis at 25°C (Figure 2.9.d); higher retention time might be required at lower temperature causing limited lipid degradation though.
- Chemical processes, such as the precipitation of ammonium as struvite, may lower degradation rates
- High concentrations (174-220 mg/L) of amino acids can inhibit protein hydrolysis; slow acidogenesis at low temperatures can lead to the accumulation of amino acids, and make inhibition more likely.

The hydrolysis of carbohydrates was also observed by Miron et al (2000):

- Hydrolysis of hydrocarbons is proportional to SRT (Figure 2.9.e). Employing high retention time to hydrolyze carbohydrates at low temperature though might result in limited lipid degradation due to acidification conditions.
- Dissolved carbohydrates were a small (1.5%) but consistent as fraction of total carbohydrates for all SRTs. This suggests that carbohydrates hydrolysis might become limited, especially at low temperature as COD_{dis} may be hard to remove increasing the percentage above.

Kinetics of wastewater hydrolysis is much debated. Pabon Pereira et al (2009) suggest that hydrolysis does not follow first order kinetics, which can be applicable in only batch studies where the inoculum is well balanced. Velasquez-Orta et al (2011), Veeken and Hameless (1999) and Eastman and Ferguson (1981) suggest that first order kinetics can describe hydrolysis irrespective of the reactor. Eastman and Ferguson (1981) also found that hydrolysis limits anaerobic wastewater treatment as the solubilisation the organic material is slow (hydrolysis rate constant: 0.125 hours^{-1}). The same study showed that carbohydrates are extensively degraded first followed by proteins; lipid degradation was slow or unlikely at the acid phase due to the reasons described by Miron et al (2000).

Hills and Nakano 1984 demonstrated the relationship between particle size and hydrolysis. This study showed that hydrolysis and subsequently methane production is inversely proportional to a substrate's particle size diameter (in average) and to its shape (sphericity) (Figure 2.10.a, b). Shape seems to have a greater impact at lower temperature as liquid density increases and polyhedral crystals are formed increasing the particle diameter and asymmetry.

Rates of hydrolysis and the effect of temperature on biodegradability have been investigated by Veeken and Hamelers (1999) for various biowastes in a temperature range of 20-40°C. The results showed that hydrolysis rates can be described by first order kinetics and the enzymes participating in the process obey the Arrhenius equation. The hydrolysis constant at 20°C was 0.03days^{-1} . The results showed the rate of hydrolysis (Figure 2.11.a) and biodegradation (Figure 2.11.b) varied with temperature. The average metabolism activation energy was estimated to be 64±14 kJ/mol.

Hydrolysis in MFC has also been studied by Velasquez-Orta et al (2011). This study showed that the more complex the substrate the lower the hydrolysis rate. Substrate complexity had also an impact on COD removal efficiency and the coulombic efficiency (CE). The rate constants were calculated (assuming first order kinetics) as 0.0024 hours^{-1} for hydrolysis and 0.018 hours^{-1} for combined hydrolysis and fermentation (at 19±2°C) suggesting hydrolysis as the rate limiting step over fermentation.

From the foregoing, it is possible to gain an insight as to why hydrolysis is the rate limiting step in low temperature anaerobic treatment. Lack of adaptation of cold-adapted mesophilic biomass to low temperature, in addition to the lack of psychrophiles in the mesophilic inocula are the main reasons behind the slow wastewater hydrolysis rates. Changes in the nature of the substrate may also contribute to the limitation. Plainly the presence of hydrolytic organisms with elevated activity at low temperatures would lead to enhanced hydrolysis and more successful anaerobic treatment at low temperatures.
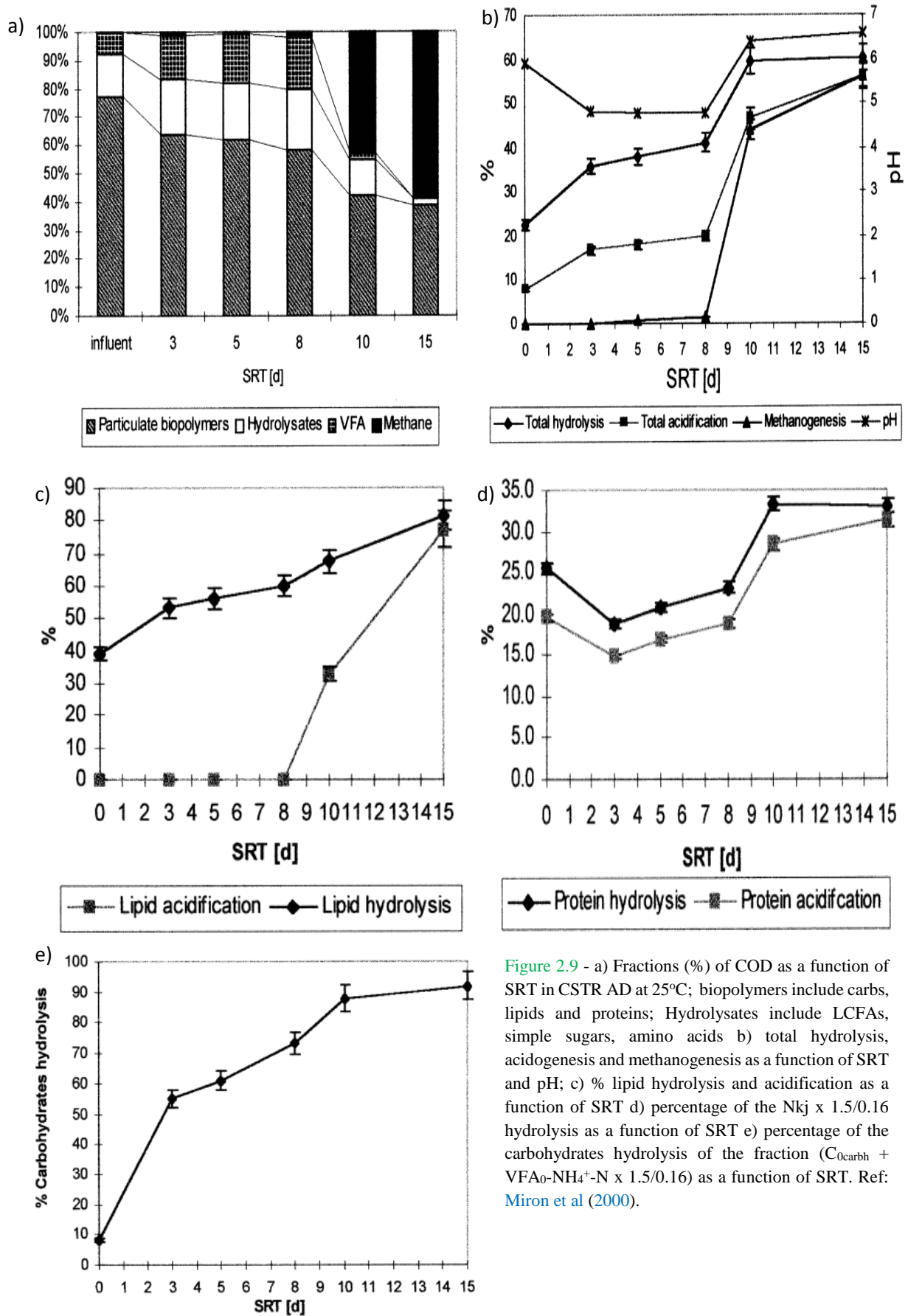
Figure 2.9 - a) Fractions (%) of COD as a function of SRT in CSTR AD at 25°C; biopolymers include carbs, lipids and proteins; Hydrolysates include LCFAs, simple sugars, amino acids b) total hydrolysis, acidogenesis and methanogenesis as a function of SRT and pH; c) % lipid hydrolysis and acidification as a function of SRT d) percentage of the Nkj x 1.5/0.16 hydrolysis as a function of SRT e) percentage of the carbohydrates hydrolysis of the fraction (C_{0carbh} + VFA_0-NH_4^+-N x 1.5/0.16) as a function of SRT. Ref: Miron et al (2000).

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
P a g e | 2 2

a)
b)
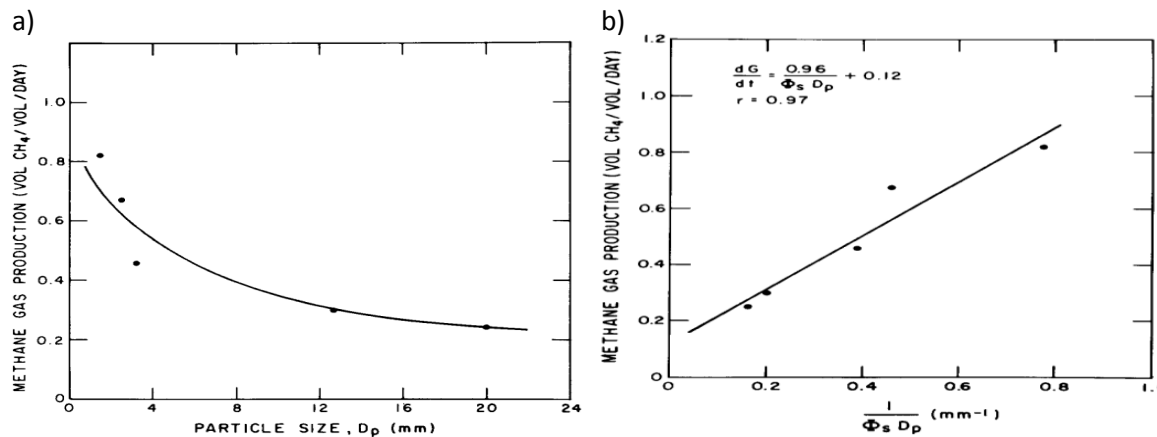
Figure 2.10 - a) Methane production as a function of substrate particle size; corrected to 0°C and 1atm; b) gas production as a function of particle sphericity (Φ_s) and average diameter (D_p); ref: Hills and Nakano 1984.

a)
b)
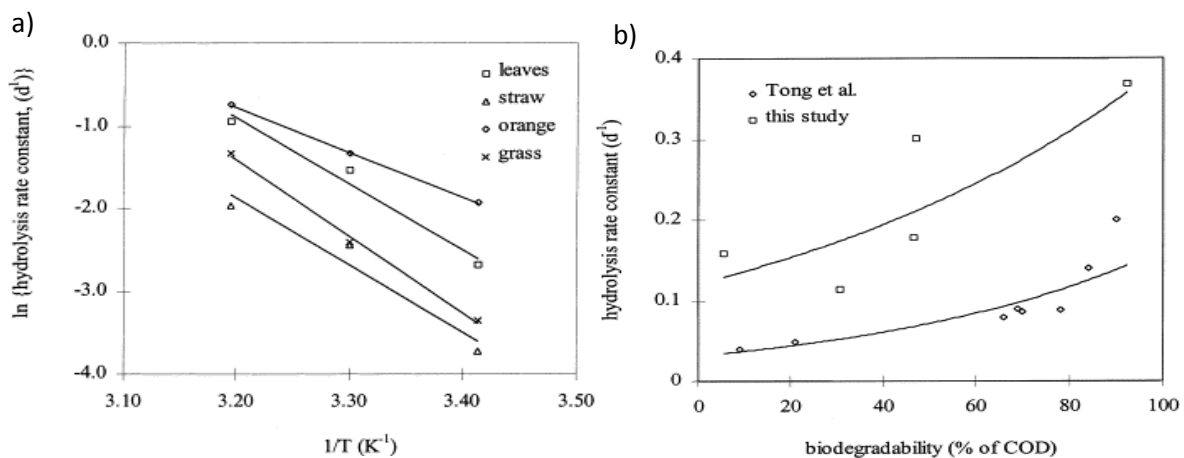
Figure 2.11- a) Arrhenius plots for hydrolysis rates of different substrates as a function of temperature; b) effect of biodegradability to hydrolysis rates (Veeken and Hamelers 1999).

2.2.9.1. Substrate preference

Irrespective, of the temperature some substrates are preferentially degraded. Consequently, some compounds accumulate in bioreactors, increasing the risk of inhibition in anaerobic treatment. Substrate preference leads to enzymes synthesis for specific substrates, this contributes to the buildup of other intermediates (Whooley et al 1983) (rates from Masse et al 2001). Breure et al 1986 studied the hydrolysis of gelatin in the presence of carbohydrates and found that carbohydrates are easier to degrade than proteins and that the biomass had greater affinity for carbohydrates than other substrates. It is believed that this preference for carbohydrates may also occur at lower temperatures. Moreover the changes in the physical nature of the substrate (e.g. lipid solidification, crystallization, fatty acids chain rigidity; Neidleman 1987) at low temperatures can make a substrate intrinsically more difficult for enzymes to break down. Thus one might anticipate that, at low temperatures, lipids are more likely to accumulate than proteins or carbohydrates.

2.2.10. Potential psychrophilic and cold-adapted hydrolysis (lipolysis)

From the foregoing, it is naturally important to consider hydrolysis and most especially lipolysis.

2.2.10.1. Lipases

The enzymes responsible for the breakdown of lipids are called 'lipases'.

Lipases (glycerol ester hydrolases - biocatalysts) are proteins secreted by bacteria to hydrolyze esters and glycerol with long chain fatty acids, based on the interface generated by a hydrophobic substrate when in liquid (Figure 2.12). Lipases don't necessarily follow the Michaelis-Menten model as activity increases with substrate emulsification (Jaeger et al 1994). At low temperature increased liquid density might lead to poor lipolysis due to,

inter alia, limited emulsification. The details of lipase synthesis and secretion depend on the bacterial taxon. The lipase catalytic site contains a serine-protease-like catalytic triad that consists of serine, histidine and aspartate amino acids. It is covered by a lid-like a-helical structure which is removed when in contact with the substrate, exposing specific hydrophobic residues at the protein's surface. This mechanism is responsible of lipase substrate specificity (Jaeger et al 1994). Specific lipid types require specific enzymes which might be specific to specific microbial genera.
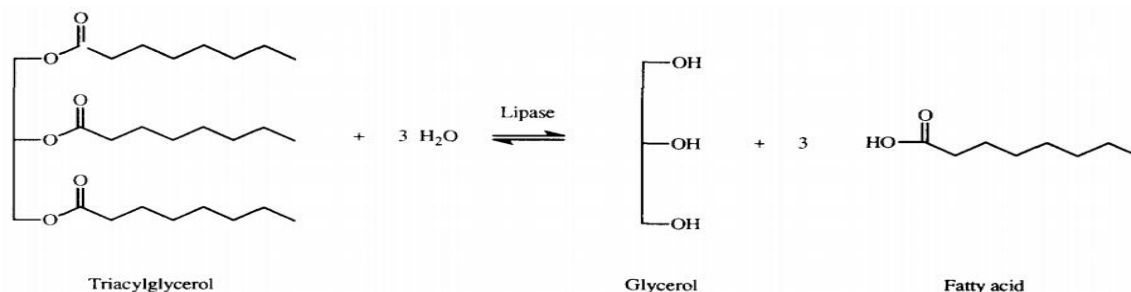
Figure 2.12 - Lipase enzymatic reaction mechanism resulting to a fatty acid and vice versa (hydrolysis and synthesis) (Jaeger et al 1994).

2.2.10.2. Psychrophilic and cold adapted Lipases

Enzymes with an optimum at low temperatures are the key proteins that a bio-reactor must have to operate at cold environments. Burgess and Pletschke (2008) estimated that for every 10ºC temperature switch the activity increases or decreases by a fold of 2. Psychrophilic and cold-adapted cells are able to produce enzymes that efficiently operate at low temperature.

Cold adapted lipases are produced by microorganisms (Table 2.1) inhabiting cold environments (≈5ºC). These organisms and their enzymes can be found in deep sea, Antarctic and Polar regions, in artificially refrigerated environments (e.g. frozen food). In addition to bacteria, a number of fungi (Table 2.2) have been also found to be able to produce cold-adapted enzymes (Joseph et al. 2007). Both aerobic and anaerobic microorganisms produce lipolytic enzymes at low temperatures (Yumoto et al 2003).

Cold adapted enzymes usually have a molecular weight in-between 30-50 kDa (Preuss et al 2001, Dieckelmann et al 1998). Water content in the substrate is of major importance for the lipolytic performance (Anderson 1980, Parfene et al 2011). Thus, as water turns into ice at lower temperatures lipolysis is decelerated. Salt concentration in the liquid also affects the lipolytic performance (Parfene et al 2011). Joseph et al (2006) showed that sodium chloride increased lipase production whereas the presence of metals in the media had an inhibitory effect. The study also suggested that lactose improved lipolysis. Performance was also boosted by the addition of 0.1-1% ($^v/_v$) detergent (Lee et al 2001).

Lipases can hydrolyze vegetable oils and lard at extremely low temperatures of (-7 to -29ºC) with great specificity (Alford and Pierce 1961). Many lipases demonstrate the ability to work at a range of temperatures (e.g. those excreted from *Aeromonas* sp.) (Pamberton et al 1997). Alquati et al (2002) and Arpigny et al (1997) investigated the mechanisms of adaptation to low temperature and found: a very low proportion of arginine residues compared to lysine, low content in proline residues, a small hydrophobic core, a very small number of salt bridges and aromatic-aromatic interactions. The same study investigated a psychrophilic enzyme from *Psychrobacter immobilis* that required half the activation energy required from a mesophilic enzyme at 37ºC when at 4ºC (from 110 to 63 kJ/mol). Breuil and Kushner (1975) showed that an *Acinetobacter* sp. excretes higher amounts of lipases at low temperatures although its optimum growth temperature is 30ºC. Lo Giudice (2006) found that 95.5% of 155 lipases had significant lipolytic activity at low temperature and one (*Pseudoalteromonas* sp.) performed higher at 4ºC than at 15ºC.

Choo et al (1998) studied a psychrotrophic enzyme showing preference to p-Nitrophenyl esters of fatty acids of short-medium carbon chains (C_4, C_6). Similar specificity was detected from Rashid et al (2001) who showed that the activity energy required was estimated at 46.9 and 32.2 kJ/mol for 5 & 35°C respectively for p-nitrophenyl butyrate hydrolysis. These values are low compared to the activation energy required for metabolism at low

temperature (110 kJ/mol) suggested by Price and Sowers (2004) or Veeken and Hamelers (1999) (64 kJ/mol) for total biowaste hydrolysis at 20-40°C. Kulakova et al (2004) suggested that only 19.2 kJ/mol is required for the same substrate at 5°C.

Table 2.1 - Bacterial cells producing cold adapted lipases (Joseph et al 2007, 2008).

Microorganism	Sources	Reference
Acinetobacter sp. strain No. 6	Siberian tundra soil	Suzuki et al., 2001
Acinetobacter sp. strain No. O16	Ns	Breuil and Kushner, 1975
Achromobacter lipolyticum	Ns	Khan et al., 1967
Aeromonas sp. strain No. LPB 4	Sea sediments	Lee et al., 2003
Aeromonas hydrophila	Marine habitat	Pemberton et al., 1997
Bacillus sphaericus MTCC 7526	Gangorthi glacier (western Himalayas)	Joseph, 2006
Corynebacterium parametabolum MTCC 6841	Naukuchiatal lake Uttaranchal	Joshi et al, 2006
Microbacterium phyllosphaerae MTCC 7530		
Moraxella sp.	Antarctic habitat	Feller et al., 1990
Morexella sp TA144	Antarctic habitat	Feller et al., 1991
Photobacterium lipolyticum M37	Marine habitat	Ryu et al., 2006 Jung et al., 2008
Pseudoalteromonas sp. wp27	Deep sea sediments	Zeng et al., 2004
Pseudoalteromonas sp.	Antarctic marine	Lo Giudice et al., 2006
Psychrobacter sp.		
Vibrio sp.		
Pseudomonas sp. strain KB700A	Subterranean environment	Rashid et al., 2001
Pseudomonas sp. B11-1:	Alaskan soil	Choo et al., 1998
Pseudomonas P38	Ns	Tan et al., 1996
Pseudomonas fluorescens	Refrigerated milk samples	Dieckelmann et al., 1998
Pseudomonas fluorescens	Refrigerated food	Andersson 1980
Pseudomonas fluorescens	Refrigerated human placental extracts	Preuss et al., 2001
Pseudomonas fragi strain no. IFO3458	BCCMTM/LMG2191T Bacteria collection, Universiteit Gent, Belgium	Alquati et al., 2002
Pseudomonas fragi Strain no. IFO 12049	Ns	Aoyama et al., 1988
Psychrobacter sp. wp37	Deep sea sediments	Zeng et al., 2004
Psychrobacter okhotskensis sp.	Sea coast	Yumoto et al., 2003
Psychrobacter sp. Ant300	Antarctic habitat	Kulakovaa et al., 2004
Psychrobacter immobilis strain B 10	Antarctic habitat	Arpigny et al., 1997
Serratia marcescens	Raw milk	Abdou, 2003
Staphylococcus aureus	Ns	Alford and Pierce, 1961
Staphylococcus epidermidis	Frozen fish samples	Joseph et al., 2006
Yarrowia lipolytica	Frozen food	Parfene et al., 2001

*Ns: Not specified

Feller et al (1991) showed that the optimum temperature can be also changed by transferring genes from a psychrophilic organism to *Escherichia* coli. Jung et al (2008) investigated a lipase secreted from *Photobacterium lipolyticum* that retains 75% of the activity at optimum conditions when exposed at 5°C. These lipases have a unique cavity beneath the lid and a wider oxyanion hole requiring lower activation energy, making them efficient lipid catalysts at low temperatures. Zeng et al (2004) studied 23 different lipolytic cells with growth temperature

ranging from 4 to 30°C. Although their optimal temperature was at 10±2°C at 4°C they could retain 60% of their optimum activity. Similar results were found by Suzuki et al (2001) who demonstrated that 57% of the optimum activity can be retained when the enzymes are exposed to 4°C.Other studies that showed noteworthy lipolytic activity at low temperature were Tan et al (1996), Abdou (2003) and Khan et al (1967).

Lipases of fungal origin (Table 2.2.) are used in the food industry for lipid degradation at ambient temperature (Coenen et al 1997). Mayordomo et al (2000) examined a fungal lipase that had a higher productivity when the mycelium was grown at 30°C, and was able to efficiently hydrolyze glycerides, showing preference towards esters of short- and middle-chain fatty acids. This lipase showed also high activity in the range of 0-20°C. One of the most widely used fungal lipases is *Candida Antarctica* A and B (Table 2.3.) with 45 and 35 kDa molecular weight (CALA and B respectively). Kirk et al 2002 and Uppenberg et al 1994 examined CALA and B estimating them of 1.55 Angstrom and 2.1 Angstrom, respectively. The structure of CALB shows that the enzyme has a Ser-His-Asp catalytic triad in its active site. Its 'architecture' appears to be in an 'open' conformation with a rather restricted entrance to the active site and is believed that this accounts for the substrate specificity and high degree of lipase stereo-specificity. The A component is shown to be more thermostable than the B component.

Table 2.2 - Fungi producing cold-adapted lipases (Babu et al 2007, 2008).

Microorganism	Sources	Reference
Aspergillus nidulans	Ns*	Mayordomo et al., 2000
Candida antarctica	Antarctic habitat	Patkar et al., 1993;
		Uppenberg et al., 1994a
		Uppenberg et al., 1994b
		Patkar et al., 1997
		Koops et al.,1999
		Zhang et al., 2003
		Siddiqui and Cavicchioli, 2005
		Kirk and Christensen 2002
C. lipolytica	Frozen food	Alford and Pierce 1961
Geotrichum candidum	Frozen food	Alford and Pierce 1961
Pencillium roqueforti	Frozen food	Alford and Pierce 1961
Rhizopus sp.	Frozen food	Coenen et al., 1997
Mucor sp.	Frozen food	Coenen et al., 1997

*Ns: Not specified

Table 2.3 - CALA and CALB enzyme characteristics (Kirk and Christensen 2002)

	CALA	CALB
molecular weight (kDa)[3]	45	33
isoelectric point (pI)[3]	7.5	6
pH optimum	7	7
specific activity (LU/mg)[4]	420	435
thermostability at 70°C[3,a]	100[100]	15[0]
pH stability[3,b]	.6-9	.7-10
interfacial activation	yes (but low)	No
positional specificity toward glycerides[5]	*Sn-2*	*Sn-3*

A: residual activity after incubation at 60oC in 0.1 M tris buffer (pH 7.0) for 20mins and [120] mins; b: pH at which more than 75% activity is retained following incubation for 20 hours at room temperature.

Plainly low temperature lipolysis is possible. Enzymes secreted either by bacteria or fungi have developed the mechanisms to efficiently operate at low temperature. Additionally, bio-engineering and biotechnology have held

the promise that we can use chemical, biological and molecular techniques to facilitate and optimize enzymatic lipolysis.

2.2.10.3. Other psychrophilic enzymes

There are a variety of other enzymes that are able to decompose either carbohydrates or proteins at low temperatures. An extracellular (40kDa) endo-polygalacturonase (PGL) enzyme from a psychrophilic fungus *Mucor flavus* able to hydrolyze polysaccharides to sugars at an optimal temperature of 20ºC was reported by Gadre et al 2003. Huang and Yang 2003 found the first cold-adapted imidase (105 kDa) (hydrolyzes cyclic imides to monoamidated dicarboxylates) with surprising high activity at low temperature. The enzyme extracted from the liver of *Oreochromis niloticus* (a fish) and in a comparative study with conventional enzymes showed 10 to 100 times higher activity from 0 to 40ºC on maleimide as substrate.

A large variety of other enzymes capable of operating at low temperature have been investigated; however this study focuses on lipases as the accumulation of lipids proved to be an issue with anaerobic treatment at low temperatures.

2.2.10.4. Enzyme pretreatment & post-treatment capacity

Lipid accumulation is a common issue in variety of industrial wastewater (for example slaughterhouse wastes (Cammarota and Freire 2006). In domestic wastewater the phenomenon is less prominent as the lipid content is usually low.

Masse et al (2001) pre-treated slaughterhouse wastewater using a *Pancreatic* lipase 250 at a concentration ranging from 125-1000 mg L^{-1} at 20-24ºC. The results showed that the enzyme assisted the reduction of the size of lipids and after 4-24 hours and LCFAs appeared after 6-7 hours of treatment. Similarly, Valladao et al 2011 showed that the use of 1%$^w/_v$ of *Penicillium restrictum* as an enzyme hydrolysis step for the degradation of poultry wastewater could increase the specific methanogenic activity at 35ºC. Many studies have recognized the feasibility of enzyme pre-treatment; however no studies have been published at temperatures of less than 20ºC.

2.3. Microbial community structure conservation

Inoculating a bioreactor with psychrophilic or cold-adapted biomass is a possible strategy for the promotion of the COD:CH$_4$ conversion at low temperature Biomass that performs at low temperatures and retains its psychrophilic characteristics might be the ideal inoculum for WWTPs situated in countries where temperature is low and seasonal temperature fluctuations are common. In such wastewater systems, numerous indigenous microorganisms will compete with the individuals from the seed for the substrate's organic carbon. This competition forms a very dynamic pattern in the bio-reactor diversity (Pender et al 2004). Curtis and Sloan (2004) suggested that 'invasion' from outside (e.g. wastewater cells) can influence an established community diversity according to:

- Diversity of the source or meta-community where the 'immigrants come from
- Distribution of the taxa in the meta-community
- Immigration rate of bacteria from the source to the local community (i.e. biological reactor)
- The population size of both invaders and system cells
- The size of the local community
- The spatial heterogeneity of the local community

Thus it is of great importance to determine whether the arrival of 'immigrants' from one source (e.g. wastewater) has a significant impact on the diversity patterns of the reactor communities, especially at low temperatures.

2.3.1. Parameters that may affect cell abundance

Engineered bio-systems allow us to study the patterns of microbial diversity which are ultimately a product of evolution (speciation) and the ecological processes of immigration and environmental selection. High rates of dispersal imply high rates of immigration; high immigration limits speciation; hence, a community once formed, is almost fixed as it would be very hard for abundant species to go extinct (Curtis et al 2006).

There are fundamental truths underlying the formation of an open local community of microorganisms. According to neutral theory (which assumes the equivalence of specific growth rate for all organisms) the community is continuously exposed to immigration from some larger source community when individuals die they are either replaced by an immigration or a birth event (Bell 2000, 2001, Hubbell 2001) (Hubbell's model Figure 2.13). Thus the community forms and develops through a continuous cycle of immigration, births and death (Sloan et al 2006b). Decay/extinction or immigration in such systems enhance (high immigration rates) or deplete diversity (low immigration rates) (Sloan et al 2006) (Figure 2.13). Although predicted taxa-abundances show agreement between theory and observation there is no doubt that the neutral theory does fail to explain some observations (Woodcock et al 2007) remaining an *anathema* to many microbiologists. Indeed Prosser et al (2007) suggested that the existing conceptual theory is not sufficient to describe biological systems in detail as they are multifactorial and cannot be fully understood.

Brock (1987) described the study of diversity as mumbo-jumbo; however **we believe that such systems can be understood if both random and deterministic factors will be analysed as they both affect the community structure**.
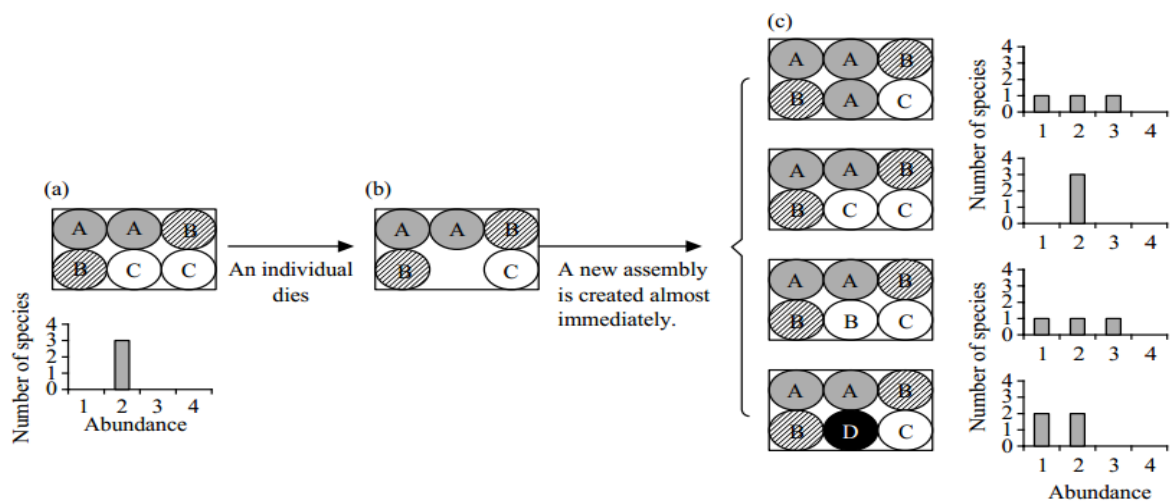
Figure 2.13 - (a) schematic representation of vacancy fulfilment of a local community of 6 individuals from 3 taxa (A,B,C); (b) an individual from C dies/extincts leaving a position vacant, the possible scenarios for the fulfilment of the vacant position may be: (c) the slot is being fulfilled from an individual of the existing population (reproduction, figures 1, 2, 3 top to bottom) or an immigrant from the meta-community (D, figure 4, bottom) (e.g. wastewater), altering the abundance distribution (Curtis and Sloan 2006; Battin et al 2007)

Neglecting the innate differences amongst species and their distinct responses to environmental factors may lead to ecosystem oversimplification. Thus, both neutral and deterministic factors such as temperature and competition for substrate respectively is needed to describe how external conditions can affect specific populations, driving them to proliferate or go extinct (Curtis and Sloan 2004). Such factors enhance growth of particular microorganisms and suppress others, changing the functionality of such systems (Kotsyurbenko 2005, Xing et al 2009), especially when communities are continuously exposed to different conditions. Analysis of a microbial community structure including both neutral and deterministic factors gives a better insight into cellular adaptation and functional redundancy. Adaptation is one of the key aspects in engineered biological systems (Rittman and McCarty 2001), especially in low temperature anaerobic treatment where the predominance of the cold-adapted/psychrophilic cells in the community matrix seems to be the key for efficient wastewater to energy bioconversion.

Chapter 3: *Experimental plan, what is the purpose for the particular experiments?*

- Batch 1: Start-up period
- Batch 2: Start-up period (2)
- Batch 3: WW treatment at all temperatures
- Batch 4: N/A
- Batch 5: Hydrolysis, cumulative phenomena
- Batch 6: N/A
- Batch 7: N/A
- Batch 8: Lipases activity assays
- Sub-culture 1: Re-inoculation from batch 4, 15-4°C sterilization experiment
- Sub-culture 2: Re-inoculation from batch 5, 15 to 4°C, 4 to 15°C sterilization experiment
- Cost & application via HRT, estimated from data collected as the arrows show

Batch 1
Batch 2
Batch 3
Batch 4
Batch 5
Batch 6
Batch 7
Batch 8
Sub-culture 1
Sub-culture 2
Cost & applicability

Figure 3.1 - Representation of the experimental sequence; from batches to sub cultures, from data collection to scaling up.

- For Batch 1-8 the experimentation was carried out using an Arctic/Alpine inoculum (the same through batches), re-fed with either raw or primary settled wastewater, incubated at the same conditions (temperatures 4, 8, 15°C; seed:substrate 1:3) for the examination of different parameters as stated on the methods.
- For the sub-cultures 1, 2 the experimentation was carried out in microcosms (serum vials) seeded with the inoculum from the batches (as indicated by the arrows), fed with either raw or primary settled wastewater (seed:substrate 1:7), set at 4 and 15°C the way it is stated on the methods, for the examination of various parameters.
- Cost & applicability included the results and findings that were extracted from the experimentation (batches and subcultures as indicated by the arrows) to create a scale up approach based on a regression analysis between the cell specific methanogenic activity and the temperature over time.

3.1. Brief description of the experiments and their conclusions.

Chapter 4: This chapter investigates the methanogenesis and wastewater treatment performance of 8 batch reactors operating at 4, 8 and 15°C, using the arctic inocula as seed and sterilized wastewater as substrate. The main aim is to collect the fundamental design parameters that would assist in the scale up process.

   4.3. This sub-chapter describes the start-up period of the anaerobic wastewater treatment at low temperatures. It depicts the size of the challenge, running a digester with low strength raw wastewater at low temperatures. It describes the boost of methane production rates between batch 1 and 2; gives an insight to molecular data of the seed (i.e. methanogenic communities) and manifests the bias that may occur between the selected molecular techniques (qPCR-FISH correlation for archaea).

   4.4. This section presents the maximum methane production achieved for the particular reactor configuration. It provides with the first estimation of the specific cellular methanogenic and hydrolytic activity and depicts the phylogenetic community structure after 400 days exposure to different low temperatures. It reveals what the rate limiting step is and how this affects the pilot scale design.

   4.5. This part presents the anaerobic treatment of primary settled wastewater at low temperatures. It shows how the COD removal increases due to substrate's higher biodegradability and further cellular adaptation. It provides with an insight into the accumulation of the un-hydrolyzed compounds after 2.5 years of operation, revealing that lipids are the components that are less degraded at low temperatures.

Chapter 5: The purpose of these experiments is to investigate the status of the microbial diversity in the seed after temperature switch (15 to 4°C and vice versa) and co-existence with the indigenous from wastewater cells.

   5.3. This sub-chapter presents the effect of temperature switch (15 to 4°C) to the cells when the latter are exposed to non-sterile raw wastewater. Hydrolysis in this case seems to be the limiting step; however the both methanogenic and hydrolytic activities increase under non-sterile conditions. Thus, a complementary interaction between the biomass in the seed and the wastewater is proposed. Insignificant differentiation to diversity suggests that the communities of the seed cannot be outcompeted from the cells in WW at low temperature, using a 1:3 seed:substrate ratio.

   5.4. This sub-section is a repetition of the previous experiment, including a temperature switch from 4 to 15°C. The wastewater in this case was primary settled and the seed:substrate ratio was 1:7. The specific cellular activity was higher at 4°C than at l5°C suggesting that the development of a psychrophilic community is likely. Additionally, higher activity at 15°C, from cells acclimatized at 4°C than at 15°C proves that the former performs better than the latter at all treatments (sterile or non WW). A 2-fold decrease (1:7 from 1:3) of the seed:substrate ratio affected the bacterial diversity acclimated at 15°C. Overall, acclimatization at cold temperatures rather than at warm leads to a highly active and robust microbial community.

Chapter 6: Lipids appeared to accumulate at low temperature. I therefore extended my investigation to lipases, the enzymes responsible for the lipid solubilization.

   6.3. This section investigates the activity of the enzymes at all temperatures, showing that the proteins at 4°C are slightly more active than at 8 and 15°C. Thus, the structure of the lipids is likely the reason for lower degradation rates at low temperatures. The activity at low temperatures (15°C) was found promising compared to activities from mesophilic treatment plants. The temperature range that triggers the degradation of lipids was also defined.

Chapter 7: In this chapter specific activity, $COD_{removed}/COD_{methanized}$ factor and temperature data for Newcastle upon Tyne region were used to simulate a reactor scale up for 20.000 people based on HRT. Regression analysis was carried out for HRT optimization and forecast. The capital cost for all HRTs was estimated.

   7.3. This chapter, after making certain assumptions, estimates of the HRT that is required for the treatment of domestic wastewater using the specific seed. The concept includes correspondence between temperature and specific rates. Regression analysis was used in HRT optimization and forecast. Finally a cost analysis for a plug-flow digester using our HRT and design volume reveals that after a certain size, application needs to consider other parameters.

Chapter 4: *Running digesters seeded with cold-adapted inocula to investigate the limits & define design parameters*
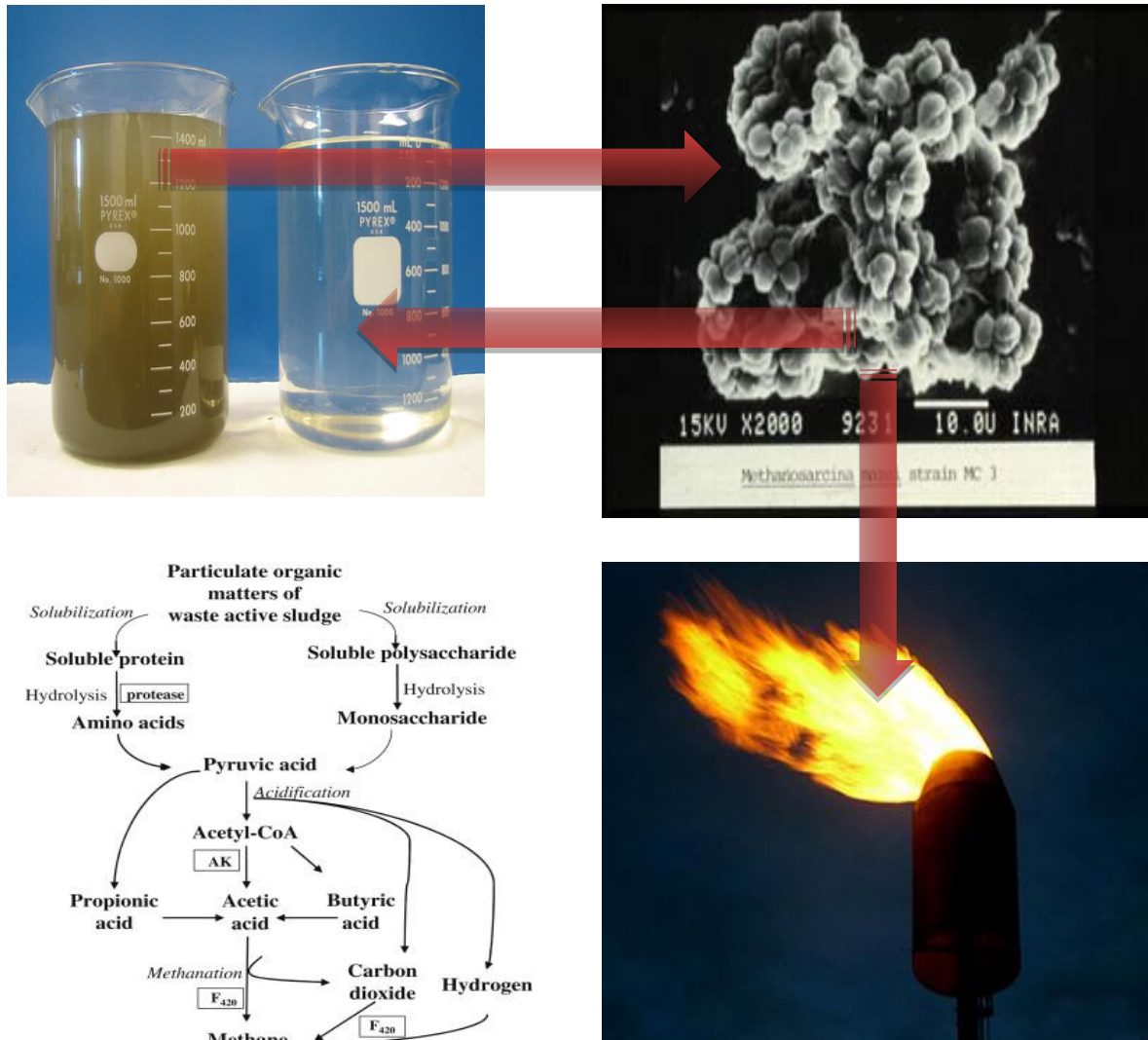

Figure 4.1 - Anaerobic digestion mechanism where sewage and its intermediates becoming substrate for microorganism (i.e. *Methanosarcina Mazei* on the figure (BacMap), converted to methane and pure water as effluent. The detailed pathway is shown on figure down-left (Mu and Chen 2011).

4.1. The start-up period – Batch 1, 2

4.1.1. Abstract

Low temperature anaerobic treatment of domestic wastewaters using conventional mesophilic biomasses, acclimatized to low temperature is challenging. I wished to determine if the anaerobic treatment of domestic wastewater at low temperatures could be improved if a cold-adapted biomass will be used to seed an anaerobic bio-reactor. Thus, 8 batch reactors were employed and inoculated with a cold-adapted inoculum (seed:substrate 1:3 ($^v/_v$)). The seed was collected from the high Arctic and an Alpine Lake to treat raw domestic wastewater. Two batch studies are presented in this sub-chapter, to describe the start-up of anaerobic WW treatment at 4, 8 and 15°C.

Batch 1 (1-230 days): inconsistent and insufficient methanogenesis was observed for the first 140 days at 4 and 8°C (0.012 and 0.013 $mgCOD_{CH4}.L^{-1}.day^{-1}$ respectively); at 15°C methane appeared from day 40 ($mgCOD_{CH4}.L^{-1}.day^{-1}$) suggesting that the initial methanogenic populations have their optimum closer to 15°C. Acidogenesis/acetogenesis was poor at 4 and 8°C, demonstrating that the bacteria responsible also grow better at high temperatures. CO_2 formation suggests that hydrolysis/fermentation takes place at all 3 temperatures, but with lower rates the lower temperature. This suggests that hydrolysis is as expected, temperature sensitive. sCOD depletion took place at 8 and 15°C after 100days. At 4°C slow hydrolysis resulted in sCOD formation even after 200 days of operation. The sequenced methanogenic taxa were *Methanomicrobiales*, *Methanosaetaceae*, and *Methanosarcina*, with the first being predominant at all temperatures. At 216 days it is not clear which taxon was dominant. A comparative study between archaeal qPCR and FISH enumeration on the same day (216) suggested the presence of a large inactive archaeal population (e.g. $FISH_{max}$: 6.7×10^3, $qPCR_{max}$: 1.1×10^7). The correlation amongst the two methods was found strong R^2: 0.896.

Batch 2 (+99 days): Higher amount of methane was formed at all 3 temperatures, compared to batch 1 ($\times 10$ at 4 and 8, $\times 2$ at 15°C). This implies that cells are better adapted to both the substrate and the temperature than in batch 1. Effluent COD met the UWWTD (91/271/EEC) standards at 15°C and showed high removal capacity at 8°C but not at 4°C. sCOD and VFAs were fully depleted at all 3 temperatures. Lower CO_2 production in the second batch demonstrates a reduction in the sinks or the seed's organic material, if present, or less/no O_2 ingress (the sampling strategy for this batch changed to ensure 100% sealed reactor for all operational days). VSS/TSS increase at 4 and 8°C reveals treatment boundaries at <15°C, due to accumulation of un-hydrolysed material (as VSS).

4.1.2. Introduction

Increase in energy demand and intensive release of carbon dioxide through rapid urban development rendered conventional wastewater treatment processes (mainly aerobic) less favourable. The British water and wastewater industry accounts for over 4 million tonnes of CO_2 per year, equal to the 1.0% of the annual UK greenhouse gas emissions, consuming 8100 GWh per annum (Environmental Agency 2009). Thus, the challenge of carbon and energy neutral/negative treatment with reduced CO_2 emissions needs to be considered (Logan 2008).

The problem can be partially solved using traditional anaerobic treatment systems. Currently almost all full-scale anaerobic treatment plants are operating at temperatures exceeding 18°C (Lettinga et al 2001). This makes the use anaerobic treatment systems problematic in cold or temperate regions (maximum average <15°C). As temperature drops, the risk of failure of both methanogenesis and hydrolysis increases. Previous studies showed that lowering the operational temperature of a digester leads to the decrease of the maximum substrate utilization rates, maximum specific growth rates and rates in biogas production (Alonzo et al 1969; Kettunen and Rintala 1997). Thus, temperature is the "Achilles heel" for anaerobic treatment systems. So far, the most efficient environmental engineering strategy was to acclimatise mesophilic organisms at low temperature; however none of the studies could adequately operate at <13°C with real wastewater as substrate (Uemura and Harada 2000, Elmitwalli et al 2002, Alvarez et al 2008, Luostarinen et al 2007, Bogte et al 1993), fact that has recently been attributed to failure in methanogenesis at temperatures below 8 °C (Bowen et al 2014).

A different approach would be the use of a cold-adapted-psychrophilic inoculum to establish the true limits of the anaerobic treatment of wastewater at low temperature. The aim of this chapter is to examine the feasibility of low temperature anaerobic wastewater treatment at 4, 8 and 15°C. At such temperatures the start-up is commonly the

I n v e s t i g a t i n g t h e t r u e l i m i t s o f a n a e r o b i c t r e a t m e n t o f w a s t e w a t e r a t l o w t e m p e r a t u r e u s i n g a c o l d a d a p t e d i n o c u l u m

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most prolonged step as lack of cells acclimated at such temperature doesn't allow the development of communities capable to function. The use of a cold-adapted inoculum though can theoretically accelerate the start-up and prove that methane production can be feasible at low temperature. A preliminary study in the performance of inoculum with regards the COD, sCOD removal in addition to gas production is presented. Finally molecular analysis reveals some fundamental characteristics of the seed such as the population and the community structure.

4.1.3. Materials and Methods

Reactors assembly; eight quick fit 1L glass batch reactors (Sigma Aldrich, UK) with 480 ml of headspace were assembled and incubated at 4, 8 and 15°C. Duplicate reactors were used for 4 and 8°C; quadruplicates at 15°C. Although the reactors were designed for CSTR purposes, mixing was only applied prior and during sampling at 70 rpm for 10 minutes using a stainless steel stirrer paddle. Mixing was avoided during operation to minimize likelihood of microbial community disturbance. Thorough mixing was only applied prior sampling for TSS, VSS. Sampling itself for both thorough and limited mixing was taking place a few seconds after mixer switch off. After preparation reactors were flushed with 99.9% N_2 to ensure anaerobic conditions and sealed.

Inoculum; The laboratory-scale batch reactors were seeded volumetrically (250 ml) with an equal mixture of putatively cold-adapted sediment from Lake Geneva ''N 46°23'04'', E 6°25'07'' (average temperature -11 – 17 °C) and soils from Svalbard, in the high Arctic at various sampling points situated at ''N78°, E11, 15,16°'' (average temperature -16 – 6 °C) and operated at 4, 8 and 15°C.

Re-inoculation; in the end of every batch (first batch) and before initiation of a new one (second batch) the mixed liquor (solid/liquid) was emptied in a plastic 1L vial and set for centrifugation (Cryofuge 5500i, Thermo Scientific, UK) at 4000rpm for 20 minutes at 10°C. After liquid-solid separation the first (supernatant) was discarded and the solid was used as inoculum for the consequent batch after volumetric measurements (250±10ml). New N_2-flushed, sterile wastewater (750 ml) was combined with the inoculum resulting to a 1L volume of mixed liquor of 1:3 seed:substrate, which was successively positioned into the quick fit reactor, re-flushed with N_2 and sealed. The re-inoculation practice was carried out into plastic bags, filled with N_2 to ensure anaerobic conditions throughout the procedure.

Wastewater substrate; wastewater collected from the Tudhoe Mill (County Durham, UK) wastewater treatment plant (WWTP) treating domestic wastewater. Screened raw influent wastewater was used to ensure high COD concentration at Batch 1, 2. The COD content was approximately 500 and 1000 mg.L^{-1} for the first and second batch respectively. The substrate composition was estimated based on COD as 45% carbohydrates 35% lipids and 20% proteins for raw wastewater with negligible variation between the two batches.

The protein content was measured via the Bradford protocol (Bradford 1976), based on the interaction between protein and Coomassie Brilliant Blue G-250, providing a blueish-brown solution depending the protein concentration. The staining reaction requires 1 minute to complete and remains stable for 30 min. The absorbance is estimated via spectrophotometry at a wavelength of 595nm. In detail, 100mg of Coomasie blue G-250 was dissolved in 50 ml of 95% ethanol. Once the dye powder was completely dissolved the volume of the solution was made up to 1L by adding deionised water. The 1L solution was then filtered until colour turned brown (from blue). 5ml of solution was added into tubes with 100μl of sample or standard. A spectrophotometer from Merck (UK) was introduced to measure the absorbance of either samples or standards with known BSA (Bovine Serum Albumin) protein concentration (Sigma Aldrich (UK)). The conversion to COD was carried out using $(C_4H_{6.1}O_{1.2}N)_x$ as a point of reference (Sanders et al 1996).

The lipids were estimated gravimetrically using a methanol:chlorophorm extraction protocol (Bligh and Dyer 1959) at a ratio 1:2. A 10 ml sample was initially mixed into a body of methanol:chloroform solution to a final volume 20 times the volume of the sample, until homogenized to form a miscible system. 1g of KCl was also added and mixed (Folch et al 1957). The homogenate is subsequently separated into two layers. Methanol phase contains all the non-lipid material located on the top, whereas lipids are dissolved in the chloroform, situated in the bottom of the vial. A Pasteur pipette was used to abstract the chloroform phase, which was subsequently evaporated in a fume cabinet so the lipids can be released. The remaining lipid after CHCl$_3$ evaporation is weighed, indicating the amount of fat that is present in the sample. 10ml of mixed liquor was used as sample volume for the

particular study's lipid content. The conversion to COD was carried out stoichiometrically using octanol ($C_8H_{18}O$) as a point of reference (main castoroil compound).

Carbohydrates content was estimated by the anthrone method, a simple colorimetric method firstly reported by Hedje and Hofreiter (1962). Polysaccharides are firstly hydrolysed to monomers from digestion via hydrochloric acid and heat. Then the solution reacts with the anthrone yielding a greenish-blue colour. The amount of total carbohydrates is determined according to its absorbance, expressed to concentration based on a glucose standard curve. In detail, 100μg samples were added into digestion tubes and hydrolysed using 5ml of 2.5N HCl at 100°C for 3 hours. After digestion the samples cooled down and neutrilized with Na_2CO_3 prior centrifugation (8.000rpm for 3 minutes). The supernatant was collected and used as the extract-sample. 1 ml sample was then mixed with 4 ml of anthrone solution (0.2g dissolved in cold 100ml iced cold 95% H_2SO_4) and set to a waterbath (Grant, UK) to boil for 8 minutes at 100°C. After boiling the samples were left to cool down at room temperature. The concentration was estimated using a spectrophotometer from Merck (UK) at 630nm, standardizing a curve with known amounts of glucose. For the preparation of the standards 100 mg of glucose were dissolved in distilled water, 10 ml of the stock was further diluted in 100 ml water, adding few drops of toluene. The glucose solution was diluted to achieve certain glucose concentrations. The procedure of HCl hydrolysis and the addition of anthrone was then repeated for the standards. The conversion to COD was implemented stoichiometrically using glucose ($C_6H_{12}O_6$) as a point of reference.

Sterilization techniques; UV irradiation was applied at a dose of 110 kJ/cm^2. The rationale behind sterilization was primarily to quantify the treatment performance of the seed, excluding the effect of the wastewater-originated cells, and secondly to preclude any competition between the cells thriving the seed and those present in the substrate. UV light (11 W; Hozelock Vorton (UK)) was selected because it had less adverse effect on the biodegradability of the wastewater (assessed by changes in the BOD_5:COD, compared to thermal methods (autoclaving at 109°C for 20 minutes). Preliminary trials showed a reduction of 16($\pm$1.4) and 8($\pm$4)% BOD_5:COD for autoclave and UV-irradiation$_{(40\ minutes)}$ respectively. Both approaches achieved an 8-log reduction of 1.73($\pm$0.01)$\times10^8$ or 2.04($\pm$0.04)$\times10^8$ CFU.100ml^{-1} (CFU: Colony Forming Unit) on R2A and Nutrient Agar respectively (chosen dilution 1:10^3, detection limit based on duplicate control petridishes as 1($\pm$1)$\times10^3$). The wastewater BOD_5 and COD were measured based on APHA 2005 to quantify the changes in the nature/composition and subsequently biodegradability of the substrate due to sterilization. The efficiency of the sterilization method was tested by counting colony forming units. After sterilization 50 μl of wastewater sample was spread onto Nutrient (NA) and R2A (APHA 2005, Reasoner and Geldreich1985) agar plates. Plates (dilutions and controls) were prepared in duplicates; additional un-inoculated plates were prepared as controls. The plates were set for incubation at 17$\pm$2°C in the dark for 5 days prior to enumeration; all calculations were based on APHA 2005.

Analytical methods; Total COD (COD) and Soluble COD (sCOD) were analysed via 'digestion tube method' following Standard Methods (APHA 2005). sCOD was measured on a 0.2μm filtered sample (glass fibre syringe filter, VWR, UK). The ratio sCOD to COD is an indicator of hydrolysis as the fate of the solubilized COD fraction (sCOD) represents the status of the organic liquidification.

VFA (Volatile Fatty Acids) analysis; samples from the liquid phase were removed from the reactors using sterile syringes, transferred to sterile 2 ml micro-centrifuge tubes and centrifuged (3 min at 13.000 rpm) to obtain a supernatant for analysis. Volatile fatty acid concentrations in the supernatant were subsequently determined by ion exchange chromatography (IEC) based on a modified method of Manning and Bewsher (1997). Briefly, aqueous samples were syringe-filtered through 0.45 μm filters, acidified 1:1 $^v/_v$ with Oxysulfonic acid and sonicated in a sonic bath for 40 minutes to remove carbonate from the samples as carbon dioxide (Manning and Bewsher 1997). The resulting samples were analysed on a DIONEX ICS-1000 equipped with an Ionpac ICE-AS1, 4x250mm column using a 1.0mM heptafluorobutyric acid eluent solution. The volume of the injection loop was 10 μl and flow rate was 0.16ml/min. The cation regenerant solution used for the AMMS-ICE II Suppressor was 5mM tetrabutylammonium hydroxide. The minimum detection limit of the particular analyte/instrument is 0.8ppm (lower values cannot be consistent). VFAs in anaerobic treatment are the products of biodegradation of sugars, amino acids long and lower chain acids. They can be used as fundamental parameters to describe the status of fermentation/acidogenesis, or estimate the prospective methanogenesis in terms of expected amount of biogas.

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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Methane; CH$_4$ was monitored in the gas phase as % by volume, using gas chromatography. Samples of gas (3ml) were removed from the headspace using a gas-tight syringe (SGE-Europe), and injected into a 3ml-volume exetainer. Subsequently 100µl of gas sample was removed from the vial and further injected directly onto a Carlo Erba HRGC S160 GC fitted with an FID detector and HP-PLOTQ column (0.32 mm diameter, 30 m length and 20 µm film). CO$_2$ gas was measured using a Mass Spectrometry (Thermo Scientific, UK) following the same sampling strategy as for methane. For both gases hydrogen was used as a carrier gas at a flow rate of 60ml min^{-1}. Gas concentrations in the liquid phase were thermodynamically calculated using Henry coefficients (Henry coef.$_{CH4}$: 433.6, 481.6, 574.8 at 4, 8 and 15°C respectively; Henry coef.$_{CO2}$: 14.7, 17.0, 21.8 at 4, 8 and 15°C) (Dolfing et al 2010, Dolfing and Janssen 1994). The Henry coefficients were used assuming equilibrium between the gas and the liquid phase,

Microbiological community analysis; microbial community structure was described using the rapid community fingerprinting method, denaturing gradient gel electrophoresis (DGGE). Biomass samples were obtained from the pellets produced during the preparation of samples for VFA analysis, as described. Total genomic DNA was extracted from biomass pellets collected during the VFA sampling using a FastDNA® SPIN for soil kit (Q-BIOgene, Cambridge, UK) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) was used to generate amplicons for use in the DGGE procedure. Bacterial 16S rRNA gene fragments were amplified using the primer pair Primer2 and Primer3 (Muyzer et al 1993) with a 5' GC clamp. A nested PCR was used to amplify archaeal 16S rRNA gene fragments: primers A20f and U1492r (Orphan et al 2001) were used to amplify a 1472 base-pair fragment (based on *E.coli* 16s rRNA position); amplicons from the first PCR were further amplified using primers Arch344 (Raskin et al 1994) with a 5'GC clamp and U522 (Amann et al 1995) to generate a 178 base pair fragment for DGGE. All PCR reactions were prepared using the PCR reagent MegaMix Blue (Microzone Limited, West Sussex, UK), according to the manufacturer's instructions. Products were analyzed by DGGE on a D-gene DGGE system (Bio-Rad, Hercules, CA, US) using a 10% polyacrylamide gel containing a denaturant gradient of 30-60%. The gel was stained for 30 minutes using SYBR green I (Sigma, Poole, UK) in the dark and viewed under ultraviolet light on a Bio-Rad Fluor-S ® Multi Imager (Bio-Rad, UK). Finally, selected DNA bands were excised from the bacterial and archaeal gel, PCR-amplified using primers Arc344f, U522r; purified using a Qiagen 502 PCR clean up kit (Qiagen, Crawley, UK) and sequenced using an ABI prism Big Dye Terminator Cycle Sequencing Ready reaction Kit and an ABI Prism 377 DNA sequencer (Applied Biosystems, USA). Sequences were compared to the GenBank database (Benson et al 2008) using the BLAST algorithm to determine nearest neighbors (Altschul et al 1990).

Quantitative PCR (qPCR) was used for the quantitation of methanogens in the reactors using primers for *Methanomicrobiales* (MMB282F, 832R), *Methanosarcinaceae* (MSC380F, 828R) and *Methanosaetaceae* (MST702F, 862R) (Yu et al 2005) which covered the dominant methanogens found in the reactors, according to the sequenced bands cut from the archaeal DGGE gel. For total archaea the Arc109F and Arc344R primers were used (Sun et al 2007; Reging et al 1998). The qPCR was carried out on a CFX96 real–time PCR system (Biorad, UK) using a 32 cycle, 2-step reaction qPCR procedure, with a reaction mixture that comprised of: 3µl DNA template, 1 µl sterile de-ionized water, 0.5 µl each of the forward and reverse primers and 5 µl of Ssofast EvaGreen Supermix (Biorad, UK). The analysis incorporated a 5-point calibration curve using standards of known genomic DNA concentration of: *Methanoculleus bourgensis*, *Methanosarcina barkeri* and *Methanosaeta harundinacea* for MMB, MSC and MST primers respectively; no-template controls were prepared from filter-sterilized de-ionized water. For total archaea standards of the last two groups were used (Yujiao et al 2007). All qPCR reactions were performed in triplicate and efficiency values were calculated based on standards and also a 4-point serial dilution of a selected sample. Starting quantity (SQ) values from the qPCR in copy no. ml^{-1} were converted to cells ml^{-1} using the formula recommended Klappenbach et al 2001 (*Eq.*11).

$$Cell.ml^{-1} = \frac{a}{b \times c} \quad (Eq.11)$$

Where: (a) SQ*dilution factor on plate
(b) /mean no. of copies of RNA gene in archaea
(c) *100 as the factor to allow for initial extraction of pellet from 1 ml into an elution vol. of 100 µl:
i.e. 1000 µl / (1000/100)

The selected mean copies of RNA gene were 2.13, 2.6, 3.0 and 2.0 for total Archaea, MMB, MSC and MST respectively (Weijers et al 2009, Klappenbach et al 2001).

For the archaeal FISH enumeration the protocol that was followed was from Coskuner et al (2005). Prior to FISH analysis, samples were fixed with 4% paraformaldehyde (Amann et al 1990), the probe that was used was ARC915, labeled with Cy3 (seq 5′-GTGCTCCCCCGCCAATTCCT-3′) (Stahl and Amann 1991), purchased from Genosys (Cambridge, UK).

For hybridization, 0.5ml of mixed liquor was set into a 2ml Eppendorf. The samples were serially dehydrated in 60%, 80%, and 96% ethanol by adding 1 ml of the corresponding concentration, followed by centrifugation at 13.000 rpm in a micro-centrifuge (MSE Microcen-taur, UK). After dehydration the remaining pellet was re-suspended in 38µl hybridization buffer (0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 0.1% SDS, 0.5 mg of poly(A)/ml, 10x Denhardt's solution; all reagents were purchased from Sigma Aldrich, (UK)). Finally 2µl of labeled probe (50 µg/µl) was added. A negative control with no probe was also prepared. Pre-hybridization step took place where samples were left in the heating block at 46ºC for 15 minutes without addition of the probe. After addition the samples were left at same temperature for 1.5 hours. Sample wash followed twice applying 0.5 ml washing buffer (20 mM Tris HCl, 0.01% SDS, 5 mM EDTA, and NaCl), incubating for 15 minutes at 48ºC, followed by a quick wash in 0.5 ml milli-q water (filtered at 0.2um-autoclaved). The samples were centrifuged removing the supernatant; extra 100 µl of milli-Q water were added. Finally 10µl sample was set on gelatin-coated slide and left to dry in a 30ºC incubator. 5-10 µl Citifluor (Citifluor, Kent, United Kingdom) was added on top of the sample followed by sealing it by coverslip. The edges were sealed using nail varnish.

The slides were examined with a Leica TCS SP2 UV confocal laser scanning microscope (CLSM) equipped with an X63 magnification na1.32 lens. The selected software for visualization was LCS 2.61 (Leica GMBH, Heidelberg). Auto-fluorescent background material was removed by setting a threshold from the no-probe control. Up to ten FOVs (fields of view) were taken, with less than 24 z axis-sections of 1.14µm average height).

The enumeration carried out via Image-J, a java-based image processing software (National Institutes of Health, Maryland USA) developed by Wayne Rasband at Research Services Branch of the National Institute of Mental Health. A 16-bit integer was used prior qualitative image analysis. A multiplication factor was introduced (×3) to compensate for the biomass that was lost through continuous pipetting during the sample preparation (FISH). This was estimated from the average weight loss of the samples, defined via weighing in the beginning of the method and in the end of the washing step.

4.1.4. Results

Batch 1 was running for 230 days. The performance in terms of gases is shown below (Figure 4.2.a, b c, d)

a)

Methane production at 15ºC and lower temperatures

$y = 0.0005e^{0.0352x}$
$R^2 = 0.9477$

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
P a g e | 3 6

b)

c)

d)
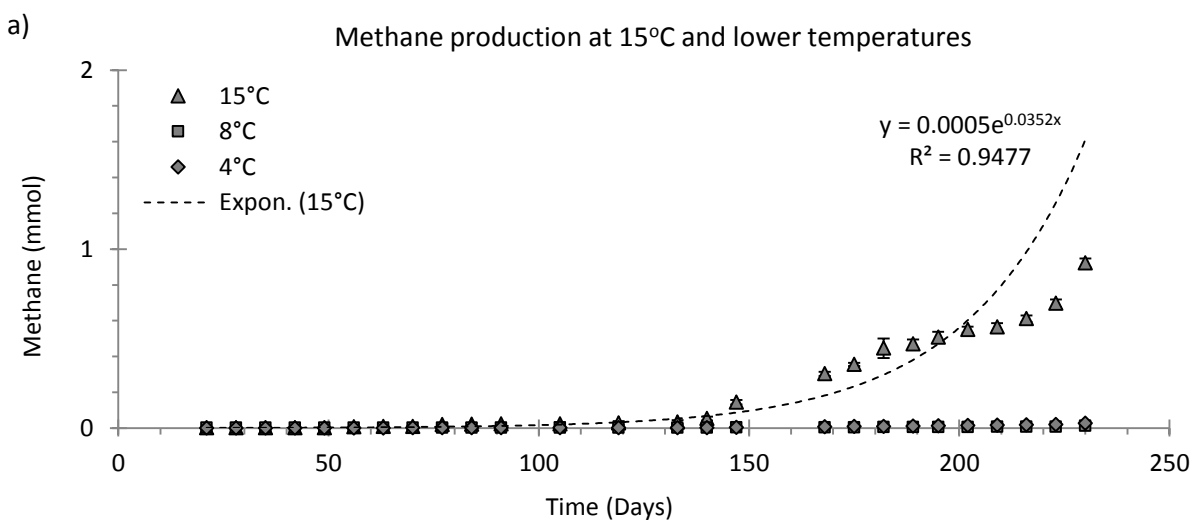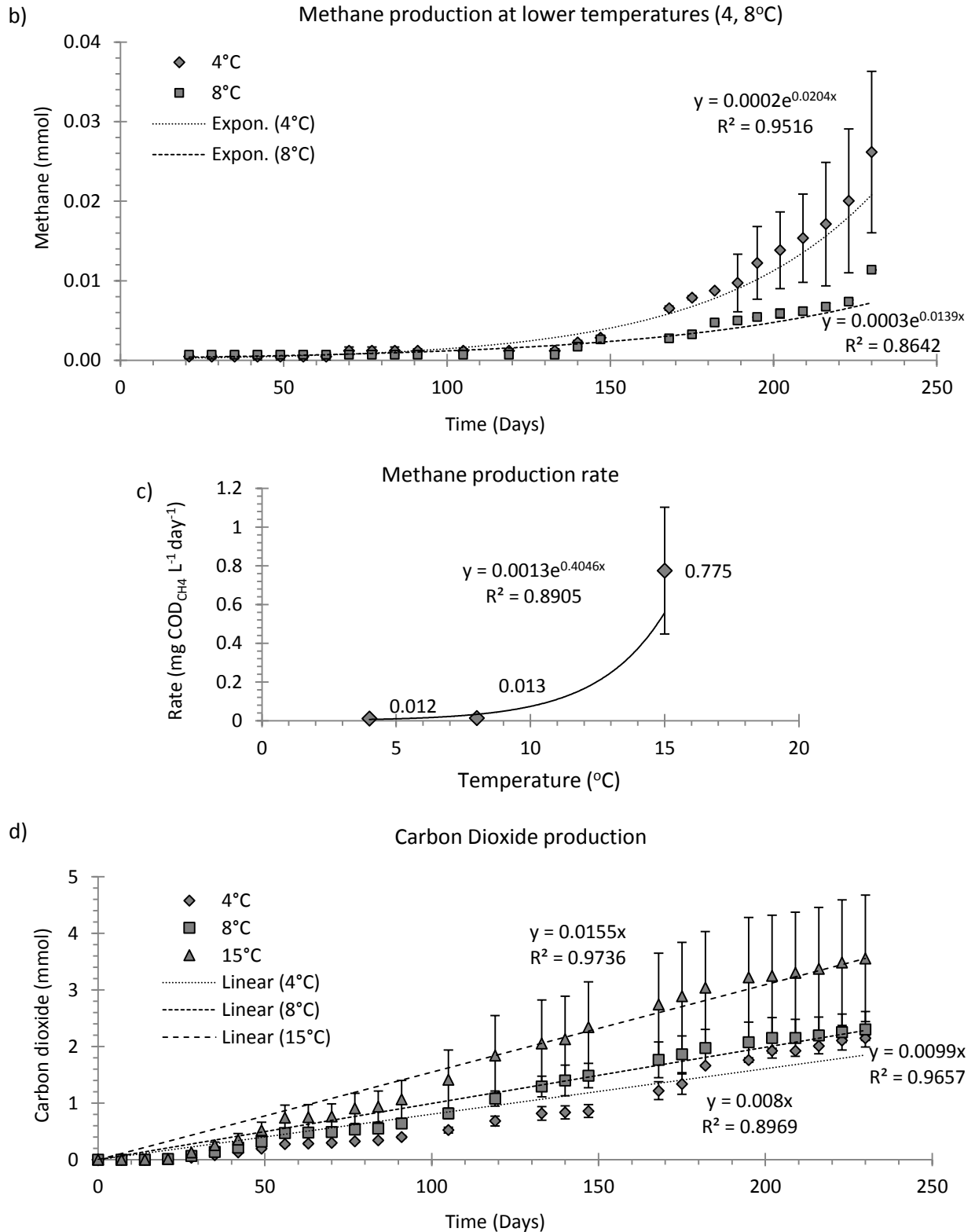
Figure 4.2 –a) Methane production at 4, 8 and 15°C with the exponential trend line for 15°C; b) similarly as 'a' at 4 and 8°C, Y axis scale different than previous; c) methane production rate in COD from CH_4 per day including a trendline to describe the effect of temperature to the methane production rate; d) CO_2 production with the corresponding trend lines at each temperatures, as a function of time; n = 2 for 4 and 8°C, n=4 for 15°C; error bars express standard error; all results are expressed as averages from duplicates and quadruplicates for 4, 8°C and 15°C respectively; all gases are expressed as mmols per volume of the headspace.

The fate of intermediates (VFAs) and sCOD is presented on the figures below (4.3.a, b)
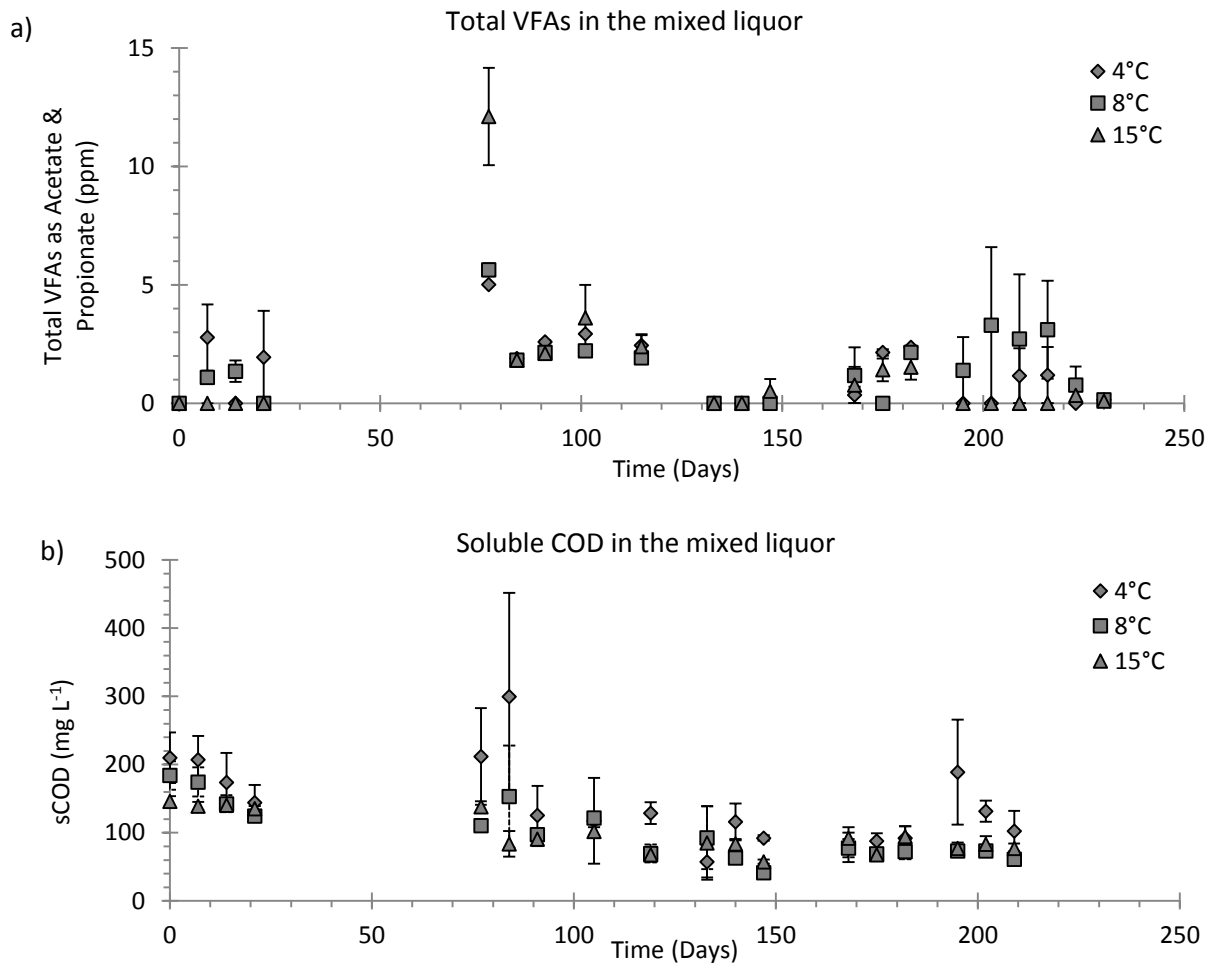
Figure 4.3 – Average a) total VFA in the reactor at all temperature as summary of acetate and propionate (no other intermediates were detected); b) Soluble COD fate at all temperature; n = 2 for 4 and 8ºC, n=4 for 15ºC, error bars indicate standard error

The molecular analysis includes an archaeal and a bacterial DGGE image (Figure 4.4.a, b respectively). Main bands are presented on Table 4.1, 4.2.
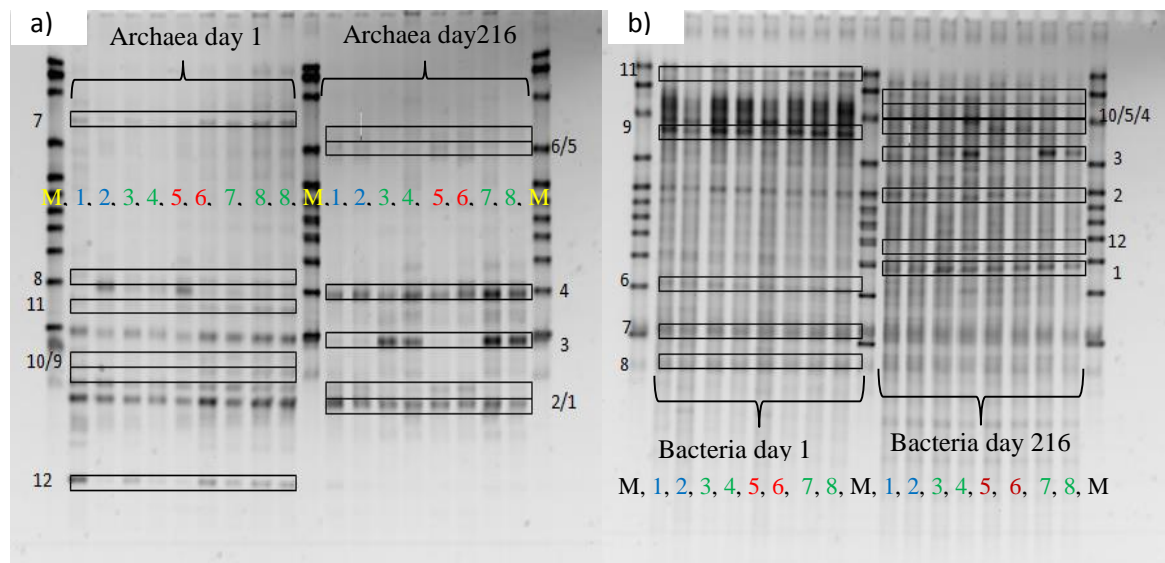
Figure 4.4 – DGGE images for a) Archaea day 1 & 216; b) Similarly for Bacteria; Sequence of columns: M, 1, 2, 3, 4, 5, 6, 7, 8, 8, M (1, 2: 4ºC; 5, 6: 8ºC; 3, 4, 7, 8: 15ºC); boxes with numbers correspond to the bands that were cut for sequencing; the affiliated genus and class is shown on the Tables 4.1, 4.2 below for archaea and bacteria respectively.

Table 4.1 – Archaeal bands sequenced via 1st generation sequencing and identified via GenBank

Band	Archaeal ID	Class	Origin	%
1	N/A (Not Applicable/unidentified)	N/A	N/A	N/A
2	N/A	N/A	N/A	N/A
3	Uncultured *Methanosaeta*	Methanomicrobia	Lake Geneva	100
4	Uncultured *Methanomicrobiales*	Methanomicrobia	Lake Geneva	98
5	Uncultured Archaeaon (*Crenarchaeote*)	Crenarchaeota	Arctic region	100
6	N/A	N/A	N/A	N/A
7	N/A	N/A	N/A	N/A
8	Uncultured *Methanomicrobiales*	Methanomicrobia	Lake Geneva	98
9	N/A	N/A	N/A	N/A
10	Uncultured Methanogenic Archaeon (*Methanosarcina*)	Methanomicrobia	Laptev Sea	100
11	N/A	N/A	N/A	N/A
12	Uncultured *Methanomicrobiales*	Methanomicrobia	Lake Pavin	100

*N/A stands for sequences that could not be sequenced at the GenBank database

Table 4.2 – Bacterial bands sequenced via 1st generation sequencing and identified via GenBank

Band	Bacterial ID	Class	Origin	%
1	Uncultured Bacterium	N/A	N/A	100
2	Uncultured Bacterium	N/A	N/A	94
3	Uncultured *Methylobacter*	Gammaproteoacteria	High Arctics	88
4	Uncultured *Cyanobacterium*	Cyanobacteria	N/A	91
5	Bacterium 081657 (*Acidovorax*)	Betaproteobacteria	Japanese paddy soil	90
6	Uncultured *Clostridia*	Clostridia	Spitsbergen - Norway	90
7	Uncultured Bacterium	N/A	N/A	84
8	Uncultured *Chloroflexi*	Chloroflexi	Roopkund Glacier - Himalayas	96
9	Acrobacter	Epsilonproteobacteria	N/A	96
10	N/A	N/A	N/A	
11	Uncultured *Paludibacter*	Bacteroidetes	N/A	82
12	Uncultured Bacterium	N/A	N/A	86

*N/A stands for sequences that could not be sequenced at the GenBank database

Based on the methanogenic species detected, a qPCR enumeration (same dates) was carried out (Figure 4.5).

The total archaeal population was quantified via qPCR (Arc109F and Arc344R)) and FISH (AR915). The results demonstrate a discrepancy between the two methods. This enabled us to correlate them for day 1 and 216 (Figure 4.6.a, b).
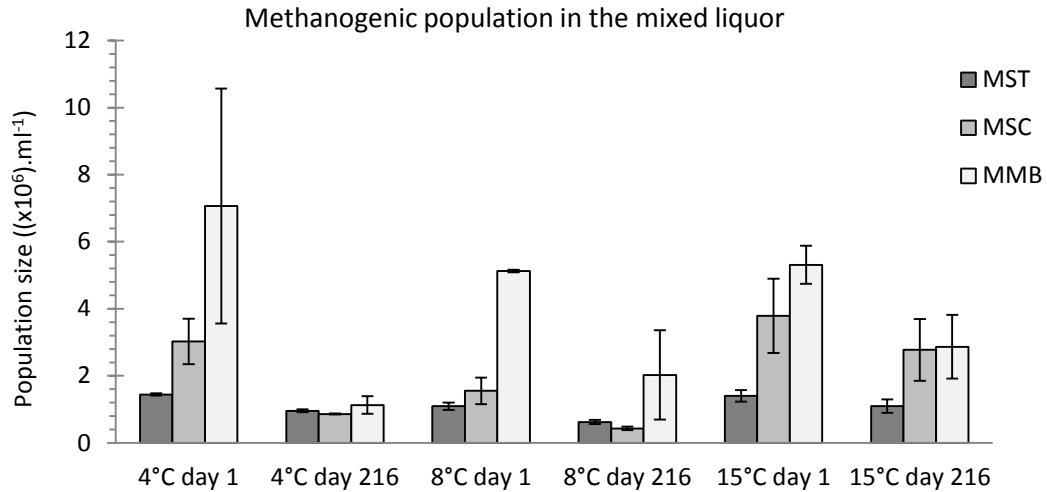
Figure 4.5 – Average methanogenic population in the reactors at all temperatures for day 0 and 216; all values expressed in 10^6; error bars indicate standard error[1]; n = 2 for 4 and 8ºC, n=4 for 15ºC; MST, MSC, MMB stand for *Methanosaetaceae*, *Methanosarcinaceae* and *Methanopmicrobiales* respectively
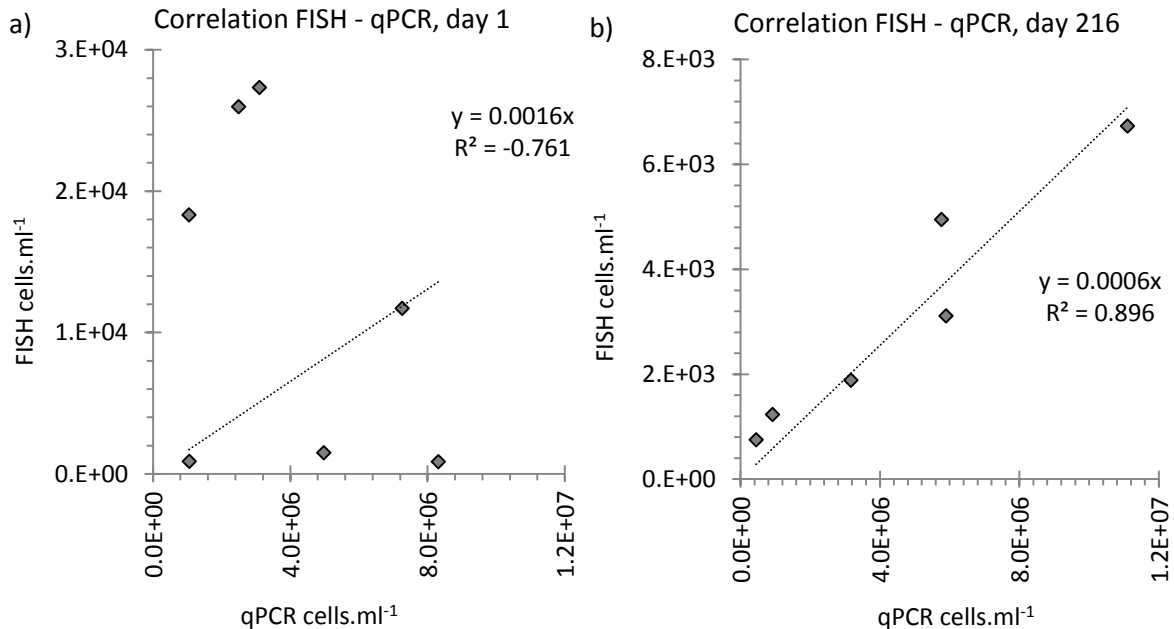
Figure 4.6 – Correlation of the archaeal qPCR and FISH enumeration for a) day 1 and b) day 216.

Batch 2: After 216 days of operation the reactors were re-fed and run for an extra 99 days at the same conditions. The performance in terms of VFAs, COD (total and soluble), methane – carbon dioxide production and MLVSS/MLSS is shown on the figures below (Figure 4.7.a, b, c, d, e, and f).

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[1] The error bars were also calculated with the log-transformed data, however the back transformation of the results could not depict the variance of the population resulting to non-representative, extremely low values (e.g. SE of 1-5 cells.ml^{-1}). A different approach for error bars was the estimation of the 95% confidence level of the log transformed mean, however the back transformation resulted to extremely high mix and max bars due to the low number of replicates (duplicates, quadruplicates).

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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a)

b)

c)

d)

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
P a g e | 4 1
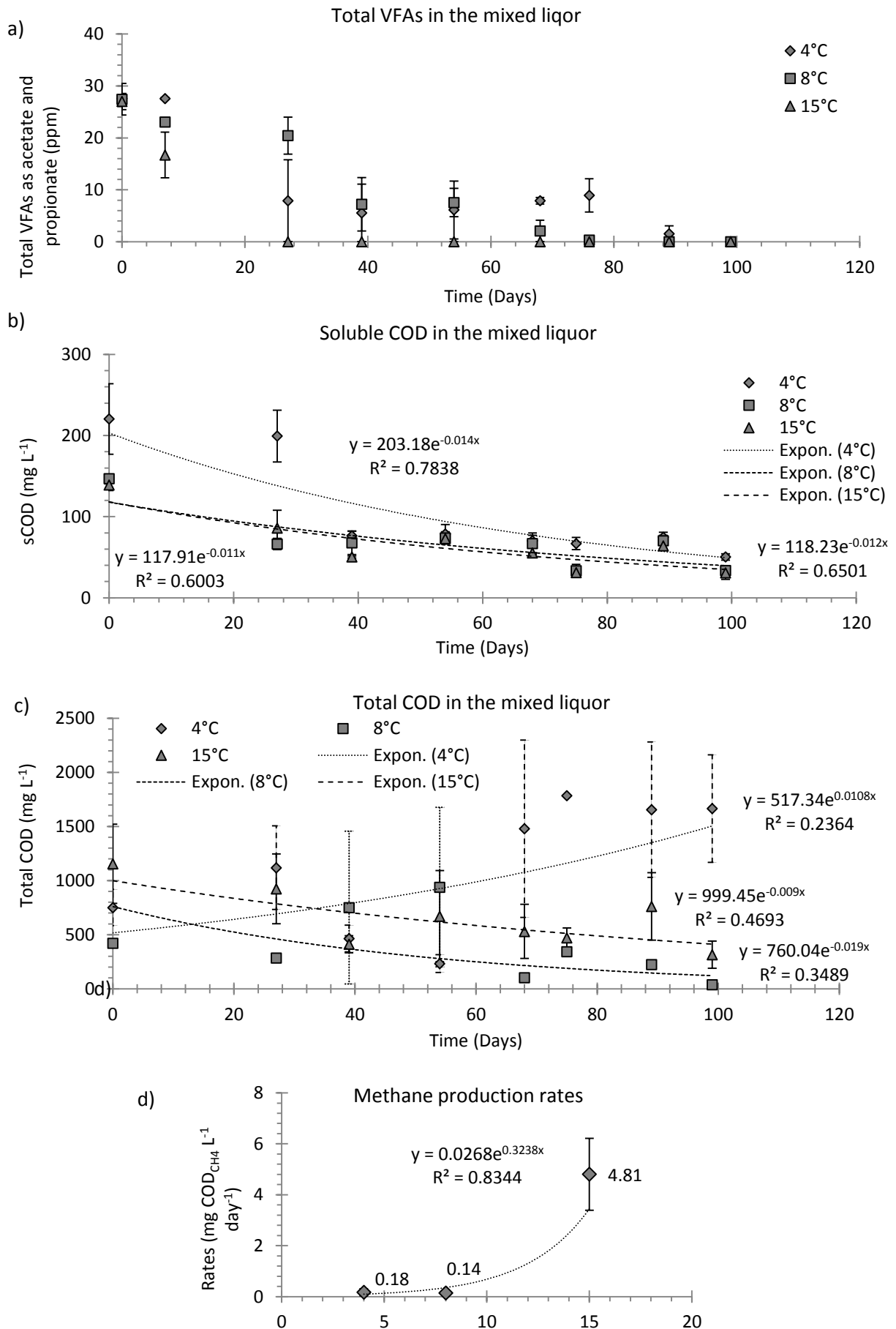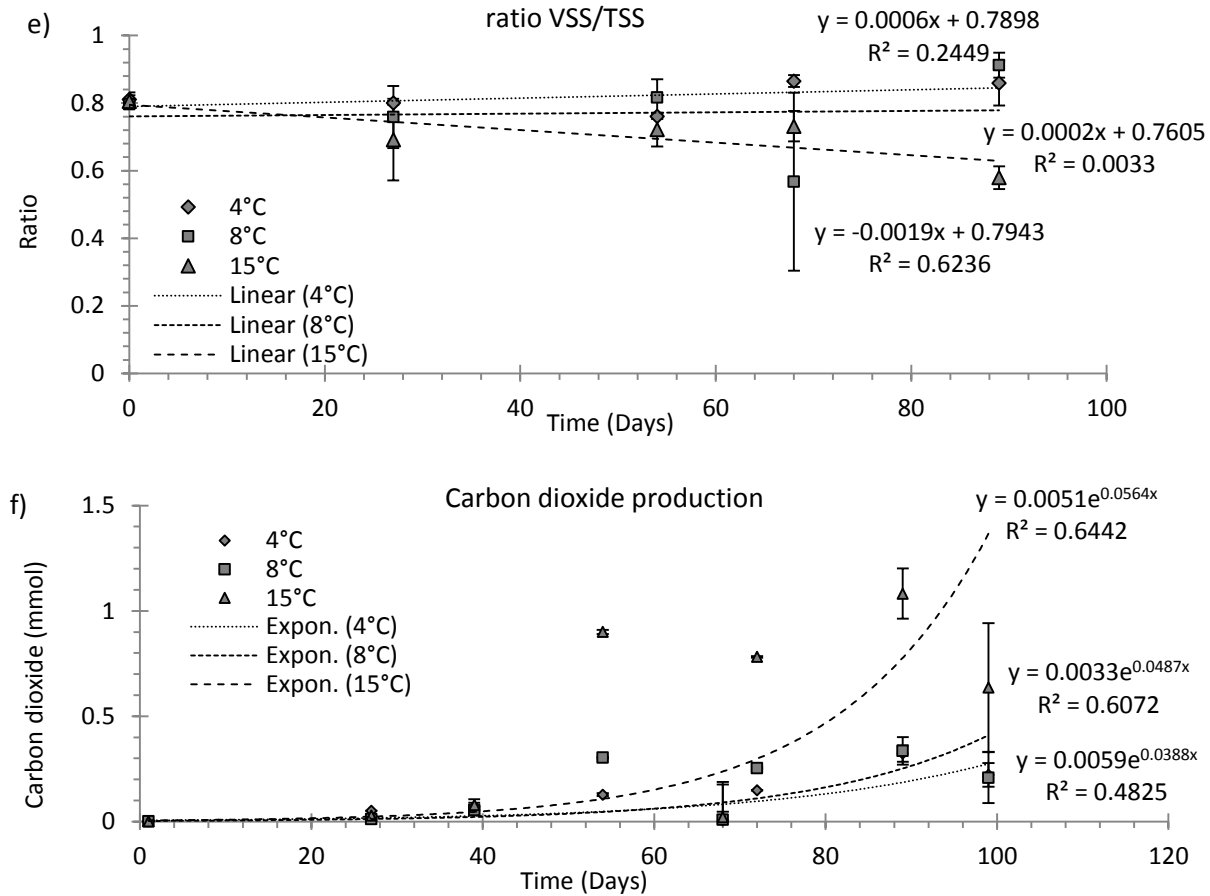
Figure 4.7 – a) VFAs as a summary of acetate and propionate (no other VFAs detected); degradation as b) soluble COD and c) total COD fate; d) CH_4 production rates with a trendline to depict the effect of temperature to methanogenesis; e) VSS/TSS ratio f) CO_2 production per had space volume; at all temperatures; all results are average of n = 2 for 4 and 8°C, n=4 for 15°C; all gases expressed in mmols per headspace volume; error bars express standard error (n=2, n=4 for 4-8 and 15°C respectively)

4.1.5. Discussion

Methanogenesis needed approximately 40 days to start-up at 15°C whereas at 4 and 8°C, 140 days were required for consistent methane production. This suggests that the cells were not acclimatized to the specific substrate and this resulted to a slow start-up period. Cellular adaptation to wastewater was found temperature dependent and methane firstly appeared at 15°C. This manifests that the bacterial and archaeal optimum temperature is closer to 15°C rather than at lower temperatures. Although the methane production rates were lower the lower the temperature (Figure 4.2.a, c) the production rate was steady fitting well to an exponential trend (R^2>0.89 at all temperatures) (Figure 4.2.c). This revealed an exponential relationship between temperature and activity, which agrees with the general idea suggested by Lettinga et al 2001 and presented on Figure 2.5.a.

In the second batch methane production rates were increased by 15, 11 and 5 fold for 4, 8 and 15°C respectively (batch 1: 0.012±0.001, 0.013±0.001, and 0.775±0.32; batch 2: 0.18±0.13, 0.14±0.01 and 4.81±1.41 mg COD_{CH4} L^{-1} day^{-1}) (Figure 4.2.a, 4.7.d). Increase in rates suggests that the cells from the seed were more adapted to raw wastewater as substrate, able to utilize it at higher rates than at the first batch. This also suggests that the bacterial/archaeal communities are more active and can operate (treat) better. The increase in methane production could not necessarily be attributed to growth of the total biomass as qPCR results on the methanogenic communities showed decay at all temperatures, especially at the two lower ones. Limited or negative growth at low temperatures (6°C) was also noticed by Lokshina et al 1999. Reductions of abundance supports that treatment at extremely low temperatures gives an advantage only to the most resilient cells to thrive and comes in contrast with what is usually considered as a thumb rule of growth in anaerobic digestion that supports that growth accounts for approximately the 3% of the COD removed (Malina and Pohland 1992, Haandel and Lettinga 1994, McCarty 1990, Stronach et al 1986). A different hypothesis for the decay of the archaeal population is the possible presence

of inactive or unable to adapt to the substrate cells that perished, especially at low temperatures. This scenario would result to vacancies in the community structures that may lead to changes in the abundance according to the theory presented on Chapter 2.3.

The first DGGE image on day 1 for both archaea and bacteria showed no difference in terms of band presence and absence (similarity <95%, P = 0.01, data not shown). Negligible differences were also present in terms of band density. After 216 days of operation, the bands are still of high similarity for both phyla and no band differentiation seems apparent. In terms of density though, visually some bands look stronger, where others seem to fade (e.g. archaea: band 3: uncultured *Methanosaeta* has its optima at 15°C). Similarly for bacteria, no noteworthy change to band pattern for both sampling days is evident; density variation though suggests different temperature optima for specific communities (Figure 4.4.a, b).

The methanogenic groups that were detected after sequencing were *Methanosaetaceae*, *Methanosarcinaceae* and *Methanomicrobiales*, with predominance of the *Methanomicrobiales* at all temperatures on day 1 (Figure 4.5). After 216 days of operation, the predominance of *Methanomicrobiales* is suggested but not proven (based on ANOVA test, P-value: 0.537, 0.397, 0.350 at 4, 8 and 15°C respectively) at 4 and 8°C where at 15°C *Methanosarcinaceae* seems to remained abundant. At cold temperatures (4, 8°C) *Methanosarcinaceae* was the second most abundant. Both *Methanosarcinaceae* and *Methanomicrobiales* are hydrogenotrophic methanogens. This suggests a preference for hydrogenotrophic methanogenesis at low temperatures, using these specific inocula. *Methanosaetaceae* was the third most abundant at all temperatures. However it is the only group with a population that was not significantly changed after 216 days of operation. The scenario of the either promoted hydrogenotrophic or acetotrophic methanogenesis requires further investigation as there is a plethora of studies supporting preference to both pathways as presented on Chapter 2.2.5, 2.2.8. Pathway to methane is important as different substrates lead to different intermediates and presence of suitable microbial communities may accelerate degradation and treatment. The reduced methanogenic populations also contributes to the uncertainty in the preferred pathway to methane as further differentiations in the archaeal communities may disturb the community dynamics and result to changes in substrate preference via high decay, immigration and colonization (Chapter 2.3). At this point tracer experiments with radio-labelled substrates could have been a more informative approach to define what the preferable pathway to methane is.

As described earlier, qPCR results revealed a decay in the methanogenic population at all temperature; the decay was apparently exacerbated at lower the temperatures. This was perhaps due to the limited hydrolysis (apparent based on VFAs and sCOD values) at lower temperatures. The limited intermediates (as VFAs) formation suggests that starvation possibly occurred, and lack of substrate resulted to minimization of the archaeal population. It is important to mention that the *Methanosaetaceae* cells were least affected (Figure 4.5). Thus it would be important to see the fate of the particular taxon in the next batch feeds.

As previously described, the VFAs level in the first batch was very modest, and essentially no net production occurred. This indicates that either hydrolysis was limited or the VFA production rate was equal to the methane production rate; however as only traces of methane were formed, it is likely that hydrolysis was limited. It is also possible that the presence of sulfur reducing bacteria (SRB) (e.g. clostridia-like, Table 4.2) contributed to low methane formation, as such scavengers might have utilized part of the intermediates (VFAs) increasing the CO_2 (Figure 4.2.d). This scenario is likely considering that the methanogenic population could have been outcompeted by the putative scavenging population. Alternatively, intermediates such as gaseous H_2 that were potentially formed by acetate oxidation (Dhaked et al 2010) escaped into the gas phase due to the sampling strategy. This strategy was based on removing liquid sample from a reactor port, allowing head space release, followed by N_2 re-flush (only for batch 1). The VFA and sCOD concentrations pattern were observed consistently low at 4°C.

The sCOD concentrations observed in the first batch at 8 and 15°C were sufficiently low and subsequently treated enough to be discharged into the environment if a secondary clarifier is present to separate the effluent from the solid fraction; at 4°C further treatment would be required, (based on UWWTD (91/271/EEC)). For the second batch all reactors after 40-60 days managed to degrade the sCOD to acceptable levels. Analyzing the trends, the second batch trend lines are steeper than the first. This suggests that the anaerobic treatment reaction rate was accelerated (sCOD rate constants (day^{-1}), batch 1: -0.003, -0.003 and -0.005; batch 2: -0.011, -0.012 and -0.013), and agrees with the accelerated methane production rates. Combining both events, COD:CH_4 conversion in the

second batch takes place at higher rates than in batch 1. This suggests that higher acclimation led to more efficient treatment and a higher rate of methanogenesis. In principle the evidently higher COD removal and methane bio conversion suggests that the specific biomass is promising and has the capacity to adapt and treat domestic wastewater at the raw phase. Thus, further batches will shed more light and help us define the details and the limits of low temperature anaerobic digestion.

In terms of COD the results in the first batch cannot lead a clear conclusion in terms of COD treatability because of high fluctuations, possibly caused from the high in solids organic content due to presence of plant material in the inoculum. In the second batch, a COD decline is noticeable for 8, 15°C, whereas at 4°C the trend suggests insufficient treatment. As the reduction in COD cannot be reconciled with the methane produced (the methane bioconversion for batch 2 estimated 0.02 and 0.07 l CH_4/$gCOD_{removed}$ at 15 and 8°C respectively, at 4°C no COD removal was observed) and it is believed that the digester partially operates as a clarifier, especially at lower temperature This event was also evident on the 1st batch, however high COD fluctuations in the mixed liquor do not lead to conclusions with confidence. This phenomenon is common in low temperature anaerobic treatment (Luostarinen and Rintala 2005, Bogte et al 1993, Elmitwalli et al 1993 etc.). This event requires further investigation as it may lead to biomass deterioration, washout phenomena and sludge instability that would result to treatment failure (Xing et al 2009, Uemura and Harada 2000). In the next batches it is important to estimate the exact ratio between the removed (methane) and settled COD and how this discrepancy may enable a new principle (as safety factor) in wastewater treatment to tackle the COD accumulation at low temperatures.

Higher production of CO_2 was observed on the first than on the second batch. This suggests that hydrolysis occurred at a higher rate on the 1st batch, possibly due to presence of high organic material in the seed. This cannot be confirmed as an unfed seed control was not prepared. Excessive CO_2 also asserts the presence of sinks (e.g. SO_4, combined with the presence of SRB) as described earlier or the introduction of O_2 during sampling. The lower CO_2 production in the second batch demonstrates a reduction in the sinks or the seed's organic material, if present, or less/no O_2 ingress (the sampling strategy for this batch changed to ensure 100% sealed reactor).

The VSS:TSS ratio appeared to be lower at higher temperatures (Figure 4.7.e), suggesting higher removal of organic solids the higher the temperatures. This agrees with the sCOD and VFA peaks that were apparent at low temperature even after 200 days, suggesting insufficient or slow hydrolysis/fermentation. Poor hydrolysis and accumulation of organic material as VSS may also be caused by the poor COD:CH_4 conversion. Excess VSS account for proteins, lipids and carbohydrates. Further investigation on these three components may define which of those is responsible for the VSS accumulation, the poor methane conversion and the discrepancy between the COD removed and methanized. As noted COD removal could not be accounted by the methane formed. VSS seem to accumulate in the mixed liquor, possibly forming agglomerates due to hydrophobicity, settling due to their weight, leading to poor methane production. VSS accumulation has been noticed in previous studies in wastewater treatment at low temperature (Alvarez et al 2008, Elmitwalli et al 2002), suggesting that hydrolysis limitation is present and needs to be engineered for successful treatment at such temperatures.

General Archaeal population was quantified both via qPCR (Arc109F, Arc344R) and FISH (ARC915). The initial relationship showed a weak correlation (Figure 4.6.a). Incorporation of the probe into the sample due to the lack of nonsense probe control could have resulted in poor correlation; however as the correlation was stronger on the later experimental days I believe that this was not the case. Hence, it is very likely that high number of inactive cells are present; the DNA of inactive cells is preserved and is detectable via qPCR but not by FISH which stains only the active cells. The weak correlation suggests that qPCR is not a preferable method for enumeration at the early days of an experiment as dead cellular material can interfere and lead to overestimation of the biomass. We believe that FISH is unlikely to overestimate biomass; however as it is a laborious and expensive method it cannot be easily used in a regular basis. For day 216 though, the correlation is high with an R^2 equal to 0.896. This suggests that presence of inactive cells is likely but lower, and it becomes even lower the more the time passes. Both methods seem to have advantages and disadvantages, thus, it is up to the researcher which one thinks more appropriate for the nature of its study. For the particular study I believe that a more representative approach would be the summation of the qPCR based methanogenic populations that were sequenced from the DGGE bands. This would exclude the archaeal cells that are not necessarily methanogens (e.g. *Crenarchaeota*) and might underestimate the methanogenic capacity of the particular seed if involved in the enumeration.

4.1.6. Conclusions

Methanogenesis start-up is highly related to temperature (40 days at 15°C, 140 for 4 and 8°C).

The inocula is adapted to this particular complex substrate at low temperatures, as the rates of CH_4 production increased up to 15 times at 4°C, soluble and total COD reduction are higher at Batch 2 than 1. Hydrogenotrophic methanogenesis during start-up appears favoured at low temperature as *Methanomicrobiales*-like seems the predominant taxon at all temperatures.

Hydrolysis was slow and inversely proportional to temperature as during the first batch sCOD and VFA peaks were observed even after 200 days at low temperatures (4, 8°C). Additionally, on the second batch a VSS:TSS increase at lower temperatures (mainly at 4°C) asserts that hydrolysis may be the limiting step.

There may have been a large inactive archaeal population present in the seed; this would explain the disagreement between FISH and qPCR, with the latter giving larger counts. The improved archaeal qPCR:FISH correlation can be achieved when the population is active, this reveals that qPCR provides with a larger archaeal coverage, and delivers with a better signal compared to FISH.

4.2. Anaerobic digestion of domestic wastewater at low temperatures (4, 8 and 15°C) in reactors with psychrophilic inocula – Batch 3.

4.2.1. Abstract

Low temperature methanogenesis is one of the most challenging aspects of the successful anaerobic treatment of domestic wastewater in temperate regions. Most previous attempts to find the lower operating temperature limits for anaerobic domestic wastewater treatment have attempted to acclimatise mesophilic sludge. An alternative approach would be the use of seed containing communities that have been adapted over evolutionary time-scales to cold-temperatures. Batch reactors were inoculated with a mixed inoculum whose sources included soils and sediments from the high Arctic and an Alpine lake to treat UV-sterilized raw domestic wastewater at 4, 8 and 15°C. Wastewater was treated to an effluent quality that met the UWWTD (91/271/EEC) standard for COD removal within 60 days. A mass balance of COD to CH_4 conversion showed that reactors at low temperatures operate partially as clarifiers with the accumulation of un-hydrolysed COD. The bacterial and archaeal communities of replicate reactors showed high similarity, whereas those at different temperatures were significantly different. *Methanomicrobiales* and *Methanosaetaceae* were equally dominant in methanogenic communities at 15°C. *Methanomicrobiales* were dominant at lower temperatures (4, 8°C) followed by *Methanosaetaceae* suggesting that at low temperature methanogenesis tends to follow the hydrogenotrophic pathway. Specific methanogenic activity at 4, 8 and 15°C were 6.3, 7.6 and 10.3 fmols CH_4 cell^{-1}day^{-1}; hydrolytic activity was estimated at 76.2, 186.6 and 250.9 fgrams COD.cell^{-1}day^{-1}. The results suggest that inoculating digesters for low temperature operation with cold-adapted communities is a promising way to treat wastewater and appropriate to investigate the limits of AD.

4.2.2. Introduction

Chapter 4.1 demonstrated that the use of a cold-adapted-psychrophilic inoculum can lower the temperature limits of anaerobic wastewater treatment proving the feasibility of methane production from the particular substrate at 4°C. Practically this asserts that the sustainable character of anaerobic wastewater treatment can be retained even for plants situated in countries with ambient temperature <15°C. The previous sub-chapter (Chapter 4.1) accounts for the start-up period of the cold adapted inoculum treating raw wastewater at low temperatures. The establishment of the efficiency of the seed with regards the CH_4 production and the COD removal from the two previous batch trials cannot be safely guaranteed. As the rates were accelerated between the first and the second batch, I am convinced that further acclimation would promote the adaptation and subsequently the capacity of the seed to treat and convert the organic material of the wastewater to CH_4. Hence, the aim of this chapter is to quantify the maximum methanogenic and wastewater COD treatment performance at low temperatures using the specific cold adapted/psychrophilic inoculum at a batch reactor setup. Additionally, the impact of temperature on the microbial community structure of the seed was evaluated. Finally, the detailed calculation of the cell specific hydrolytic and methanogenic activity were presented. These results would assist in determining if the inoculum is capable of treating anaerobically domestic wastewater at the temperatures of 4, 8, and 15°C and it will also examine if the efficiency is adequate to meet the UWWTD (91/271/EEC) COD standards.

4.2.3. Materials and Methods

Reactors assembly; the same reactors' regime as described on Chapter 4.1.3 was used for the particular batch experiment, using the same inoculum (as in Chapter 4.1.3), following the re-inoculation technique described on the same chapter. The reactors after reinoculation and re-feed were set at 4, 8 and 15°C.

Wastewater; the reactors were fed with raw wastewater of 600 ppm. The collection of the substrate took place according to Chapter 4.1.3 (treatment plant, treatment step). Its composition was found similar to the one estimated for the first and second batch based on the carbohydrates, lipids and protein content (45% carbohydrates 35% lipids and 20% proteins – protocols on Chapter 4.1.3). The substrate was UV-sterilized following the same sterilization procedure as described on Chapter 4.1.3. Presence of sulphate (SO_4^{-2}) and nitrate (NO_3^-) in the wastewater was determined after sample filtration from a 0.45μm syringe filter. Filtration was required to protect the column of the Dionex, ICS-1000 Ion Chromatograph fitted with AS40 Automated Sampler from impurities. The sample volume was 5 ml. The data analysis was carried out on Chromeleon software (Dionex, Corporation).

Analytical methods; total and soluble COD (tCOD, sCOD respectively) were estimated as described on Chapter 4.1.3 From the initial and final COD values for a specific time period Δt the k (removal coefficient for COD) values were estimated (1st order kinetics; $Eq.12$).

$$COD_t = COD_0\ e^{-k\Delta t}\ (Eq.12)$$

VFA (Volatile Fatty Acids) analysis in the mixed liquor in addition to the gas analysis for methane and carbon dioxide content in the head space were estimated following the methods described on Chapter 4.1.3.

Rates; Hydrolysis and methanogenesis rates were estimated using the equations below ($Eq.$ 13, 14 and 15):

- If $sCOD_{t1} > sCOD_{t0}$ then **Hydr. Rate = [ΔsCOD$_{(t0,t1)}$) + ΔCOD$_{CH4(t0,t1)}$] / Δt** $Eq.13$
- If $sCOD_{t1} < sCOD_{t0}$ then **Hydr. Rate = [(ΔCOD$_{CH4(t0,t1)}$) + (ΔCOD$_{VFA(t0,t1)}$*] / Δt** $Eq.14$
- **Methanogenesis rate = ΔCOD$_{CH4(t0,t1)}$ / Δt** $Eq.15$

* for $sCOD_t < sCOD_0$, VFAs present ≈0ppm to avoid including Acidogenesis in the calculations.

Where $sCOD_{t0}$ and $sCOD_{t1}$ stand for the initial and final sCOD concentration at a specific time period respectively, $\Delta sCOD_{(t0,t1)}$ stands for the difference amongst them. $\Delta COD_{CH4(t0,t1)}$ stands for the methane production in a specific time period expressed in COD; $\Delta COD_{VFA(t0,t1)}$ stand for the production/reduction of the VFA status in a specific period of time expressed in COD; Δt stands for the time required for any of the above differences to occur. Typically the hydrolysis rate equals the solubilisation rate that is the amount of the soluble organic material (sCOD) formed in a specific period of time Δt plus the amount of methane formed in that same period ($Eq.13$). However, in some cases sCOD (and VFA) decreased over the relevant time period (Δt). Then the hydrolysis rate was calculated as the sum of the amount of VFAs and the amount of methane that were formed in Δt, minus the VFAs present at t_0 (all expressed in units of COD; $Eq.$ 14. The $Eq.15$ was used to simply describe methanogenesis rate as the amount of methane that was formed in a specific Δt.

Microbiological community analysis; microbial community structure was described using the rapid community fingerprinting method, denaturing gradient gel electrophoresis (DGGE) as described on Chapter 4.1.3. Briefly, this includes the extraction of genomic DNA from biomass pellets, amplification of the extract via PCR (Polymerase Chain Reaction) and DNA band visualization via DGGE on a D-gene DGGE system (Bio-Rad, Hercules, CA, US); selected DNA bands were excised, sequenced and compared to the GenBank database to determine nearest neighbors (all steps are in detail described on Chapter 4.1.3).

Diversity of the microbial communities from different temperatures were analyzed based on band pattern using gel images obtained from DGGE, processed with the software package Bionumerics (Applied Maths, Austin, Texas, US). The image after band matching was quantified and re-examined in Primer6 (Multivariate statistics for ecologists, Luton, UK) for microbial community analysis at all temperatures. The data were transformed by 'presence or absence' transformation and examined using the 'Bray-Curtis' similarity test. Analysis was carried out using non-Metric Multidimensional Scaling (MDS) and analysis of similarity (ANOSIM). The selected test was ´one way´ with temperature as factor. The significance of the similarities were evaluated based on P-value. P-value is a number used to determine if a result is statistically significant, practically is a function of the observed

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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results that is used for testing a statistical hypothesis (e.g. similarity). The confidence level was chosen as 90% which corresponds to a P-value = 0.1.

Quantitative PCR (qPCR) was used for the quantitation of the dominant methanogens found in the reactors, according to the sequenced bands cut from the archaeal DGGE. The methodology is described on Chapter 4.1.3. A correction factor was included to normalize the results based on the samples' minimum DNA content. The value was estimated via Spectrophotometer - Fluorospectrometry (Nanodrop – Thermoscientific UK) so populations with non-equal DNA content can be compared. The minimum amount estimated as 2.8 ng.μl^{-1} and was used for normalization purposes.

For bacterial FISH enumeration the protocol from Coskuner et al (2005) was followed. Prior to FISH analysis, samples were fixed with 4% paraformaldehyde (in ethanol) (Amann et al 1990). The probe was labeled with FITC (fluorescein) and was purchased by Genosys (Cambridge, UK). The type was Eub338 (S-D-Bact-0338-a-A-18) – (sequence: GCT GCC TCC CGT AGG AGT) – specificity: 16S rRNA gene of many eubacteria (338–355) (Aman et al 1990). The hybridization, visualization and enumeration methodology is described on Chapter 4.1.3.

The rate of change of a reaction as a function of temperature, Q_{10}, (temperature coefficient) was estimated from the cell specific activity following the Eq.16. Where R1, 2 account for the specific methanogenic or hydrolytic activities corresponding to the T1, 2 temperatures (K). The Q_{10} was calculated as an average of the temperature coefficients estimated from the developed activities between 4°C-8°C, 4°C-15°C and 8°C-15°C.

$$Q_{10} = (\frac{R_2}{R_1}).10^{10/(T2-T1)} \quad (Eq.16)$$

For the specific activity the conversion of the cells (both bacteria and methanogens) to grams of cells was carried out based on the assumption that 1 cell weighs 26.24 f.grams (Trousselier et al 1997). For a more conservative approach the cellular weight suggested from Rittmann and McCarty (2001), equal to 10^{-12} gram cell, was also taken into account and involved in the discussion part.

4.2.4. Results

The collected wastewater was low in organic matter content (COD of 600mg.L^{-1}). Anions (SO_4^{2-}, NO_3^-) were found able to scavenge a negligible amount of methane (1.4% of the theoretically expected). The influent BOD$_5$ accounted for the 40% (240 mg.L^{-1}) of the influent COD suggesting a biodegradability ratio (BOD:COD) of 0.4. The reactors were monitored for 102 days. The starting pH at all temperatures was 7.0. During the process no values less than 6.8 and 7.0 or no more than 7.3 and 7.0 for 15 and 4°C respectively were observed; at 8°C pH laid in-between these two values. The reactors' performance was described following the three fundamental steps of AD, hydrolysis (fermentation), acidogenesis-acetogenesis and methanogenesis (Figure 4.8.a, b, 4.9. and 4.11). The fate of the organic material in the reactor as COD and sCOD is described on Figures 4.8.a, b.
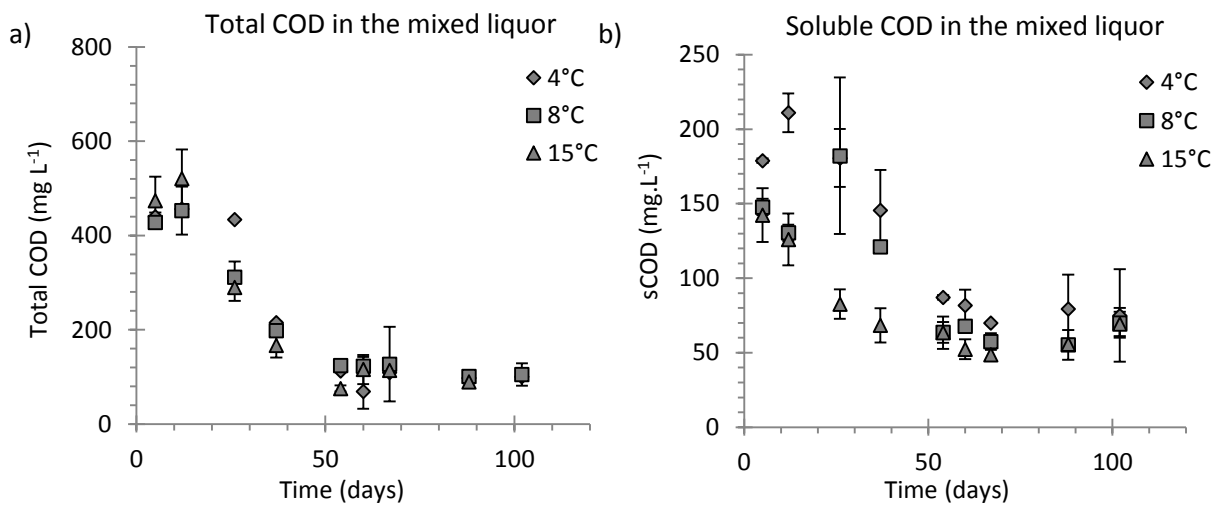
Figure 4.8 – a) Total COD in the mixed liquor after 102 days of incubation at all temperatures; b) Soluble COD for the same time and conditions: n=2 for 4 & 8 degrees and n=4 for 15°C, error bars indicate standard error

I n v e s t i g a t i n g t h e t r u e l i m i t s o f a n a e r o b i c t r e a t m e n t o f w a s t e w a t e r a t
l o w t e m p e r a t u r e u s i n g a c o l d a d a p t e d i n o c u l u m
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The VFAs were also poor, their status is shown on Figure 4.9.
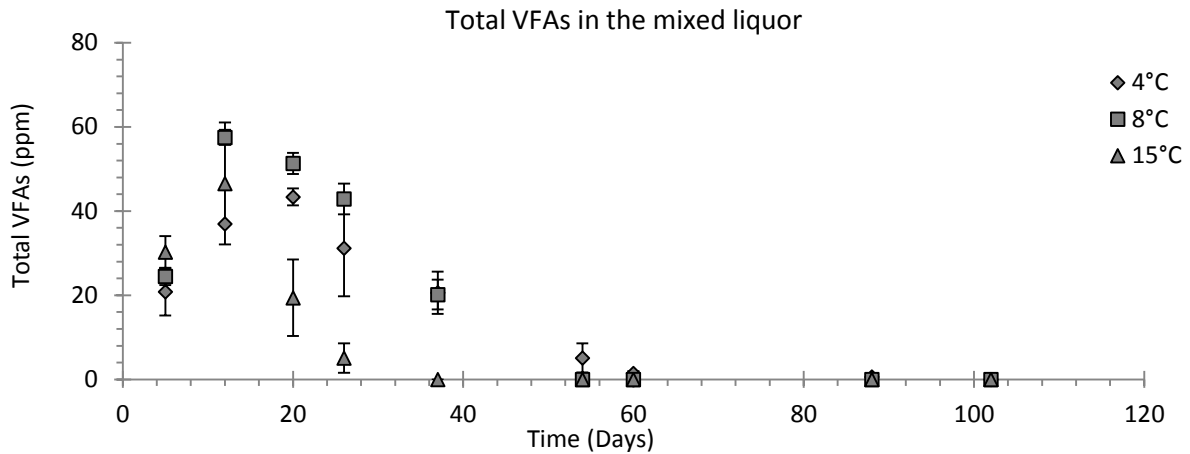
Figure 4.9 – Volatile fatty acids in the mixed liquor as the sum of acetate and propionate; longer chain acids never detected; IEC minimum detection limit = 0.8ppm; n=2 for 4 & 8 degrees and n=4 for 15°C, error bars indicate standard error

From Figure 4.8.a and *Eq.*12 the 'k' removal coefficient was calculated (Figure 4.10)
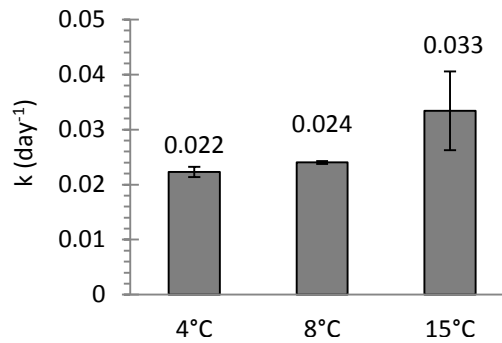
Figure 4.10 - k COD removal coefficient (day^{-1}) for the reactors seeded with cold adapted microbial communities at 4, 8 and 15°C (day^{-1}); n=2 for 4 & 8 degrees and n=4 for 15°C; error bars express standard error.

The methane production at all temperatures is shown on Figure 4.11.
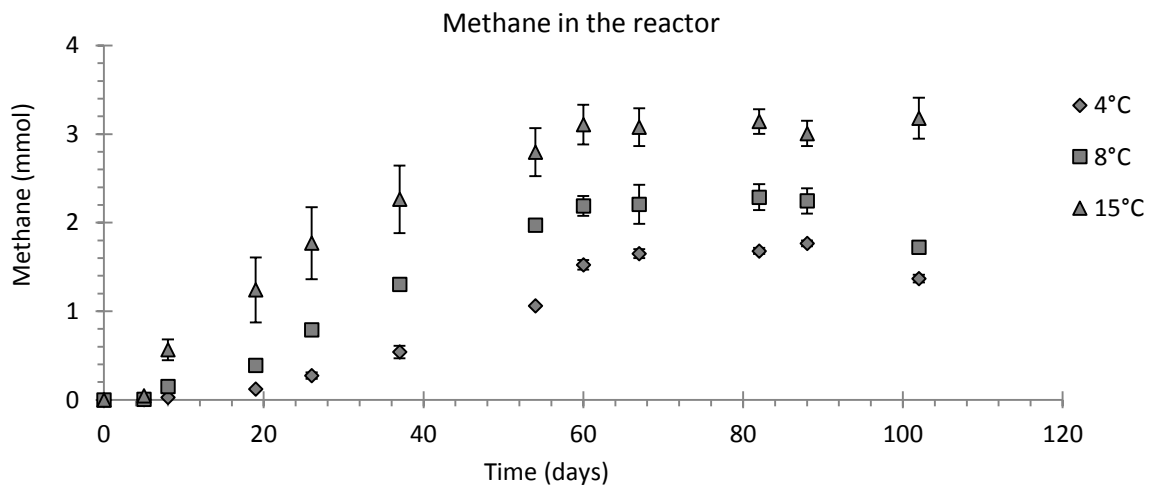
Figure 4.11 – Methane formation from wastewater for the particular seed at 3 temperatures for 102 days of incubation; n=2 for 4 & 8 degrees and n=4 for 15°C. The volume is expressed as mmol in the headspace, error bars indicate standard error

From the DATA collected and the equations 13, 14, 15 the daily rates for hydrolysis and methanogenesis were estimated (Figure 4.12); from *Eq.*12 and COD fate (Figure 4.8.a) the COD removal rates were calculated.

Removal, Hydrolysis and Methanogenesis rates
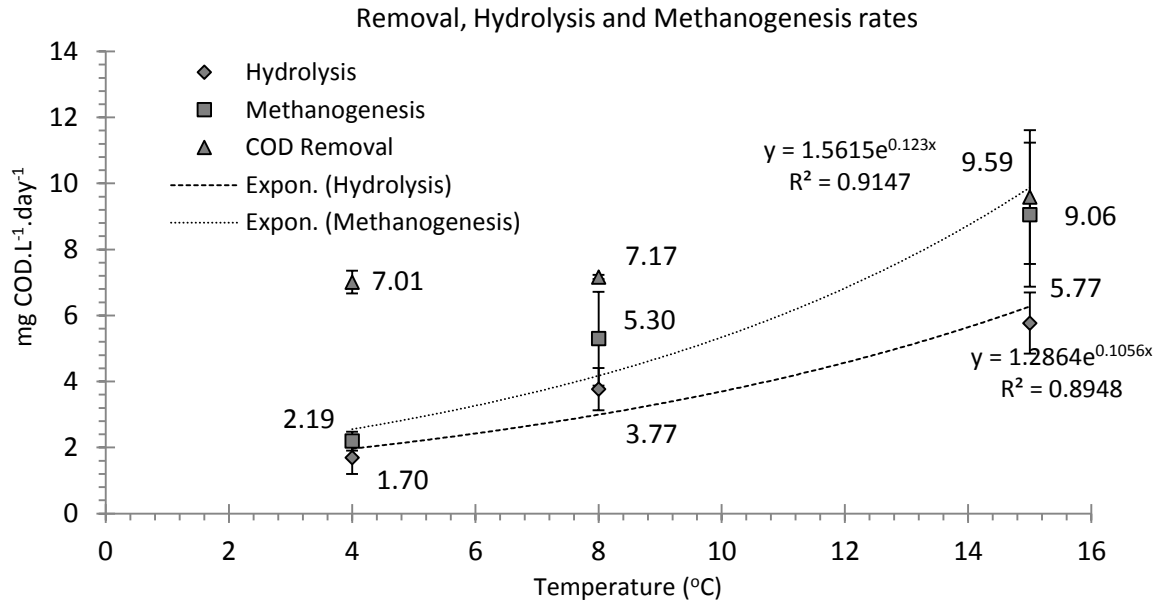
Figure 4.12 - Hydrolysis, methanogenesis and COD removal rate expressed as mg COD. $L^{-1}.day^{-1}$ for wastewater as substrate; error bars indicate standard error (n=2 for 4, 8°C, n=4 for 15°C), all results are expressed as averages from duplicates and quadruplicates for 4, 8°C and 15°C respectively calculated between the days: 5-37, 8-37 and 5-37 for hydrolysis methanogenesis and for COD removal rates respectively; polynomial trendlines show the effect of temperature to the reaction rate.

The volumetric rates above enable the estimation of the size of an anaerobic treatment facility. An equivalent person usually releases 100L of wastewater, containing 50g COD day^{-1} in daily basis, or wastewater of 500 mgCOD L^{-1} per day. This value may change as the physicochemical properties of wastewater vary depending on socio-economic factors. The wastewater driven to a conventional wastewater treatment plant may reduce its concentration by 50% from the primary physical separation processes. Hence, approximately 250mgCOD L^{-1} is remained to be treated until the COD reach the 125mg L^{-1}, according to the UWWTD 91/271/EEC. Thus, the reactor volume that is required to efficiently hydrolyse, biomethanize or treat the wastewater per person in one day to cope with the directive standards, using the 1:3 seed:substrate ratio, based on Figure 4.12 is shown on Table 4.3 below:

Table 4.3 – Required volume estimations for all processes based on the observed volumetric rates (hydrolysis, methanogenesis and COD removal), using the specific seed at a 1:3 seed to substrate ratio

	m^3 person^{-1}.day		
Temp.	Hydrolysis	Methanogenesis	COD Removal
4°C	7.4 (2.2)	5.7 (0.8)	1.8 (0.1)
8°C	3.3 (0.6)	2.4 (0.6)	1.7 (0.0)
15°C	2.2 (0.3)	1.4 (0.3)	1.3 (0.3)

*values in the parenthesis express the standard error; n = 2, 2, 4 for 4, 8 and 5°C respectively

Temperature differentiation affected both bacterial and archaeal seed communities. After analysis of the DGGE image at Bionumerics and further statistical analysis in Primer6 the effect of temperature to archaea and bacteria is shown on Figure 4.13.a and b respectively. The analysis was carried out on the 56th day of the experiment.

a)

b)
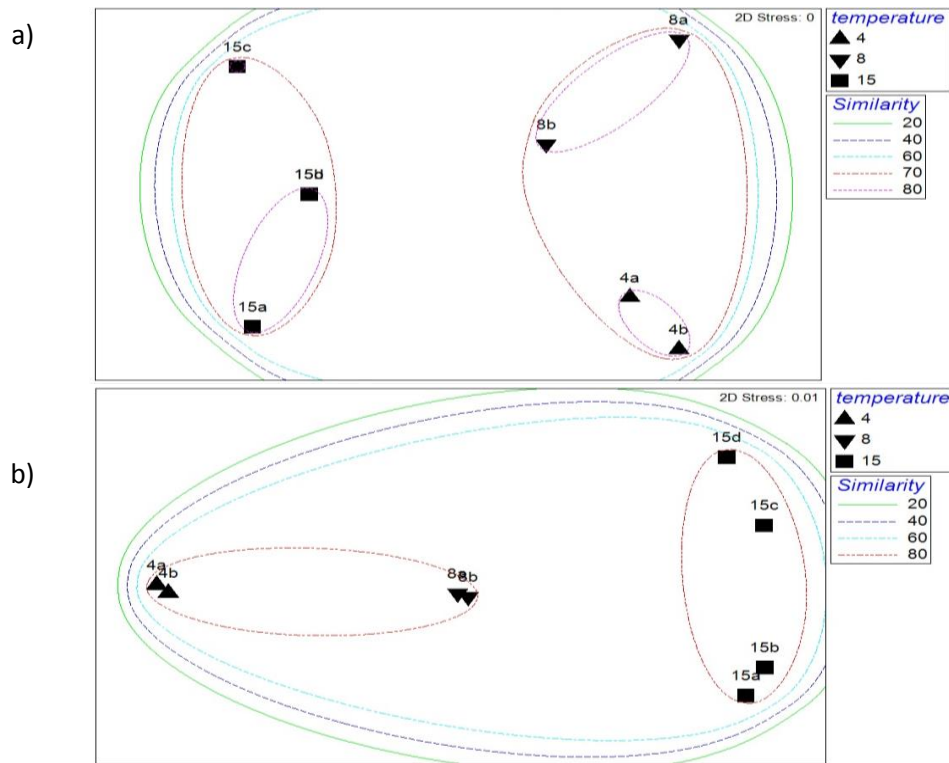
Figure 4.13 – MDS ordination plots based on Bray-Curtis similarity of presence-absence data for communities of a) archaea and b) bacteria in reactors operating at 4, 8 and 15 °C for the treatment of raw domestic wastewater; sample titles correspond to temperature (4, 8, 15°C) and the number of replicate (a, b, c, d).

To get a better insight of the methanogenic community structure within the reactors at all operational temperature a qPCR analysis was carried out (Table 4.4).

Table 4.4 - Methanogenic taxa populations per ml detected at the experiment, day 1, 56, 102;

	day 1			day 56			day 102		
	MST	MSC	MMB	MST	MSC	MMB	MST	MSC	MMB
4°C	3.96 (2.16)	0.22 (0.08)	3.54 (1.92)	0.64 (0.05)	0.01 (0.08)	0.54 (0.24)	0.48 (0.16)	0.02 (0.02)	0.62 (0.29)
8°C	4.98 (1.58)	0.18 (0.1)	3.51 (1.67)	1.08 (0.07)	0.17 (0.1)	0.75 (0.6)	0.60 (0.12)	0.04 (0.01)	0.78 (0.13)
15°C	4.57 (0.88)	0.51 (0.24)	4.8 (0.77)	0.41 (0.16)	0.09 (0.04)	0.73 (0.46)	1.22 (0.18)	0.31 (0.31)	1.29 (0.47)

*all values expressed in 10^6 per ml; abbreviations stand for: MMB *Methanomicrobiales*, MSC *Methanosarcina*, MST *Methanosaetaceae*; n=2 for 4 & 8 degrees and n=4 for 15°C; values in parenthesis express the standard error (n 2 for 4 and 8°C, n=4 for 14°C)[2].

Similarly, the bacterial population trend at all operational temperatures was examined through enumeration via FISH (Table 4.5). Representative FISH images can be also found in Appendix (Appendice 1, Figure a.1a, b, c for 4, 8 and 15°C).

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[2] The error bars were also calculated with the log-transformed data, however the back transformation of the results could not depict the variance of the population resulting to non-representative, really low values (e.g. SE of 1-5 cells.ml^{-1}). A different approach for error bars was the estimation of the 95% confidence level of the log transformed mean, however the back transformation resulted to extremely high mix and max bars due to the limited number of replication (duplicates, quadruplicates)

Table 4.5 – Total bacterial cells enumerated by FISH

	day 5	day 60
	Total bacterial cells	
4 ºC	3.71 (1.54)	0.01 (0.00)
8 ºC	1.12 (0.37)	1.52 (1.51)
15 ºC	2.32 (0.69)	0.1 (0.05)

*all values expressed in 10^7 per ml; n=2 for 4 & 8 degrees and n=4 for 15ºC; values in parenthesis indicate standard error (n = 2 for 4 and 8ºC, n=4 for 15ºC)[3]; counts took place on day 5 and 60.

SCOD, VFAs and methane data (Figure 4.8.b., 4.9, 4.11) were combined with the methanogenic population (Table 4.4) and the bacterial cell enumeration from FISH (Table 4.5), to enable the calculation of the specific activity per cell at low temperature for raw domestic wastewater treatment (Figure 4.14.a, b). The specific activity was also expressed gCOD (removed or reduced) per gram of methanogenic and hydrolytic biomass per day for further comparison purposes with previous studies. The conversion based on Trousselier et al 1997 (1 cell = 26.24 f.grams C) (Figure 4.14.d). The specific activity also enabled the calculation of the Q_{10} (Figure 4.14.c) for both reactions.
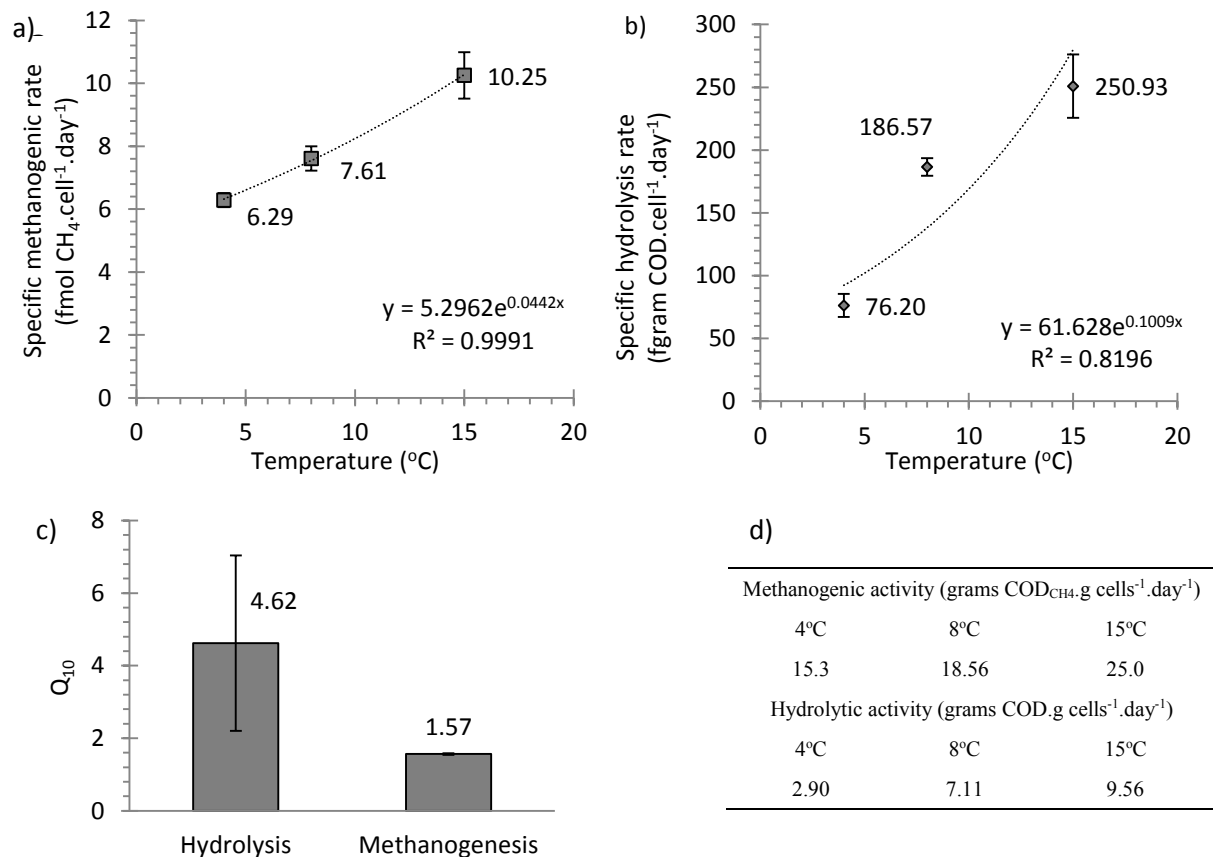
Figure 4.14 – a) Specific methanogenic activity per methanogenic cell at all temperatures; b) specific hydrolytic activity per bacterial cell at all temperatures; values referred to raw domestic wastewater as substrate; n=2 for 4 & 8 degrees and n=4 for 15ºC; error bars express standard error. c) Q_{10} for hydrolysis and methanogenesis reaction as an average of the values calculated amongst temperatures; n = 3, 4-8, 4-15, 8-15; d) methanogenic and hydrolytic activity expressed per gram of methanogenic and hydrolytic biomass per day – conversion based on Trousselier et al 1997 (1 cell = 26.24 f.grams C).

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[3] The error bars were also calculated with the log-transformed data, however the back transformation of the results could not depict the variance of the population resulting to non-representative, really low values (e.g. SE of 1-5 cells.ml-1). A different approach for error bars was the estimation of the 95% confidence level of the log transformed mean, however the back transformation resulted to extremely high mix and max bars due to the limited number of replication (duplicates, quadruplicates)

The specific activity is required to accurately calculate the maximum wastewater OLR that can be applied. This, is especially useful when considering scale up of bench scale observations. Based on this study's activities for hydrolysis and methanogenesis, the desired OLR was determined for each process assuming that there are $\sim 10^9$ and $\sim 10^8$ bacterial and methanogen cells/ml respectivley (Table 4.6). The rationale behind this population size selection is that these numbers are relatively common in real scale conventional anaerobic wastewater treatment plants.

Table 4.6 - Suggested Organic Loading Rate (OLR) for hydrolysis and methanogenesis processes based on specific activity

	4°C	8°C	15°C
Hydrolysis (kg COD/m^3.day)	0.08	0.19	0.25
Methanogenesis (kg COD/m^3.day)	0.04	0.05	0.07

*OLR values were estimated for ideal conditions where 10^9 cells can be retained in the digester at a 'methanogens:total cells' ratio is 1:10.

4.2.5. Discussion

COD is a key parameter in wastewater treatment (Rittman and McCarty 2001), an approximate estimate of the potential energy contained in wastewater (Heidrich et al 2011) and is commonly used to describe AD performance and effluent quality; thus, anaerobic reactor performance (Figure 4.8.a, b.). At all three temperatures both COD and sCOD were reduced to levels lower than the effluent standard of 125 mg L^{-1} required in Europe (UWWTD 91/271/EEC) within a period of 56 days (Figure 4.8.a, b). sCOD of the preceding wastewater batches (first and second batch) were also sufficiently removed but more time was required; COD was similarly removed at 15°C to levels below the UWWTD standard of 125 mg COD L^{-1}, except at lower temperatures (4, 8 °C) where the standard was only met in this third batch. Therefore a satisfactory effluent COD quality can in principle be obtained by AD, even at temperatures as low as 4 °C with cold-adapted inocula.

Methane production occurred at all temperatures (Figure 4.11). Organic substrate in the wastewater was converted to methane at ratios of 0.12, 0.15 and 0.18 L CH$_4$/g COD$_{removed}$ at 4, 8 and 15 °C respectively in the absence of VFA accumulation. These values are distinctly lower than the theoretical value of 0.35 L CH$_4$/g COD$_{removed}$. No methane COD was observed in the liquid phase considering that the COD values were low. Though, it is important to mention that the wastewater was raw and limited COD to methane conversion was expected. This renders the biomass promising considering that a higher COD:CH$_4$ is likely for feed with primary settled wastewater, in absence of heavy, solid, hard to hydrolyse organic material. After approximately 56 days of operation the difference in methane production between the highest and the lowest temperature was only two-fold (Figure 4.11) although the temperature was 3.75 times lower; at 8°C the methane lies in between these 2. This suggests that an increase of 'seed:substrate' ratio from 1:3 (selected) has the potential to theoretically boost the performance in terms of methane production as more cells would be introduced into the reactor. The quality of the biogas was high in CH$_4$ content at all temperatures (93.3($\pm$0.02)%, 95.1($\pm$0.3)% and 91.9($\pm$1.1)% (CH$_4$:(CO$_2$-CH$_4$)) at 4, 8 and 15°C respectively).

Only low amounts of VFA were, produced which presumably reflects the low COD concentration in the waste. Moreover, the VFA consumption rate was equal or higher to the VFA production (Figure 4.9), resulting to values close to 0 after the 20th, 40th and 40th day for 15 and 4, 8°C respectively. The stable pH is also indicative of satisfactory VFA consumption rates. This event ensures high methanogenic activity, but on the other hand reveals potential of slow hydrolysis.

The data in Figure 4.8.a were used to calculate the first order rate coefficient k. The values ranged between 0.022 day^{-1} and 0.033 day^{-1} at 4 and 15 °C respectively (Figure 4.10). 'k' is a parameter that assists in predicting the time that is required for sufficient wastewater treatment, and preliminarily estimates the volume of a pilot scale AD reactor (Marais and Shaw 1961). Additionally, the daily COD removal rate was estimated and compared with the CH$_4$ production rate (Figure 4.12). The daily removal rate is useful for further comparison with the methane formation rates, to calculate how efficiently the organic material is bio methanized. These two rates clearly depict how much of the COD accumulates as a function of temperature and inform how anaerobic treatment is being affected by temperature drop.

COD removal rates ranged between 7.0 and 9.6 mg COD.L^{-1}day^{-1}. These values were higher than the COD-equivalent CH$_4$ production rates, which ranged between 2.2 and 9.1 mg COD.L^{-1}day^{-1}, and higher than the hydrolysis rates which ranged between 1.7 and 5.8 mg COD.L^{-1}day^{-1} (Figure 4,12). The difference between rates of hydrolysis and methanogenesis increased with decreasing temperature. The discrepancy between the daily removal and methanogenesis rate (mg COD day^{-1}) was negligible at 15 °C but 3.2-fold at 4 °C and 1.35-fold at 8°C. Taken together with the lower than theoretical conversion of COD to methane and absence of VFA accumulation, these data suggest that a substantial fraction of the COD removal occurred via settling, that is that the reactors acted partially as clarifier, as shown by Luostarinen and Rintala 2005, Bogte et al 1993, Elmitwalli et al 1993 etc. These results are in line with previous observations that hydrolysis is the rate limiting step in anaerobic sewage treatment at low temperatures (Elmitwalli et al 2003); however the difference between hydrolysis and methanogenesis decreases with temperature. Further evidence for hydrolysis limitation can be deduced from the sCOD:COD ratio that increased with decreasing temperature, presumably due to the slow COD lysis combined with slow uptake of the already hydrolysed compounds. The low VFAs that were formed at all temperatures (Figure 4.9) also provide with evidence of hydrolysis limitation. This phenomenon was also likely to batch 1 and 2; batch 3 assisted in quantifying the effect of COD settlement as a function of temperature. In general the volumetric COD removal and COD$_{CH4}$ rates are realistic and modestly lower than those Rebac et al (1999) applied to treat domestic wastewater at 10-15°C (Chapter 2.2.5.2).

In terms of wastewater engineering this means that the system that only operates at 4°C would need to be de-sludged at least 3 times more often with less stabilised sludge than the one operating at 15°C. However, in practice temperatures fluctuate over the year and part of the settled COD may be subsequently hydrolyzed during periods of increased temperature. To avoid the phenomenon of COD accumulation the reactor configuration needs to be designed including a multiplication factor for COD content in the system, equal to the division of 'COD$_{removed}$ rate/COD$_{CH4}$ rate', as a function of the period of time that temperature follows the pattern of 4, 8 or 15°C. A different approach to tackle this issue would be the increase of the seed:substrate ratio (from 1:3 current study) according to this factor. This would introduce larger microbial populations, ready to contribute in the biodegradation of the COD, by excreting additional quantities of enzymes to promote hydrolysis and treatment at these low temperatures. Further research into the nature of the COD fraction that was not degraded may allow the development of engineering strategies to overcome this issue. In practice increase of the seed takes place by adding new active inoculum or it occurs from the anabolic reactions of the biomass. The first option although it is more expensive is recommended as growth in anaerobic systems at low temperatures is low.

In short, the particular inoculum is able to treat wastewater to a satisfactory level at low temperatures. Although the time taken to achieve a satisfactory effluent concentration was long, the reactor kinetics (a batch fed system) was favourable. The effluent quality reflects the intrinsic properties of the biological material; in particular the affinity for substrate which in turn represents the k$_s$ (half saturation coefficient) for an equilibrated effluent concentration at theoretically unfavorable conditions (e.g. low temperature). The rate of removal reflects, in part, the active biomass concentration, which can be manipulated (e.g. membrane reactors) to maintain its content to dictate the factor m (decay). It is no surprise therefore that some success has been reported with ambient temperature wastewater treatment in membrane bioreactors; however, low temperature anaerobic wastewater treatment is in principle possible in any reactor format (Krzeminksi et al., 2012, Smith et al, 2013, Garcia et al, 2013). It is clear though, that anaerobic treatment is slower at lower temperatures and self-evident that temperatures vary throughout the year in temperate climates. It should be possible to accommodate lower temperatures by having larger concentrations of biomass and longer retention times; however, this might be more difficult if the winter temperature biomass was different from the summer temperature biomass.

Temperature differentiation affected both bacterial and archaeal seed communities. Plausible reasons could be natural selection, decay, dominance and microbial immigration trends based on the preferable for the organisms' conditions. Statistical analysis (Figure 4.13.a, b) was carried out on day 56 as all reactors were at the peak of their activity.

The bacterial DGGE image at day 1 (data not shown) demonstrated no significant difference in terms of presence and intensity between communities-bands (similarity >90%, P = 0.01). Therefore, the inoculum with the mixed-biomass was essentially uniform in all reactors. By day 56 the differences in band intensity suggested that

different taxa were dominating at different temperatures. The differences in intensity were observable, but less pronounced on the final sampling date (day 102) Statistical analysis of the DGGE image on day 56 (Figure 4.13.b) showed that the bacteria at 4 and 8 °C were ~85 % similar. This suggests that 4 degrees difference in temperature does not cause important changes between bacterial communities at the lower temperature range. At 15°C the bacterial communities differed from the previous two with a similarity <75%, P = 0.07 (Figure 4.13.b).

This implies that switching from 8 to 15°C implies far more biological change than switching from 8 °C to 4 °C. This in turn explains why attempting to adapt mesophilic sludges to psychrophilic conditions has proven difficult in the past (Bowen et al 2014).

The higher similarity between the communities developed at lower temperatures can be roughly visualized from Appendix - Appendix 1, Figure a.1 a, b, c where the cells from 4 and 8°C seems more similar in terms of shape and size (smaller) compared to those that were developed at 15°C.

Likewise the differences between archaeal communities were also negligible on day 1. However, over time temperature related differences became apparent for the bands associated with *Methanosaetaceae* that appear more intense at higher temperature and the bands associated with *Methanomicrobiales* that were stronger at lower temperatures (differentiation occurred in previous batch as the experiment was re-fed for 400 days). On day 56 and day 102 of analysis the band intensity differences were even stronger than at the early experimental days. By day 56 of the third batch the statistically differences were apparent between the reactors at 4 and 8°C (>70%) and those at 15°C (Figure 4.13.a) (60%; P=0.01).

The archaeal communities appear even more temperature sensitive than the bacterial communities as they differ at a higher degree compared to bacteria, i.e. at 4-8°C, with 70 and 80% similarity for archaea and bacteria respectively. In both cases there appears to be an important difference between the reactors at 4-8 °C and those at 15 °C.

The archaeal sequences had a generally low similarity with those that were compared in the database. The methanogenic taxa that were sequenced were similar to those detected in the first batch and belong to the groups of *Methanomicrobiales*, *Methanosarcinaceae*, and *Methanosaetaceae*.

The abundance of key genera was evaluated using qPCR analysis (Table 4.4). After 102 days the marginally dominant genus at 4 and 8°C is *Methanomicrobiales*, a strictly hydrogenotrophic methanogen, followed by *Methanosaetaceae*, a strictly acetoclastic methanogen. At 15°C the difference between the two taxa is not distinguishable. The genus *Methanosarcina* was detected at low abundances at all temperatures. In earlier samples (day 0 and 56) the abundances of *Methanomicrobiales* and *Methanosaetaceae* populations were not significantly different (Table 4.4). The hydrogenotrophic methanognesis scenario agrees with the observations of Simankova et al (2003) where *Methanomicrobiales* seemed to have an important role at low temperature anaerobic digestion. However, many studies have found that acetoclastic methanogenesis and homoacetogenesis are favoured over H_2/CO_2 methanogenesis (Schulz et al 1997, Fay et al 2004, Kotsyurbenko et al 1993, Nozhevnikova et al 2007). An authorative answer to this question would require a long term time series with varying temperatures.

From Table 4.4, 4.5. a decline in the abundance for both bacteria and methanogens is evident. This is probably due to reduction of the available substrate up to day 56, event that is also obvious from Figure 4.8.a,b, 4.9 and 4.11 where organic carbon is low and methane production plateaus.

As previously observed (in the first batch) where *Methanomicrobiales* seemed to be the most important genus at low temperatures. However, it is also noticeable that *Methanosarcinaceae* cells, which were initially relatively abundant, compared to other groups are undetectable. *Methanosaetaceae* were also almost as important as *Methanomicrobiales* with the difference between the two genera becoming less evident at higher temperatures.

Bacterial sequencing revealed at least 14 taxa (out of 31) to organisms associated with cold environments (data not shown). This suggests that cold adapted bacteria are present in the seed and hydrolyse organic material at temperatures lower than 15°C. Like the methanogenic population, the abundance of the bacterial one also declined (Table 4.5). The cause seems likely to be the limited organic material available.

The hydrolytic and methanogenic cell specific activity (Figure 4.14.a, b) can be used for design as it provides a detailed representation of the performance of the selected seed. The vast majority of research studies express activity per gram of VSS. This increases the potential of error especially when the seed is originated from sediments as other carbon sources other than microbial cells may contribute to VSS leading to an underestimation of its methanogenic capacity. Specific activity combined with certain assumptions (e.g. a COD OLR, plausible cellular population (10^8/ml and 10^9/ml for methanogens and bacteria respectively) may lead to a plausible scale up model for sufficient wastewater treatment OLR (Table 4.6). These values are realistic (compared to those stated on Chapter 2.2.5) and correspond to sufficient wastewater treatment in 1 day HRT. The unresolved question is how to achieve a 10^9 and 10^8 active bacteria and methanogens respectively. I believe that these specific activities are underestimated due to presence of inactive cells that affected the qPCR and potentially higher cellular adaptation and affinity to the substrate in future batches. Furthermore, the bacterial activity is likely to increase if a more biodegradable (as opposed to settled) wastewater was used.

Specific activity also enabled the estimation of the Q_{10} and the clear demonstration that hydrolysis is easier affected by temperature than methanogenesis. Consequently understanding hydrolysis may be the key in understanding and optimising the relationships between treatment and temperature. Specific activities of methanogens under reactor conditions ranged between 6 and 10 fmol CH_4 $cell^{-1}.day^{-1}$ (Figure 4.4.a). These values are only slightly lower than typical maximum specific activities of mesophilic methanogens if we assume a "typical" cell mass of 10^{-12} gram cell (Rittmann and McCarty 2001) and specific activities of 35 mmol CH_4 g^{-1} day^{-1} and 450 mmol CH_4 g^{-1} day^{-1} for acetoclastic and hydrogenotrophic methanogens respectively (Dolfing and Mulder 1985). Being less conservative, using the proposed cellular mass based on Trouselier et al 1997 the activity between the specific cold adapted cells and typical mesophilic cells tends to become almost equal (Figure 4.14.d). This underlines that this biomass offers potential to make anaerobic treatment of municipal wastewater at low temperatures a viable process. My findings on the relative temperature sensitivity of hydrolysis and methanogenesis support earlier suggestions by Zeeman and Lettinga (1999), Elmitwalli et al (2003), Rebac et al (1999) that the anaerobic treatment of domestic wastewater at lower temperatures would be more efficient if hydrolysis/fermentation were separated from methanogenesis. They reasoned that a two-phased configuration might reduce risk of the inhibition of methanogenesis by intermediates.

I recommend designing reactors on the basis of specific cell activity to avoid the underestimation of activity because of the presence of plant biomass. To provide space for COD_{sludge} accumulation and allow a higher retention time a multiplication factor should be included as a design parameter equal to the ratio between $COD_{removed}$ and COD_{CH4} for the corresponding time period of 4, 8 or 15°C (appropriately interpolated for other temperatures). The archaeal qPCR data showed that anaerobic treatment can potentially follow the H_2/CO_2 pathway. Thus, to further promote gas-liquid exchange the 2-phase chambers should have connected head spaces so the CO_2 formed from hydrolysis/fermentation and the H_2 from acidogenesis/acetogenesis would be easier accessible from the methanogens. A key unresolved question is if it will be possible to sustain a reactors psychrophilic properties during the warmer months of the year and if the reactor could transition from moderate to low temperatures in the winter. This will depend on the effect of temperature on the long term dynamics of the microbial community (Ofiteru et al 2010). Therefore engineers should be able to manage the microbial population to ensure they have the treatment capacity required for the temperatures they anticipate to encounter.

4.2.6. Conclusions

Anaerobic wastewater treatment at low temperature (4°C to 15°C) was successful. This suggests that the selected biomass can, in principle, cope with extreme temperature conditions and after 56 days of biological reaction the effluent at 4, 8 and 15°C is able to meet the UWWTD (91/271/EEC) COD standards for discharge on water body.

The amount of methane at 15°C was only two-fold higher than the amount measured at 4°C, no process failure observed at all three temperatures.

A safety factor for design purposes was estimated from the discrepancy between $COD_{removed}$ and COD_{CH4} (3.2, 1.35 and 1 for operation at 4, 8 and 15°C respectively). This disagreement suggests that the digester works partially as a clarifier the lower the temperature gets.

Hydrolysis is the rate limiting step of the process; the limitation becomes less evident the lower the temperature.

Temperature affects archaeal communities; lower temperatures favoured hydrogenotrophs. Temperature associated differences in the bacterial community were also observed.

Specific rates for hydrolysis and methanogenesis of raw wastewater at 4, 8 and 15°C were calculated so they can be further used as fundamental parameters for applied engineering purposes.

4.3. Cumulative phenomena, what is responsible for poor COD:CH4 conversion at low temperature - Batch 5

4.3.1. Abstract

Conventional anaerobic wastewater treatment plants treat primary settled wastewater. In this study 8 batch reactors were employed to treat primary settled wastewater at 4, 8 and 15°C, using the seed from the previous 3 batches. During treatment with primary settled sewage peaks of sCOD and VFAs were observed, with larger variety of intermediates, in marked contrast to the results observed using raw wastewater. This suggests that hydrolysis is not as limiting as when raw sewage is used. Methane production rates were still affected by temperature. The primary settled sewage first order COD removal coefficients 'k' (day^{-1}) were higher than of raw sewage. This suggests that the easier to degrade substrate combined with potentially further acclimation of the seed accelerates treatment. Wastewater influent (COD: 190mg L^{-1}) attained UWWTD (91/271/EEC) COD standards in 15 days. As in previous batches, hydrolysis seemed to be the limiting step. Analysis of the effluent for proteins, lipids and carbohydrates revealed that lipids formed a major part of the un-degraded fraction. Thus, this research study needs further focus on the enzymes (lipases) responsible for lipid degradation.

4.3.2. Introduction

So far, Chapters 4.1, 4.2 demonstrated that wastewater treatment in terms of COD and its CH4 bioconversion can be achieved at temperatures of 4, 8 and 15°C using a cold-adapted inoculum, meeting the COD standards (UWWTD 91/271/EEC, Chapter 4.2) regardless the raw and hard to degrade nature of the substrate. This manifests that the biomass can treat and bioconvert organic substrate originated from domestic wastewater to methane regardless the low temperature range.

Practically, real scale wastewater treatment plants rarely treat wastewater at its raw phase as this may cause problems not only in the quality of the inoculum (Uemura and Harada 2000, Elmitwalli et al 2000, Xing et al 2009) but also in the electro/mechanical equipment of the plant. As anaerobic treatment is frequently positioned in treatment plants after the physical separation units (e.g. clarifier, (Metcalf and Eddy 2002; Hammer and Hammer 2002)), the incoming wastewater stream to the biological process is usually primary settled. Treatment of a pre-treated effluent reduces the chance of hard-to-hydrolyze or un-hydrolyzed COD accumulation and subsequently minimizes the probability of biomass deterioration, low settleability and sludge washout as described on Chapter 2.2.5. On the contrary, low-strength substrate (e.g. primary settled) may limit mass transfer and good contact between the seed and substrate (Lettinga et al 2001). Analysis of the composition of the raw wastewater showed that the reactors were consistently fed with substrate rich in lipids. This agrees with what Raunkjaer et al (1993) observed in similar tests trying to identify the wastewater nature. Chapter 4.2 showed that there is a fraction of removed COD which cannot be methanized. This gap in COD:CH4 mass balance is inversely proportional to temperature. Stability of the pH and lack of VFA peaks suggest that this material remains unhydrolyzed. Hence, accumulation of unhydrolyzed material is inversely proportional to temperature. Further literature review commonly supports that lipids account for the higher fraction of the un-hydrolyzed material (Eastman and Ferguson 1981, Miron et al 2000). Possibly, this is due to the hydrophobicity of lipids that renders them able to be associated on the surface of the anaerobic sludge (Rinzema et al 1993).

Thus, the aim of this study is to investigate the feasibility of the anaerobic treatment of primary settled wastewater using the particular cold adapted inoculum after long acclimation with raw wastewater at low temperatures (4, 8 and 15°C), and evaluate its efficiency with regards to the COD removal and CH4 production. Additionally, a quantification and a comparison of the unhydrolyzed material in the seed (lipids, proteins and carbohydrates) amongst the operational temperatures would reveal which of the wastewater compounds is less likely to be hydrolyzed and can potentially accumulate at lower temperatures.

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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4.3.3. Materials and Methods

Reactors assembly; the same reactors' regime as described on Chapter 4.1.3 and 4.2.3 was used for the experimentation, using the same inoculum (as in Chapter 4.1.3 and 4.2.3), following the re-inoculation technique as described on Chapter 4.1.3. The reactors after re-inoculation and re-feed were set at 4, 8 and 15°C.

Wastewater; the reactors at this batch were fed with sterile primary settled wastewater of ≈220 mgCOD L^{-1}. The collection, pre-sterilization and re-feed of the substrate was carried out similarly to Chapter 4.1.3. Its composition was found to be 60% carbohydrates and 38% lipids; proteins were less than 2% – the protocol is on Chapter 4.1.3.

Analytical methods; total and soluble COD (tCOD, sCOD respectively) were estimated as described on Chapter 4.1.3. The k removal coefficients for COD were estimated from the initial and final COD values for a specific time period Δt using 1st order kinetics (*Eq.*12) as on Chapter 4.1.3. VFA (Volatile Fatty Acids) analysis in the mixed liquor in addition to the gas analysis for methane content in the head space were estimated following the methods described on Chapter 4.1.3.

Thorough mixing was only applied prior sampling for lipid, carbohydrates and proteins tests to accurately depict the properties of the biomass and to quantify the insoluble COD that may accumulate in the reactor. The quantification of these compounds, expressed in COD, would assist in the determination of the least easy to hydrolyze compound between lipids, carbohydrates and proteins. The estimation and conversion to COD method is described on Chapter 4.1.3.

4.3.4. Results

The treatment performance of the anaerobic reactor can be described by the VFAs (Figure 4.16.a) and the soluble COD (Figure 4.16.b) in the mixed liquor. Methane production (Figure 4.17) was evident supporting that methanogenesis at very low COD concentrations is feasible.
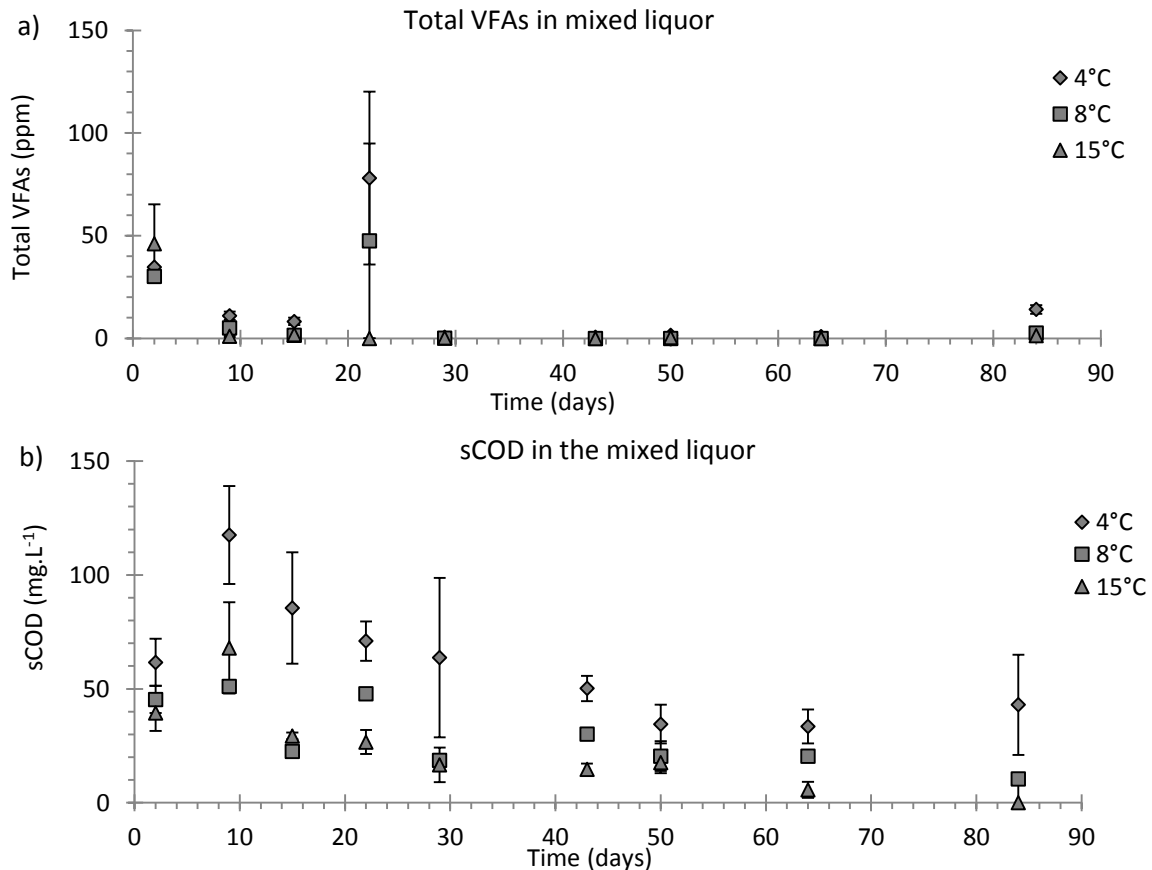
Figure 4.16 –a) VFAs status at all temperatures as a summary of acetate, propionate, butyrate, valerate and isovalerate; b) Soluble COD in the mixed liquor; at all temperature for primary settled wastewater; error bars indicate standard error , n=2 for 4 & 8 degrees and n=4 for 15°C

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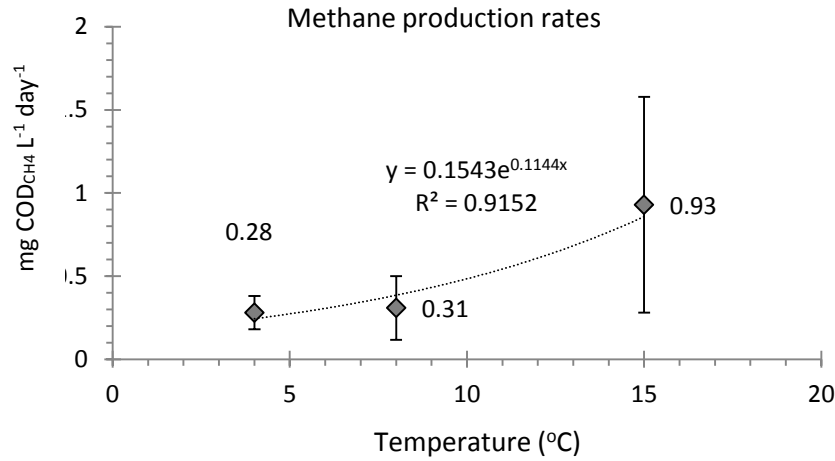
Figure 4.17 – Methane production rates at all temperature for primary settled wastewater; n=2 for 4 & 8 degrees and n=4 for 15°C; error bars express standard error.

Lipids, carbohydrates and proteins concentrations were quantified and expressed as COD (Figure 4.18.a, b, c). This clearly manifests which one accumulates in the bioreactors as in previous batches hydrolysis seemed to be the limiting step with an evident discrepancy between the COD removal and COD_{CH4} production rates.

c)
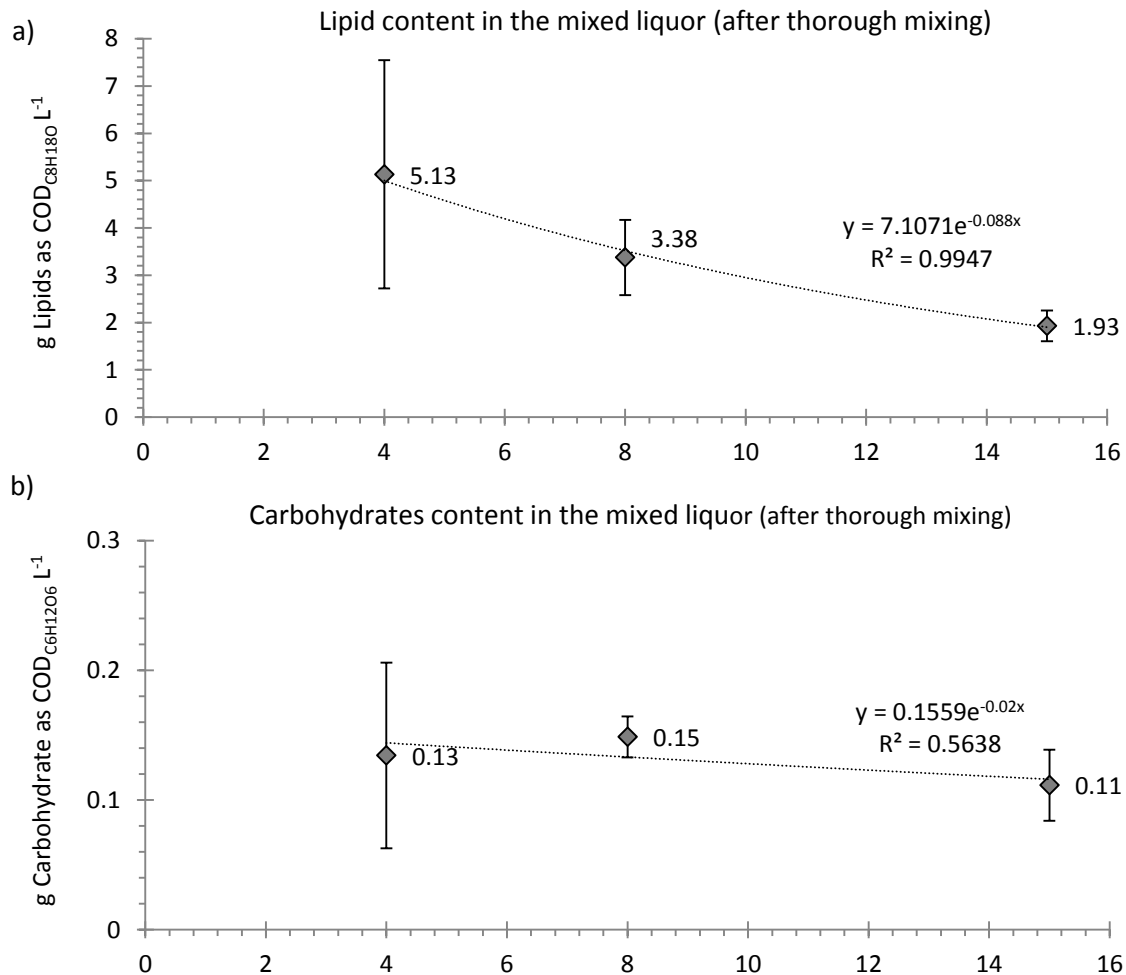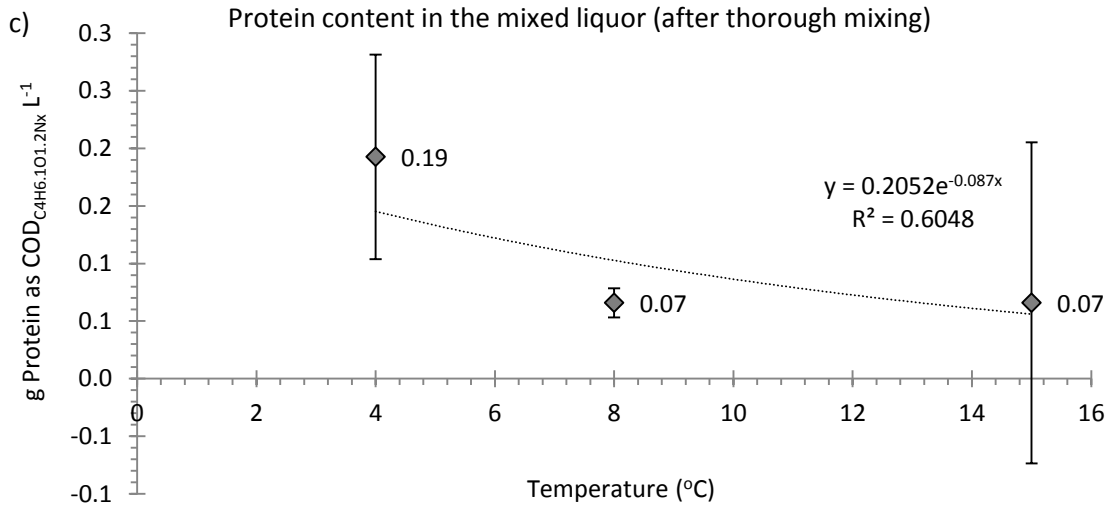
Figure 4.18 – Cumulative phenomena after thorough mixing for a) lipids; b) carbohydrates; c) proteins; in the mixed liquor at all 3 temperatures; error bars indicate standard error, n=2 for 4, 8°C, n=4 for 15°C

With regards the wastewater treatment, the substrate COD was reduced to levels able to meet the UWWTD (91/271/EEC) standards (Figure 4.19a). Additionally, the COD fate time series enabled the calculation of the 'k' COD removal coefficient (Figure 4.19b).
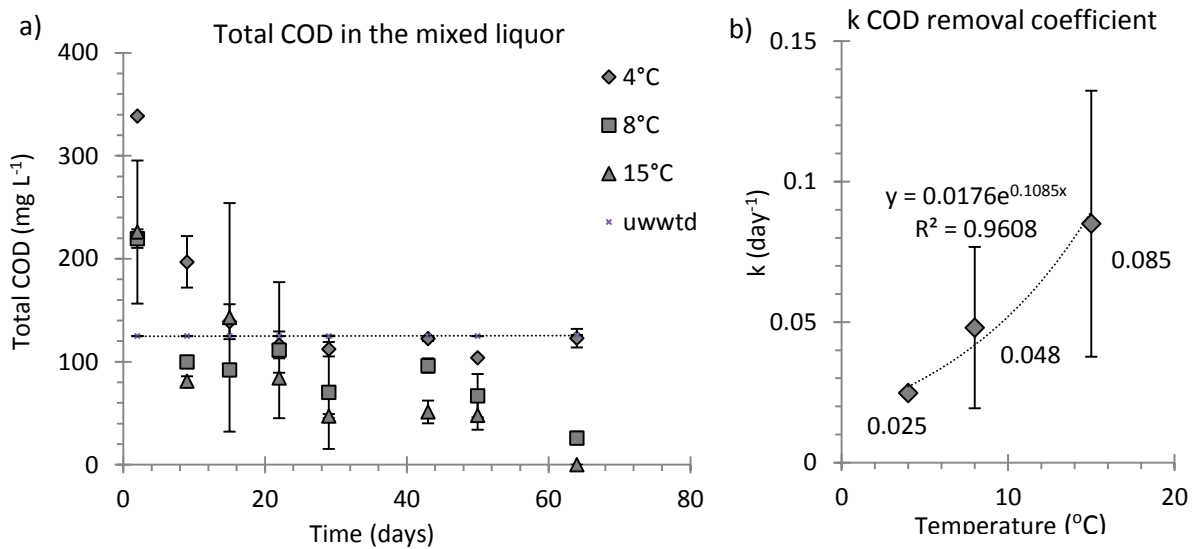
Figure 4.19 – a) COD fate in the reactor; k COD removal coefficient; for primary settled wastewater at 4, 8 and 15°C; n = 2 for 4 and 8, n = 4 at 15°C, error bars express standard error.

4.3.4. Discussion

Successful wastewater treatment in terms of COD was achieved at all operational temperatures (4, 8 and 15°C), meeting the COD standards (UWWTD 91/271/EEC) although the substrate was low in strength. This demonstrates high biomass acclimation into wastewater after four batches with a similar substrate. A scenario that requires further investigation is that acclimation with raw wastewater may have promoted adaptation and natural selection of only the resilient, highly active communities (Chapter 4.1, 4.2 – reduction of bacterial and archaeal abundances) to prosper. Further research on the microbial communities that develop on specific substrates at these temperatures would assist in the pre-selection of cells (or enzymes) to accelerate anaerobic wastewater degradation.

Methane production rates were lower than those achieved for raw sewage at the third batch (Chapter 4.2.); however this is to be expected due to the low COD content. The agreement between first order COD removal coefficient and methane production rates, as exponential trend lines (Figure 4.17, 4.19.b) indicates a stronger relationship

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l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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between COD removal and methane formation, event that was not evident for raw wastewater. This, as expected, asserts that the primary settled wastewater can be easier biomethanized than raw wastewater and is perhaps less prone to settling. In terms of wastewater engineering this is highly desirable, as less settling promotes use of smaller tanks, due to lower demand of biomass (cells) for COD degradation. Additionally it signifies, less chances of VSS accumulation, absence of unhydrolyzed COD and subsequently minimizes the probability of biomass deterioration, low settleability and sludge washout as shown in previous studies (Uemura and Harada 2000, Elmitwalli et al 2000, Xing et al 2009).

The temperature sensitivity of methanogenesis (as rates' exponential trendline coef.) was found to be similar to those previously observed for reactors fed with raw wastewater (Chapter 4.2). This suggests that substrate biodegradability may hold a secondary position in wastewater treatment at low temperatures, and further investigation needs to focus to the microbial communities that participate into the anaerobic treatment process (optimum growth, operational temperature, presence of enzymes, activity) rather than the substrate itself.

The current study better represents the normal scale WWTPs due to the use of primary settled wastewater as substrate, which is common to WWTP designation that usually includes a primary clarifier prior the biological reaction tank for numerous reasons (Metcalf and Eddy 2002). The use of primary settled WW at this study mainly ensures that treatment will be accelerated compared to what we have previously seen in the batches with raw wastewater. It also reveals that low temperature anaerobic treatment using the specific inoculum can be applied for effluent polishing purposes, event that was not evident in a similar study with a cold adapted mesophilic sludge implemented by Bowen et al (2014). Effluent polishing was also shown by Rebac et al 1999 who treated VFA at low temperature that were generated in the first compartment of a two-phased unit. The current study though has the advantage that achieved effluent polishing using only one compartment to fulfil all 3 anaerobic reactions.

No VFAs were detected after 30 days of operation, thus all easily-degradable compounds were hydrolyzed or the rate of methane production was equal or greater than the rate of hydrolysis and acidogenesis, with an inexplicable peak of acids on day 22 (Figure 4.16.a), a peak was simultaneously detected in the sCOD (Figure 4.16.b), which suggests that this was not a measurement error. The peak may signify the highest observed rate of acidogenesis-acetogenesis in the system that could have been promoted by rapid hydrolysis/fermentation (justified by the lowest observed pH prior acids´ peak-data not shown, (Hwang et al 2001)). The VFA peak decreased the next days, supporting the scenario that the optimum pH for hydrolysis/fermentation is not necessarily the same with the one required for methanogenesis, with the first usually lower than the second (Hwang et al 2001). From an engineering point of view, this scenario supports that a two chamber reactor configuration for anaerobic treatment of intermediates can be used to overcome the limited hydrolysis and guarantee different pH between hydrolysis and methanogenesis.

As described in the previous batches, the anaerobic reactors that were employed for this study so far were running with raw wastewater as substrate, and hydrolysis seemed to be the limiting step. As shown on Chapter 4.2 (Figure 4.12) there is a discrepancy between COD removed and COD methanized. This discrepancy leads to COD accumulation that contributes to accumulation of insoluble organic material within the reactor. The accumulation is highly dependent on temperature as showed on Chapter 4.1 as VSS/TSS (Figure 4.7.e). Similar evidences of VSS accumulation due to limited hydrolysis has been also reported by Uemura and Harada 2000, Elmitwalli et al 2000, Xing et al 2009.

Detailed analysis for carbohydrates, proteins and lipids in the mixed liquor shed light on the fate of hydrolysis of these specific compounds. As it can be clearly observed not all the rates of hydrolysis are equally affected by temperature.

The concentrations of carbohydrates were low (110-150 mg COD L^{-1}) at all temperature (Figure 4.18,b). Protein concentrations were also low, (70 mg COD L^{-1} approximately) at the warmer temperatures with only modest accumulation at 4ºC (Figure 4.18,c). The signal for lipids was considerably higher (2-5 grams COD L^{-1} for 15 and 4ºC) (Figure 4.18,a).

Clearly, lipids account for the higher fraction of the un-hydrolyzed material. Similar results were found by Eastman and Ferguson (1981) and Miron et al (2000). The conclusion of methanogenesis limitation through lipid degradation is also supported by the good agreement between the exponential coefficients of methane production

rates and lipid content at all temperatures (current study and Chapter 4.2). The exponential trends have a coefficient of 0.114 and 0.123 for primary settled and raw wastewater respectively, where the lipid content has a coefficient of -0.885, suggesting that lipids may notably account for the poor COD to methane conversion at low temperatures.

At this point it would be good to clarify why the effluent COD was much lower than the COD lipid signal. As described on Chapter 4.2 thorough mixing was only applied before sampling for carbohydrates, proteins and lipids measurements, where particles of biomass were abstracted for analysis. For liquid effluent analysis less mixing and avoidance of biomass particles ensured a consistent analysis for other tests (VFAs, COD, etc.), parameters that could have had high fluctuations if biomass was involved. Additionally the hydrophobicity of lipids helps them being adsorbed on the surface of the anaerobic sludge, hindering the transport of soluble substrates to the biomass, decreasing their degradation rate (Rinzema et al 1993), event that also renders them hardly able to keep in suspension for long after mixing at the low selected speed of 70 rpm.

Subsequently, as lipids evidently account for the largest part of hydrolysis limitation, the investigation needs to focus on the catalysts that cells are using to solubilize the specific compounds.

Lipases (glycerol ester hydrolases - biocatalysts) are enzymes secreted by bacteria to hydrolyze glycerol with long chain fatty acids. This suggests that either the production or activity of the relevant enzymes (lipases) is temperature sensitive or perhaps the biphasial nature and structure (1, 2, 3 acid chains) of the lipids change with temperature rendering them difficult to biodegrade (Neidleman 1987).

An investigation into the activity and presence of such enzymes at the corresponding temperatures might explain why lipids accumulate in the reactors at low temperatures.

4.3.5. Conclusions

Low strength primary settled wastewater can be successfully treated in 15 days, in terms of COD, according to UWWTD (91/271/EEC) standards at all temperature (4, 8 and 15°C). The first order removal coefficient for settled wastewater was higher than that previously observed for raw wastewater. The hydrolysis of lipids appears to be more temperature sensitive than the hydrolysis of proteins and carbohydrates. The failure of lipids to hydrolyze could be a key factor in the temperature sensitivity of methane production rates.

An investigation into the presence and activity of lipases in this context would be valuable.

I n v e s t i g a t i n g t h e t r u e l i m i t s o f a n a e r o b i c t r e a t m e n t o f w a s t e w a t e r a t
l o w t e m p e r a t u r e u s i n g a c o l d a d a p t e d i n o c u l u m
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Chapter 5: *What is the impact of temperature switch and WW-cell invasion to the structure and treatment performance of a microbial community developed in low temperature anaerobic reactors*
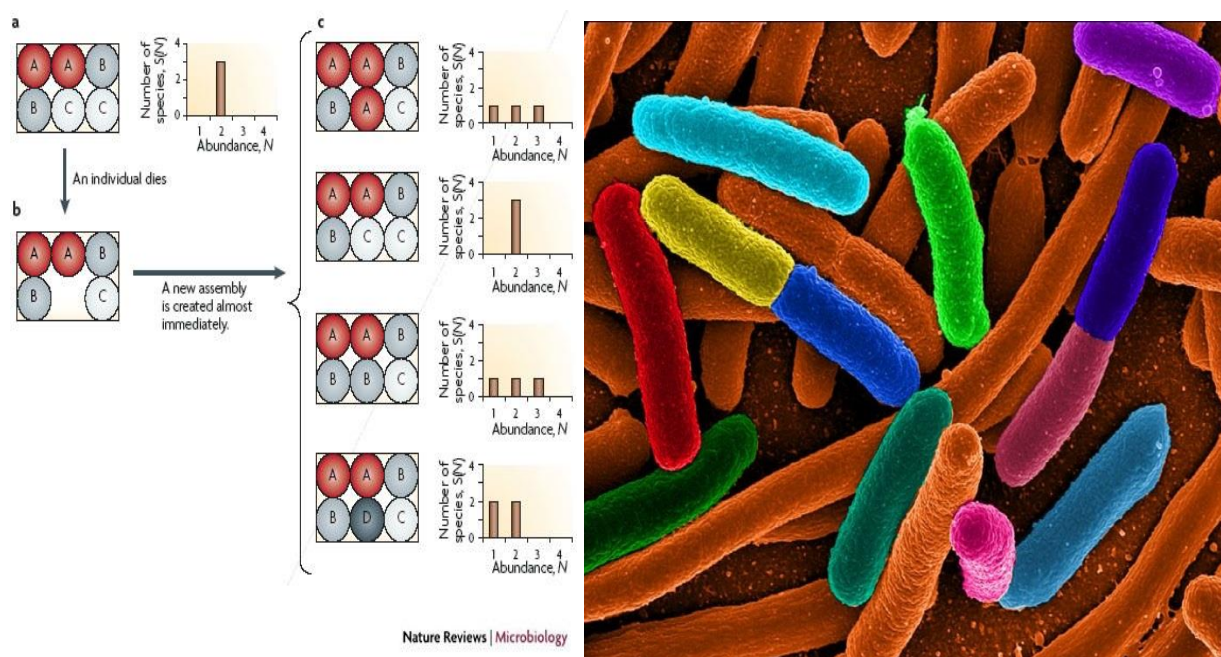
Figure 5.1 - Left: Microbial community dynamics based on neutral theory (Battin et al 2007); Right: visualization of colorized bacterial cells from a SEM image, representing different taxa in a saturated from microorganisms' community.

5.1. Psychrophilic microbial communities; who wins the competition for dominance for successful anaerobic wastewater treatment?

5.1.1. Abstract

Perhaps the most important parameter for successful anaerobic domestic wastewater (DWW) treatment at low temperature is the selection of the biomass, as seed. An ideal biomass would operate at low temperature, converting COD to methane, and exclude the competition from the indigenous from wastewater (WW) microorganisms. To achieve the above, I believe that the use of a cold-adapted seed is the key. The main aim of this study is to introduce a new psychrophilic/cold-adapted biomass for anaerobic treatment of low strength DWW, which can be used in digesters, situated at countries where temperature can rapidly fall at 4°C, leading to decline of hydrolysis and methanogenesis rates. 12 microcosms were inoculated with a cold-adapted seed, acclimatised at 15°C, fed with UV-sterile and non-sterile WW (350 mgCOD L^{-1}), and incubated at 4 and 15°C at a 1:3 seed:substrate ratio. The results showed that anaerobic treatment continues when temperature falls to 4°C, ensuring high COD to CH$_4$ conversion (>80%). The diversity (Bacteria and Archaea) of the inoculum did not significantly change regardless the treatment (sterile, non-sterile) after 96 days of operation. Additionally, wastewater communities seem to improve the hydrolytic activity. I conclude that the addition of autochthonous wastewater bacteria increases the treatment performance and that these bacteria co-exist with the cold-adapted hydrolytic cells. Lower temperatures had no significant effect on microbial communities. Hydrolysis and methanogenesis rates were adversely affected by temperature decrease (drop from 6.9 to 1.35 mgCOD.L^{-1}.day^{-1} and from 4.8 to 0.03 mgCOD.L^{-1}.day^{-1} for methanogenesis and hydrolysis respectively for the seeded and fed with sterile wastewater reactors). However, the cells from wastewater contributed to the hydrolysis/fermentation of the substrate, accelerating wastewater treatment even at 4°C (drop from 9.76 to 1.39 mgCOD.L^{-1}.day^{-1} and from 7.4 to 0.8 mgCOD.L^{-1}.day^{-1} for methanogenesis and hydrolysis respectively for the seeded and fed with non-sterile wastewater reactors). In general this Arctic inoculum may have a promising role in the anaerobic treatment of domestic WW in temperate climates where typical temperatures vary between 15 and 4°C.

5.1.2. Introduction

The need for carbon neutral or carbon positive domestic wastewater treatment needs to be re-considered due to the increase of CO$_2$ emissions from the rapid urban development (Environmental Agency 2009). The current high carbon footprint of wastewater treatment (aerobic treatment) can be reduced using traditional methanogenic systems. Currently almost all full-scale anaerobic treatment plants operate at temperatures >18°C (Lettinga et al 2001). Attaining this temperature threshold would require energy consumption for heat generation especially in countries with cold or temperate climates where the ambient temperature is often low (<15°C). Previous studies showed that lowering the operational temperature of a digester leads to decrease in the maximum substrate utilization rates, maximum specific growth rates and rates in biogas production (Alonzo et al 1969; Kettunen and Rintala, 1997) or failure. Previous studies though used mesophilic inocula, acclimatized to low temperature. In nature methanogenesis does occur at low temperatures (Metje and Frenzel 2007). I therefore sought to use of cold-adapted/psychrophilic biomass as an inoculum for a reactor treating domestic wastewater in the hope that it might improve treatment at low temperature.

The aim of this study was to see how anaerobic treatment is affected when a cold adapted biomass acclimatized at 15°C is exposed to 4°C. This would assist in depicting the behaviour of the digesters situated in cold climate countries, where minimum and maximum average temperature lies in between 4 and 15°C respectively.

A secondary objective was to understand the impact of the ´invasion´ of the autochthonous from wastewater microorganisms to the inoculum diversity.

Seeding can be understood using a neutral approach (Bell 2000, 2001), where decay or immigration at such systems is equally likely for all individuals (Sloan et al 2006). The treatment efficacy of the cold-adapted matrix might be compromised by autochthonous wastewater bacteria which are not cold adapted leading to treatment rates deceleration at low temperatures. This problem can be potentially tackled if the seed:substrate ratio is increased with no obvious limits to this approach. I therefore sought to determine if the cold-adapted biomass can outcompete the autochthonous wastewater bacteria and retain its dominance in the bio-reactor.

5.1.3. Materials and Methods

Microcosm Assembly; 12 microcosms were set up in sterile 160 ml glass Wheaton vials (Sigma Aldrich, UK), sealed with butyl rubber caps and flushed with 99.9% N_2. Eight of them were inoculated with 20 ml of seed material and 60 ml of raw domestic wastewater allowing a headspace volume of 80ml; four additional bottles were inoculated with just domestic wastewater (DWW) as un-seeded controls. All microcosms were prepared in duplicates and incubated at 15 and 4°C. Further replication was avoided to maintain the inoculum to the reactors (where the seed was originated from) relatively stable.

Microcosms were seeded with biomass from laboratory-scale batch reactors treating DWW which had been operating at 15°C for 400 days. These reactors were originally seeded with a mixture of putatively cold-adapted sediment from Lake Geneva and soils from Svalbard, in the high Arctic (Chapter 4.1, 4.2). The re-inoculation was carried out in parallel with the re-inoculation of the 1L reactors (after finalization of batch 3, Chapter 4.2). Equal volumetrical abstraction of the desired amount of biomass was extracted from the pellet generated after centrifugation (at 4000rpm for 20 minutes at 10°C) of the four 1L reactor replicates (15°C). The extracted seed was combined and used as inoculum for the current experiment.

Raw wastewater was collected from Tudhoe Treatment works at Spennymoor (UK) wastewater treatment plant. The substrate was pre-sterilized using an autoclave and UV irradiation, the effect of sterilization was evaluated by its impact on biodegradability (as the BOD to COD ratio). For UV irradiation a UV lamp (400mm UV lamp T-514, Semtec Flow Water Sterilization, China) was selected. The wastewater was passing through the chamber where the lamp was situated using a peristaltic pump (Watson and Marlow, Poole UK) for 5, 10, 15, 20 and 40mins. For thermal sterilization an autoclave was employed (Ensign Rodwell, UK, set), operating at various retention times and temperatures (109-121°C for 10-20mins). After sterilization BOD_5, COD, total cell counts were quantified as previously described (Chapter 4.1.3).

BOD_5, COD, soluble COD, VFA, SO_4^{-2}, NO_3^- hydrolysis and methanogenesis rates, CH_4, CO_2, microbiological community analysis were also evaluated as previously described (Chapter 4.1.3). The coefficient of variation was calculated for the sCOD, VFAs and methane production time series. Average variation coefficient is the quotient between standard deviation divided by the mean (n=2), averaged for all data points in the time series that correspond to a specific temperature/treatment setup.

The DNA samples were taken on days 3, 32 and 96. The molecular analysis was similar as described on Chapter 4.1.3. The statistical analysis on them was carried out on day 96 that corresponds to the initiation of the plateau phase in COD concentration and CH_4 production. The statistical test was a two-way test (pairwise) using Primer6 (Multivariate statistics for ecologists, Luton, UK) (as described on Chapter 4.2.3) selecting temperature and treatment as factors. The statistical significance of the results were also evaluated via ANOSIM (ANalysis Of SIMilarity) and expressed as statistical significance (P-value) and possible relationship level % between variables (R-value). As previously stated (Chapter 4.2.3), P-value is the number that defines whether a result is statistically significant. The confidence level was chosen as 90% which corresponds to a P-value = 0.1. R-value (Global R) examines how strong the difference between the compared samples is by measureming the separation between groups. R can be from 1 to 0, R rarely is negative (0: indistinguishable; 1: all similarities amongst groups are less than any similarity between groups; -1: usually coupled with statistically insignificant comparisons by P value).

5.1.4. Results

Wastewater was low in organic matter, COD of 353mg L^{-1}; providing 0.33 mmols of CH_4. Analysis for anions (SO_4^{-2}, NO_3^-) suggested minimum competition for methanogens from cells using other electron acceptors (CH_4 loss <0.0012 mmols). BOD_5 estimated as 60% of COD providing a biodegradability ratio BOD:COD ~0.4. The bacterial population was quantified as $3.0E8 \pm 1.6 \times 10^8$ & $1.2E8 \pm 5.8 \times 10^7$ CFU (colony forming unit)/ml on NA and R2A respectively.

The effect of UV and thermal sterilization to biodegradability is shown on Figure 5.2.a, b. For UV irradiation, X axis represents the retention time as the power was constant (11 Watts). For autoclaving the variable is energy, estimated by the power that was introduced to the liquid, to reach the desired temperature, starting from 4°C

multiplied by the retention time. Figure 5.3.a, b shows the UV sterilization efficiency to the wastewater microbial communities. The performance of the thermal sterilization of wastewater is also on Figure 5.3.c, d.
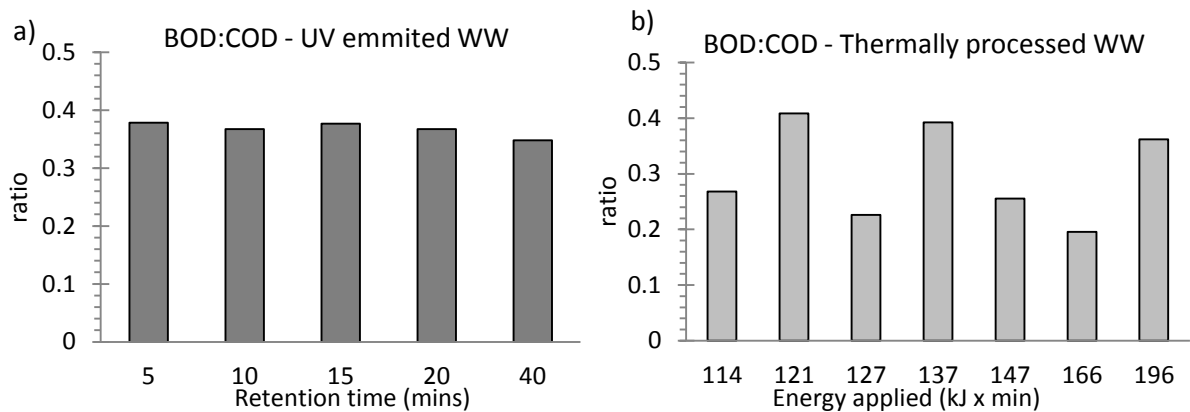
Figure 5.2 - (a) Biodegradability as BOD:COD ratio after sterilization via UV irradiation at different retention times; (b) Biodegradability as BOD:COD ratio after autoclaving.
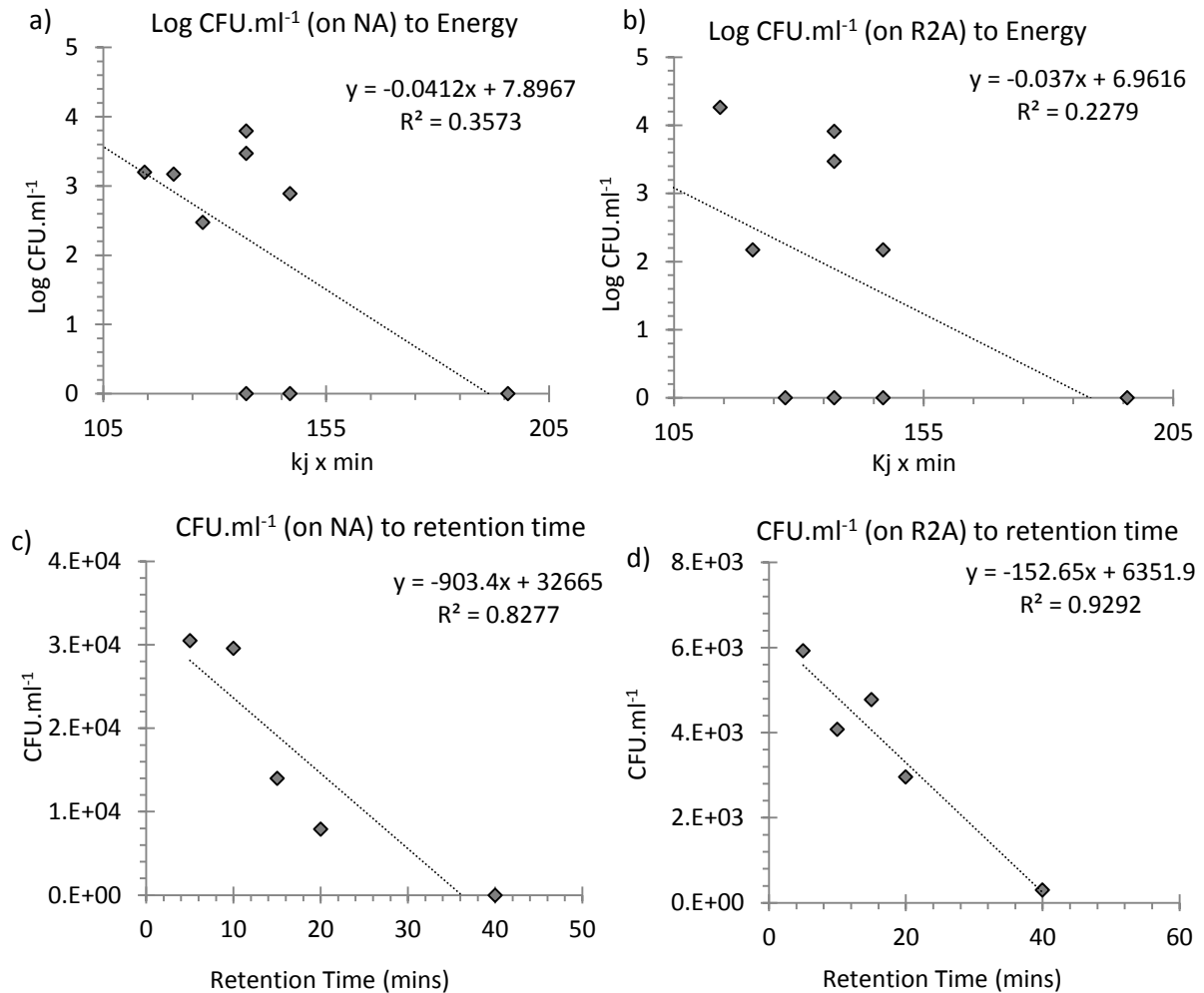
Figure 5.3 – Bacterial enumeration after sterilization of wastewater using UV irradiation – enumeration on (a) nutrient agar (NA), (b) R2A agar; Bacterial enumeration after sterilization of wastewater via autoclaving, evaluated on (c) nutrient agar (NA), (d) R2A agar; thermally processed samples were Log- transformed for better visualization.

The anaerobic wastewater treatment performance is described following the three fundamental steps of AD, hydrolysis-fermentation, acidogenesis-acetogenesis and methanogenesis (Malina and Pohland 1992; van Haandel and Lettinga 1994 etc.). The representative parameters are shown on Figure 5.4.a, b, c.
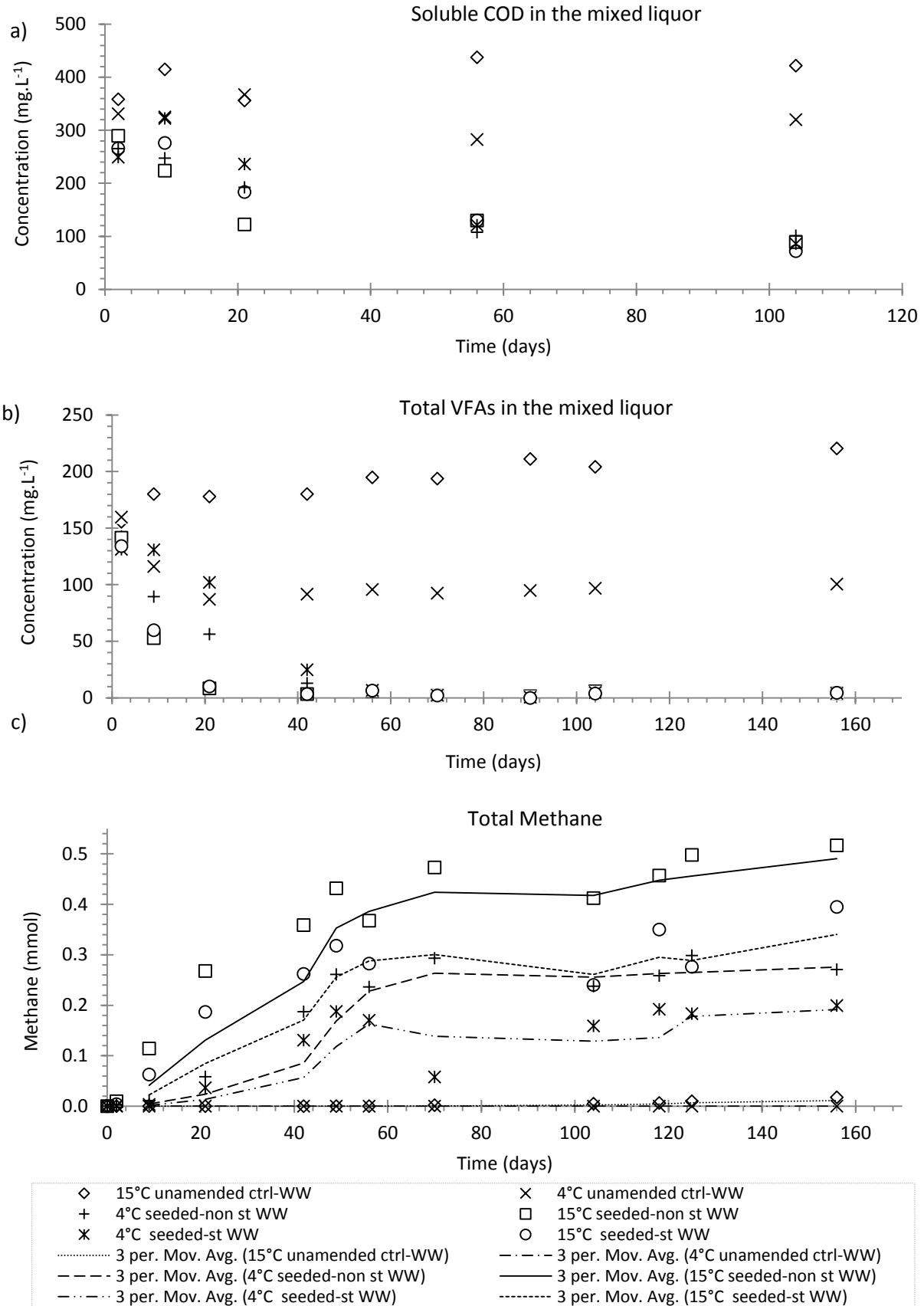
Figure 5.4. – Average (a) sCOD present in the microcosms at all conditions; (b) total volatile fatty acids as summary of acetate, propionate, butyrate, valerate, isovalerate and isobutyrate; (c) methane production at the same conditions; moving average trend was used for illustration purposes; n = 2 for all samples; the average variation coefficients for '15°Cunamended control-

WW', '4°Cunamended control-WW', '4°C seeded-non st WW', '15°C seeded-non st WW', '4°C seeded-st WW', '15°C seeded-st WW' are, plot a: 0.04, 0.36, 0.17, 0.09, 0.02, 0.09 respectively; plot b: 0.07, 1.14, 0.66, 0.19, 0.24, 0.15 respectively; plot c: 0.54, 0.0, 0.29, 0.08, 0.14, 0.04 respectively

Combining the data above, using *Eq.* 12, 13, 14, the hydrolysis and methanogenesis rates were determined. (Figure 5.5.).
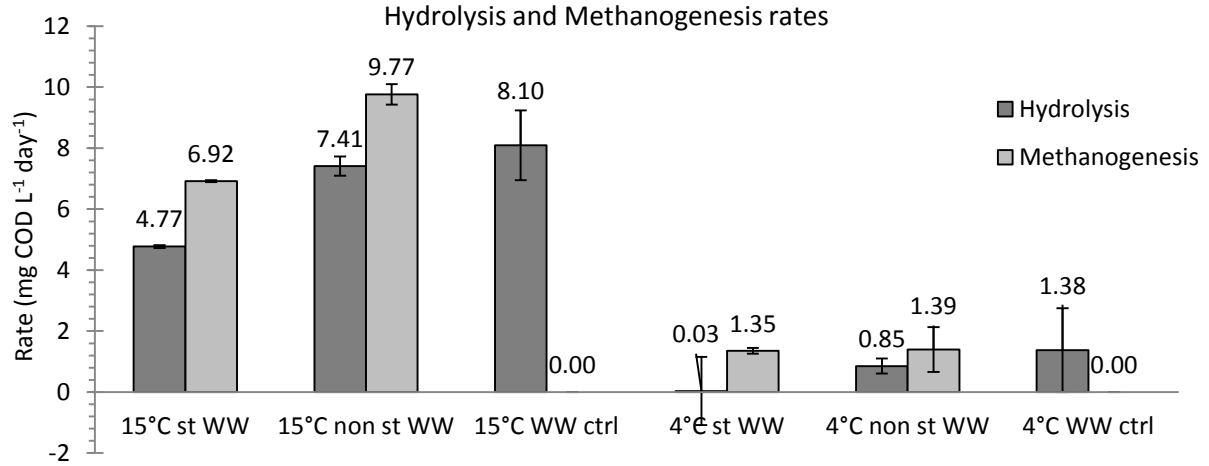
Figure 5.5. - Hydrolysis and methanogenesis rate for wastewater as substrate at all treatments and temperature conditions; st WW stands for seeded and fed with sterile wastewater, non st WW stands for seeded fed with non-sterile wastewater, WW ctrl stands for unseeded wastewater control; error bars represent the standard error, n = 2 for all samples.

The bacterial DGGE (Figure 5.6) and the statistical analysis of the bacterial community structure for different treatments and a temperature is shown on (Table 5.1). The Table 5.1 represents the ANOSIM results of a two-way (pairwise) test for temperature and treatment (sterile or non) as factors. It includes the statistical significance (P-value %), the possible level (R-value). As described on Chapter 5.1.3, 4.2.3, P-value is the number that defines whether a result is statistically significant (confidence level chosen as 90% which corresponds to a P-value = 0.1), while R-value (Global R) examines how strong the difference between the compared samples is by measureing the separation between groups; R can be from -1to +1; commonly from 0 to 1 (more details on Chapter 5.1.3).

Similarly, the DGGE image for Archaea (Figure 5.7), in addition with the statistical analysis (Table 5.2).

ID:Temp - Charact.
72.3: 15 - sterile
71.3:15 - sterile
70.3: 4 - sterile
69.3: 4 - sterile
68.3: 4 - non sterile
67.3: 4 - non sterile
66.3: 15 - non sterile
65.3: 15 - non sterile
64.3: 4 - WW control
63.3: 4 - WW control
62.3: 15 - WW control
61.3: 15 - WW control
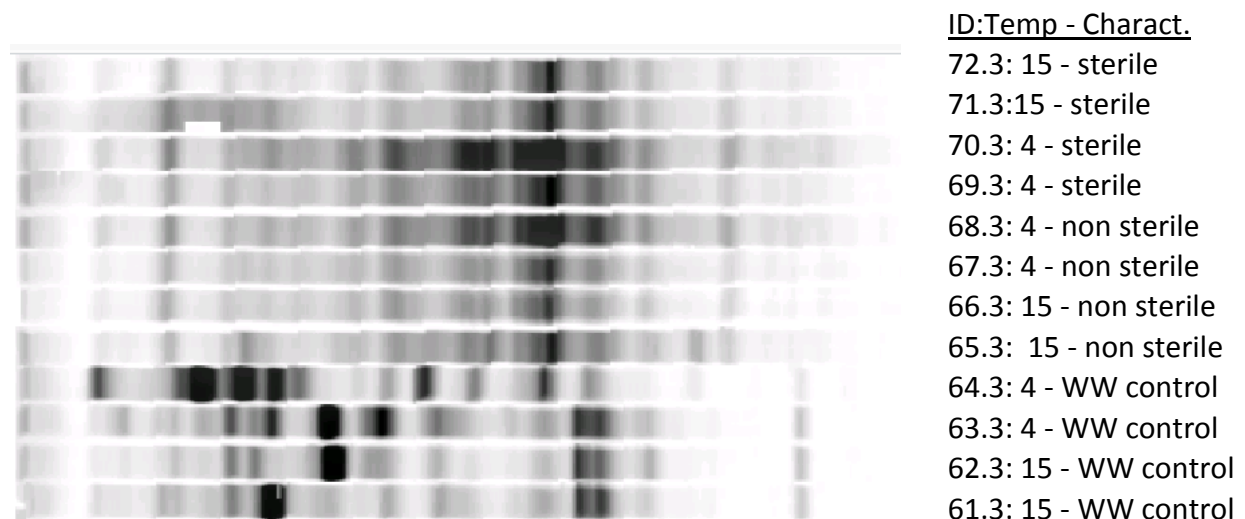
Figure 5.6 - Bacterial DGGE gel image after 96 operational days for all temperatures (4, 15°C) and treatments (sterile wastewater fed, non-sterile wastewater fed and WW controls (un-seeded); image taken via Bionumerics software; the labels correspond to the code name of the microcosm reactor (e.g. 72.3 or 71.3.), the operational temperature 4 or 15°C and the treatment type (sterile or non or un-seeded wastewater control)
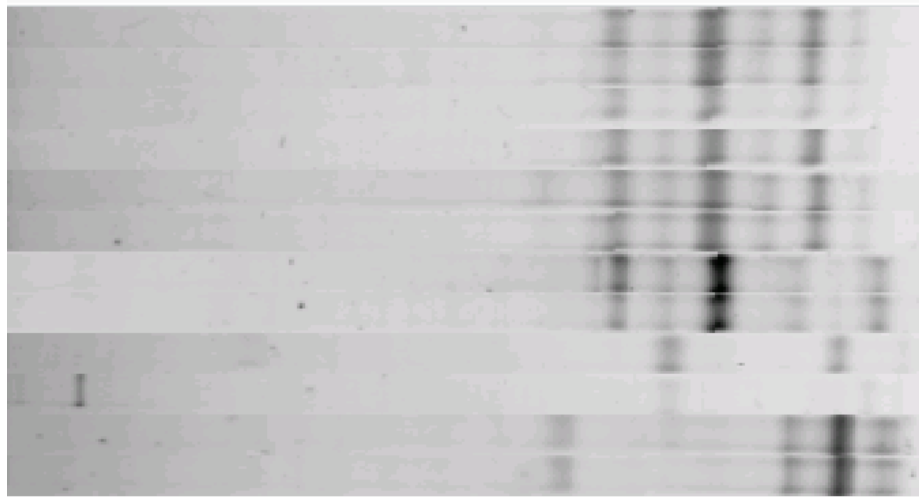
ID: Temp - Charact.
70.3: 4 - sterile
69.3: 4 - sterile
68.3: 4 - non sterile
67.3: 4 - non sterile
66.3: 15 - non sterile
65.3: 15 - non sterile
64.3: 15 - WW control
63.3: 15 - WW control
62.3: 4 - WW control
61.3: 4 - WW control
71.3: 15 - sterile
72.3:15 - sterile

Figure 5.7 - Archaeal DGGE gel image after 96 operational days for all temperatures (4, 15ºC) and treatments (sterile wastewater fed, non-sterile wastewater fed and WW controls (un-seeded); image taken via Bionumerics software; the labels correspond to the code name of the microcosm reactor (e.g. 72.3 or 71.3.), the operational temperature 4 or 15ºC and the treatment type (sterile or non or un-seeded wastewater control)

Table 5.1: ANOSIM table from the Bacterial Pairwise Test between treatments

Groups observed	R value, Possible level	P value, Statistical significance (%)
Non sterile - sterile	0.25	33.3
Non sterile – WW control	1	11.1
Sterile – WW control	1	11.1

*sterile, non-sterile and WW correspond to the treatments that were selected to each microcosm

Table 5.2: ANOSIM table from the Archaeal Pairwise Test between treatments

Groups observed	R value, possible level	P value, Statistical significance (%)
Non sterile - sterile	0.25	20
Non sterile – WW control	0.667	2.9
Sterile – WW control	0.667	2.9

*sterile, non-sterile and WW correspond to the treatments that were selected to each microcosm

For better interpretation the microbial community structure is presented in dendrogram and MDS plots for both bacteria and archaea (Figure 5.8 a, c and b, d for bacteria and archaea respectively).

C)

d)
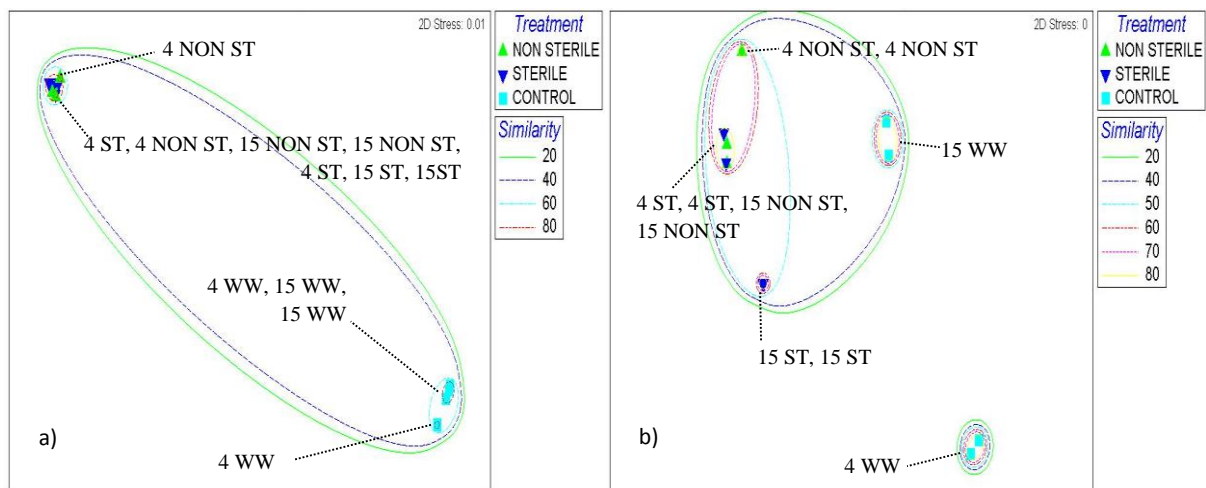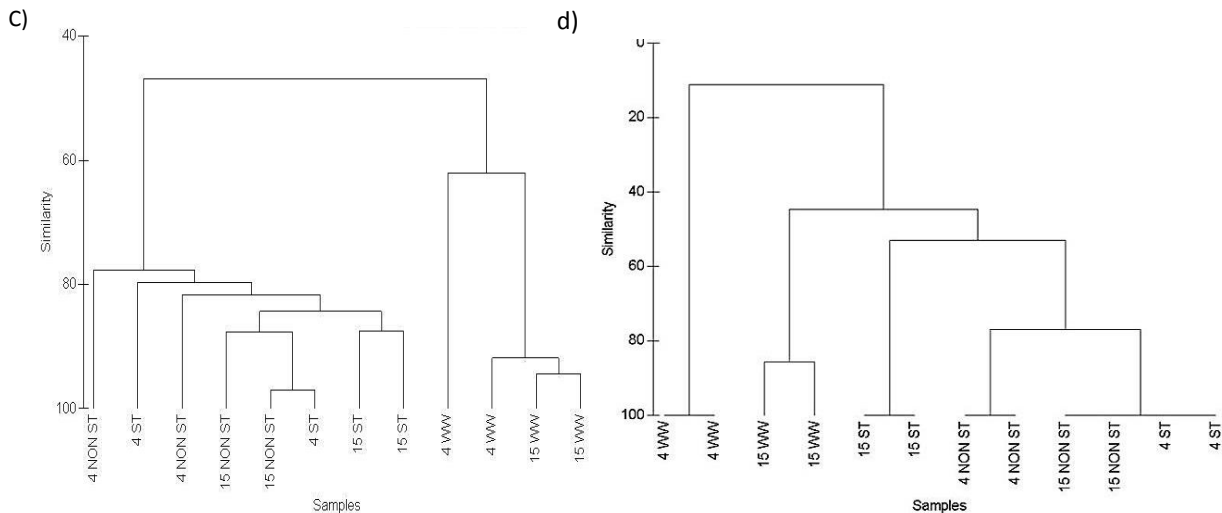
Figure 5.8 – Community structures on day 96, (a) Bacterial MDS; data were pre-transformed according to band to presence (as 1) –absence (as 0); (b) Similarly for archaea MDS. (c) Cluster analysis for bacteria presented as dendrogram. (d) Similarly for archaea; 4 and 15 correspond to the operational temperatures; WW: wastewater un seeded controls; ST and NON ST correspond to the treatment type of the inoculated reactors, sterile and non-sterile respectively

5.1.5. Discussion

COD (chemical oxygen demand) is a key parameter in wastewater treatment (Ritmann and McCarty 2001; Henze et al 2008) as it gives an insight on how efficient the organic removal is. The sCOD was successfully degraded at all seeded microcosms, meeting the UWWTD (91/271/EEC) requirements for COD (<125 mgL^{-1}) (Figure 5.4.a). This manifests that the inoculum is efficient in terms of COD treatment, and low strength wastewater of 350 mgCOD L^{-1} can be hydrolysed, fermented and converted to methane even at its raw phase (Figure 5.4.a, b, c). No sCOD depletion occurred in the 'wastewater only' controls, presumably due to the absence or small numbers of active methanogens (no bands or extremely faded before day 96), though the accumulation of organic intermediates (sCOD, VFAs, (Figure 5.4.b)) implies that hydrolysis took place.

The rates were poor at lower temperature as it required more time for the substrate to be hydrolysed and bio-methanized. Similar results were obtained previously on Chapter 4.1 and 4.2 and are common in literature (Krzeminski et al 2012, Lettinga et al 2001 etc.). Hydrolysis was the rate limiting step, in all seeded treatments (sterile or non) and temperatures, similarly to previous studies (Elmitwalli et al 2003 etc.), or as shown on Chapter 4.2. This suggests that a ratio between hydrolysis and methanogenesis equal to 1:1 is hard to be achieved. As methanogenesis occurs faster the research endeavours need to focus on ways to promote engineered hydrolysis (mechanically, chemically or biologically engineered, e.g. pre-mixing, fenton process, enzymatic hydrolysis respectively), otherwise methanogens are 'convicted' to survive in conditions of starvation that may risk their presence in the community matrix in the long run.

COD is an indicator of the amount of energy contained in wastewater that might be recovered as methane (Heidrich et al 2011). At both 4 and 15°C the time that is required for the substrate to reach the methane plateau phase is the same Figure 5.4.c. This perhaps happens due to the effect of temperature to the substrate itself, the first limits the second and this reveals that there is a relationship between the not easily available substrates and temperature. Nonetheless, the COD:CH$_4$ conversion was lower at 4°C, reached approximately the 80% and 60% (theoretical 100% equals to 0.35 LCH$_4$/gCOD$_{removed}$) for non-sterile and sterile substrate respectively (Figure 5.4.c). At 15°C the conversion reached and exceeded (non sterile) the 100% in both treatments. The total amount expected was 0.33mmol/head space. The surplus of methane in the microcosms with non-sterile WW at 15°C originates presumably from the organic material that could have been present within the seed (previously un-hydrolysed material). 80% COD:CH$_4$ conversion is really close to the theoretical one (100% or 0.35 LCH$_4$/g COD$_{removed}$). Thus, the introduction of non-sterile wastewater would decelerate the accumulation of the insoluble COD and subsequently reduce the likelihood of biomass deterioration as reported by Uemura and Harada 2000, Elmitwalli et al 2000, Xing et al 2009. The further treatment capacity of the non-sterile wastewater fed reactors would ensure

sufficient treatment at low temperatures in any likelihood of slow transition from 4 to 15°C, where the conversion is ~100%.

On Chapter 4.3 the results showed that there is excess COD the lower the temperature, which was also detectable on Chapter 4.1 expressed as VSS:TSS ratio. COD tests in the soil (seed) at the end of the experiment showed that the excess amount of methane is close to the average COD difference between non-sterile and sterile seed. In detail, the COD_{seed} difference between sterile and non-sterile at 4 and 15°C was 98 and 82 mg COD.L^{-1} respectively. This corresponds to an estimated difference between sterile and non-sterile COD_{CH4} at 4 and 15°C of 65 and 105 mgCOD$_{CH4}$/L respectively. High standard errors of ±1937 and ±1284 (test in triplicates) for the COD_{seed} at 4 and 15°C due to the nature of the sample do not confidently support this conclusion. In any case the methane from the non-sterile wastewater fed reactors was consistently higher at both temperatures whereas the COD in the microcosm reactor was higher than expected due to the presence of an insoluble fraction (also shown on Chapter 4.3). Hence, the accumulated organic fraction (mainly lipids), combined with the wastewater originated´ bacterial organisms that operated in the reactor explain the increased methane volumes at non-sterile conditions. The fact that only non-sterile treatments provided with higher than expected amounts of methane suggests that there should be some hydrolytic activity from the cells habiting the wastewater that promotes anaerobic wastewater treatment. It is not clear why lipids accumulate, a plausible scenario could be due to changes in their structure at low temperatures (Neidleman 1987) or reduced enzymatic expression or activity.

In terms of engineering, 60 days of retention time is a long period as an HRT in a conventional WWTP; thus optimizations or further acclimation and growth need to be considered, otherwise it can only be applicable in limited treatment plants where volume is of minor importance. The time that was required for treatment was similar with the one needed on Chapter 4.2. This was expected, as the experiments were using the same seed (type and acclimation to wastewater time), had the same seed:substrate ratio and were exposed to similar temperature conditions (4, 15°C).

The notable acceleration in VFA and methane production observed in the microcosms inoculated with non-sterilized wastewater is presumably due to the presence of beneficial autochthonous bacteria that promote hydrolysis. This subsequently led to higher methane formation rates. This supposition is supported by the amount of VFAs that was generated to the un-seeded controls (especially at 15°C), where the substrate was hydrolysed and the intermediates accumulated as there were no methanogens to bio-methanize them.

Thus, it seems that microorganisms from both the seed and the wastewater are complementary. In particular, the modest hydrolytic and excellent methanogenic capacity of the seed was matched by excellent hydrolytic (I suspect lipolytic - a lipase assay to the seed and wastewater would shed light into the hypothesis, Chapter 6) and modest methanogenic capacity of wastewater. Clearly the best treatment will be obtained by using "live" wastewater as in practice higher numbers of bacteria are introduced in the system, capable to contribute in wastewater hydrolysis at low temperature.

There was no detectable difference in the bacterial diversity amongst the seeded microcosms, irrespective of the wastewater used (sterile or non-sterile) at day 3. This suggests though, that either the DNA of inactivated microorganisms from UV irradiation might still be present the first days of the experiment at these temperatures or that the wastewater DNA was undetectable against the background of the seed.

Differences between the wastewater only controls and the Arctic seed microcosms were apparent by day 32. The differentiation indicates that the bacterial communities of the Arctic seed predominated whereas the microbial community structure was not evidently affected from the wastewater originated cells. The phenomenon was stronger at day 96 (Figure 5.8.a, c). The clear cluster formation between the microcosms seeded with adapted biomass and those with wastewater alone indicate that the indigenous WW bacteria could not become dominant, at least over a 3 month period.

Temperature decrease could not affect the communities qualitatively as the cells at 4°C cluster with those at 15°C (similarity ≈80%) regardless the treatment. Statistical analysis (Table 5.1) showed no significant difference amongst them (R = 0.25, P = 0.33). Wastewater controls at both 4 and 15°C were nearly significantly different to the microcosms inoculated with the seed at both temperatures, whether sterile or not (R = 1, P = 0.11); Temperature had little effect on bacterial community composition in microcosms containing only wastewater (>90% similarity

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
P a g e | 70

- excluding the '15WWctrl (1)' (un-seeded wastewater fed control at 15°C) due to O$_2$ ingress after malfunction of a syringe during gas sampling). This suggests that the bacterial communities in the seed are resilient to invasion from the microorganisms in the wastewater at "high" and low temperatures.

The gene sequences found in the seeded microcosms were similar to those previously retrieved from cold environments, and dissimilar to any of the predominant sequence present in the wastewater.

The archaeal groups that were found at the original Arctic seed were retained in the microcosms fed with, either sterile or non-sterile wastewater (Figure 5.7). The sequences were similar to those previously retrieved from cold environments and belong to the groups of *Methanosaetaceae, Methanomicrobiales* and *Methanosarcinaceae*. This explains why COD to methane conversion worked well (Figure 5.4.c). The archaeal communities in WW controls, that were detected at the end of the experiment were significantly different (Table 5.2) to those from the Arctic seed at all temperature and treatments (R = 0.667, P = 0.029). Temperature decrease affected the communities in WW controls (similarity <90%), as archaea (putative methanogens) started appearing at 15°C but in low numbers, as only traces of methane appeared. The archaeal communities of the seed were not significantly affected by temperature (R = 0.25, P = 0.2 between treatments at both temperatures, Table 5.2).

It is concluded that the cold adapted inoculum performed adequately after the rapid temperature decrease. The biomass retains its original diversity structure, and operates in synergy with the indigenous wastewater microorganisms. Pre-sterilization of the substrate to "protect" the seed from the putative wastewater mesophiles is not required.

I conclude that a well-established community might be difficult to disturb. Interpreting the phenomenon based on neutral dynamics (Bell 2000, 2001) it can be said that the established community in the seed did not allow the wastewater communities to predominate in the bio-reactor community matrix.

This conclusion though was based on a seed:substrate ratio of 1:3. It would be interesting to investigate if changes in the ratio, promoting the wastewater originated cells, would retain the microbial community unchanged. In real scale quantitative and sometimes qualitative retention of the biomass in desirable levels can be achieved using anaerobic membrane reactors.

Finally in terms of sterilization both methods were successful. Autoclaving can potentially cause inconsistency to the substrate altering its nature expressed as BOD:COD ratio. This is perhaps due to excess heat that causes caramelization of disaccharides and monosaccharides (Butler 1913), main biodegradable carbon sources, which can be easily utilized from the biomass. However, even if a thermal approach operated it would have been impossible to apply thermal sterilization in a normal scale wastewater treatment plant. Thus, UV irradiation is the recommended method for wastewater sterilization.

5.1.6. Conclusions

Conversion of COD to methane reached the 80% and 100% at 4°C at 15°C respectively. This suggests that the inoculum is able to treat wastewater in terms of COD, based on UWWTD (91/271/EEC), at low temperature.

Decrease of temperature from 15°C to 4°C decelerated the reaction but no failure occurred. Both hydrolysis and methanogenesis were reduced.

Sterilization as pre-treatment step is not required. It might only be useful when the communities in the seed are less than the autochthonous organisms and one wishes to confer an advantage to the seed.

For the microcosms with a cold adapted inoculum, the bacterial and archaeal diversity remained apparently unchanged by the microbial communities from the wastewater even though the presence of unsterile wastewater assisted in the hydrolysis organic material.

Archaeal communities were negligible in wastewater, as only faded bands were detected; thus, the higher rates of methanogenesis from non-sterile microcosms can be attributed to the improved hydrolysis rates.

Examining sterilization techniques it was found that UV irradiation is preferable to thermal sterilization, as it has the less effect on biodegradability.

It is believed that this particular inoculum can make the assist in anaerobic wastewater treatment at low temperatures and may be robust to fluctuations in temperature especially when operated in conjunction with the biomass originated in the wastewater.

However it would be interesting to examine whether the inoculum would function at lower seed:substrate ratios and test the effect of temperature raising from 4-15°C .

5.2. Low temperature for psychrophilic AD - seed is the key

5.2.1. Abstract

Microcosms were inoculated with acclimatized seed from long term 4°C and 15°C batch 'mother' reactors containing un-hydrolyzed material from previous batches and fed with UV-sterilized and unsterilized wastewater (WW). Wastewater contained inhibitory compounds and decelerated methanogenesis in comparison to un-amended samples (just seed) especially when operated at 4°C. Specific methanogenic activity at 15°C was higher for inocula acclimatized at 4°C than inocula acclimatized at 15°C when both operated at that temperature. Molecular analysis showed that bacterial communities from 4°C retain their structure better when temperature fluctuates or when challenged by indigenous organisms from WW; no significant change occurred to the archaeal community at both temperatures (4°C, 15°C. Thus, digesters operating at low temperature would be preferably inoculated with biomass acclimatized to a lower temperature.

5.2.2. Introduction

As previously mentioned, numerous studies showed that lowering the operational temperature of a digester leads either to a decrease of the maximum substrate utilization rates, maximum specific growth rates and rates in biogas production or to a failure (Alonzo et al 1969; Kettunen and Rintala, 1997). So far we proved that methanogenesis can occur at low temperatures (4°C, Chapter 4) from communities that are acclimated both at 4 and 15°C (Chapter 4, 5.1). But what if the temperature rises?

The aim of this study was to investigate how anaerobic treatment is affected when a cold adapted biomass acclimatized at 15°C is exposed to 4°C and vice versa using a lower seed:substrate ratio (1:7) compared to the previously selected one (1:3). Similarly to Chapter 5.1, this would assist in depicting the behaviour of the digesters situated in cold climate countries and further understand the impact of the ´invasion´ of the wastewater-originated microorganisms to the inoculum diversity.

For an expanded interpretation of the impact of the temperature/treatment conditions to the treatment/methanogenic performance of the seed, the results were expressed in cell specific activity. This introduces a more detailed parameter ($CH_4.cell^{-1}.day^{-1}$) that depicts the effect of the conditions to the methanogenic cells. The methanogenic population was selected instead of total cells due to the importance of the former in anaerobic treatment. The presence of active methanogens supports that all previous steps including bacterial populations were successfully carried out. An expression per total cells wouldn't necessarily be comprehensive (with the means we used) as it is not certain which cell is active and which is not.

The cell specific methanogenic activity can more accurately describe the treatment and methanogenic capacity of the biomass as it excludes the presence of non-bacterial organic compounds of the seed. This can potentially lead to inoculum underestimation when expressing the treatment efficiency per gram of VSS.

5.2.3. Materials and Methods

Microcosm Assembly; 28 microcosms were set up in sterile 160 ml glass Wheaton vials (Sigma Aldrich, UK), sealed with butyl rubber caps and flushed with 99.9% N_2. 16 microcosms were inoculated with 10ml of seed material and 70ml of raw domestic wastewater allowing a headspace volume of 80ml (seed:substrate 1:7 or seed:total vol.: 1:8); four additional microcosms were inoculated with just DWW, as un-seeded controls. 8 were inoculated with just seed and de-ionized water as 2nd un-amended control (details on Figure 5.9). All microcosms were prepared in duplicates and incubated at 15 and 4°C for 70 days.

Microcosms were inoculated with with biomass from laboratory-scale batch reactors treating DWW acclimatized to domestic wastewater at 4 and 15°C for 2.5 years (from Chapter 4). These reactors were originally seeded with

a mixture of putatively cold-adapted sediment from Lake Geneva and soils from Svalbard, in the high Arctic (Chapter 4.1.3). The re-inoculation was carried out according to the process described on Chapters 4.1.3 and 5.1.3.

Wastewater was collected from Tudhoe Treatment wastewater treatment plant (Spennymoor, UK). The substrate was taken after the primary clarifier. Its composition was found similar to the one at Chapter 4.3, the analysis was carried out similarly to the one described on Chapter 4.1.3. Presence of chloride Cl⁻ in the wastewater was determined via Ion Chromatography as described on Chapter 4.2.3 for other anions (e.g. SO_4^{-2}, NO_3^-).

Where necessary, UV-sterilization was undertaken as previously described (Chapter 5.1.3)

CH_4, Microbiological community analysis was evaluated as described on previously (Chapter 4.2.3, 5.1.3). The average variation coefficient was calculated for the methane production time series (average variation coefficient is the quotient between standard deviation divided by the mean (n=2), averaged for all data points in the time series that correspond to a specific temperature/treatment setup.).
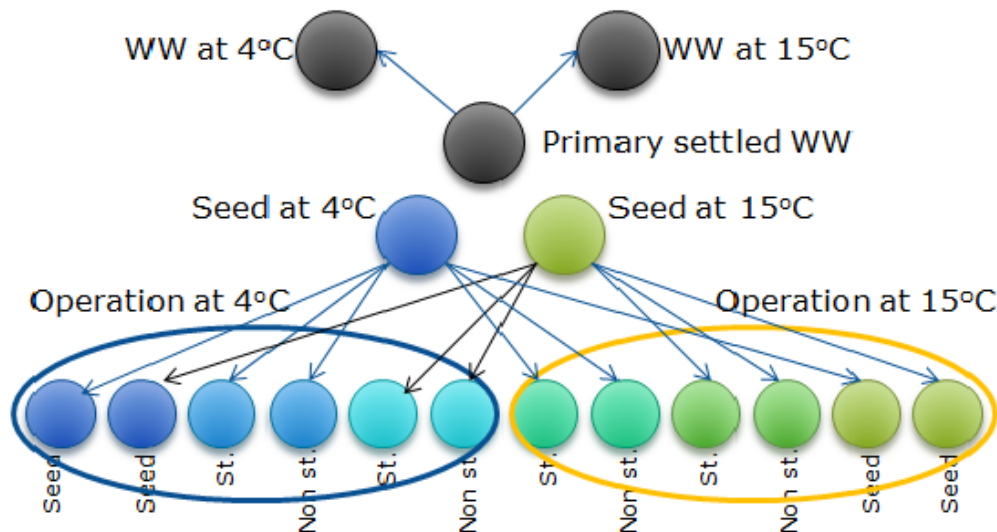
Figure 5.9 - Graphic representation of experimental setup; inoculum from acclimatized cold adapted reactors (seed at 4°C and seed at 15°C) was equally distributed to the microcosm reactors in the circles (4°C: blue circle; 15°C: yellow circle). Additionally controls with unsterile wastewater were prepared and set at 4 and 15°C (black spots); Abbreviations: Seed: just biomass control; St.: Seeded with sterile wastewater; Non St.: Seed with non-sterile wastewater; WW: Wastewater un-amended control; all microcosms were examined in duplicates

Quantitative PCR (qPCR) was carried out following the same protocol as Chapter 4.2. The minimum DNA content was 4.75ng/μl. This is required for normalization of the cellular population to compare it with others of different DNA extraction efficiencies.

Bacteria enumeration from cell counts: The enumeration had been carried out following the Kepner and Pratt (1994) protocol for DAPI, modified according to Meynet et al (2012) who used SYBr Gold (Invitrogen Life Sciences, UK) as stain for better resolution on the image. In detail, 1ml of 1:10.000 diluted stain in Mili-Q water (filtered-autoclaved distilled water) added to a pelleted sample after centrifugation of 1 ml sample (discarded supernatant). Sample and stain were applied on a polycarbonate membrane filter (Merck Milipore, UK) and set onto vacuum. The remaining sample was set on microscope slides and was covered with Citifluor prior sealed with a cover slip and glued with nail varnish. Slides were examined with an Olympus BX40 Epifluorescence microscope; 20 randomly chosen fields of view were counted measuring the fluorescent cells that have a clear outline and finite cell shape. To define the bacterial the methanogenic population (qPCR) was abstracted from the total cellular population.

The DNA samples were taken on days 0 and 70. The community structure was statistically analysed on day 70. The analysis was similar as described on Chapter 4.2.3. The statistical test includes one way and two-way tests (pairwise) depending on the comparisons (one way to compare only temperature or treatment switch, pairwise to compare the overall effect of both factors). The tests were carried out on Primer6 (Multivariate statistics for ecologists, Luton, UK) selecting temperature and treatment as factors (similarly to Chapters 4.2.3, 5.1.3).

5.2.4. Results

The substrate was weak in organic content (COD of 170 mg L^{-1}); the methane produced from seeded microcosms is shown on Figure 5.10.a, b. Methanogenesis was observed at both 4 and 15°C, the rates were higher at higher temperature.
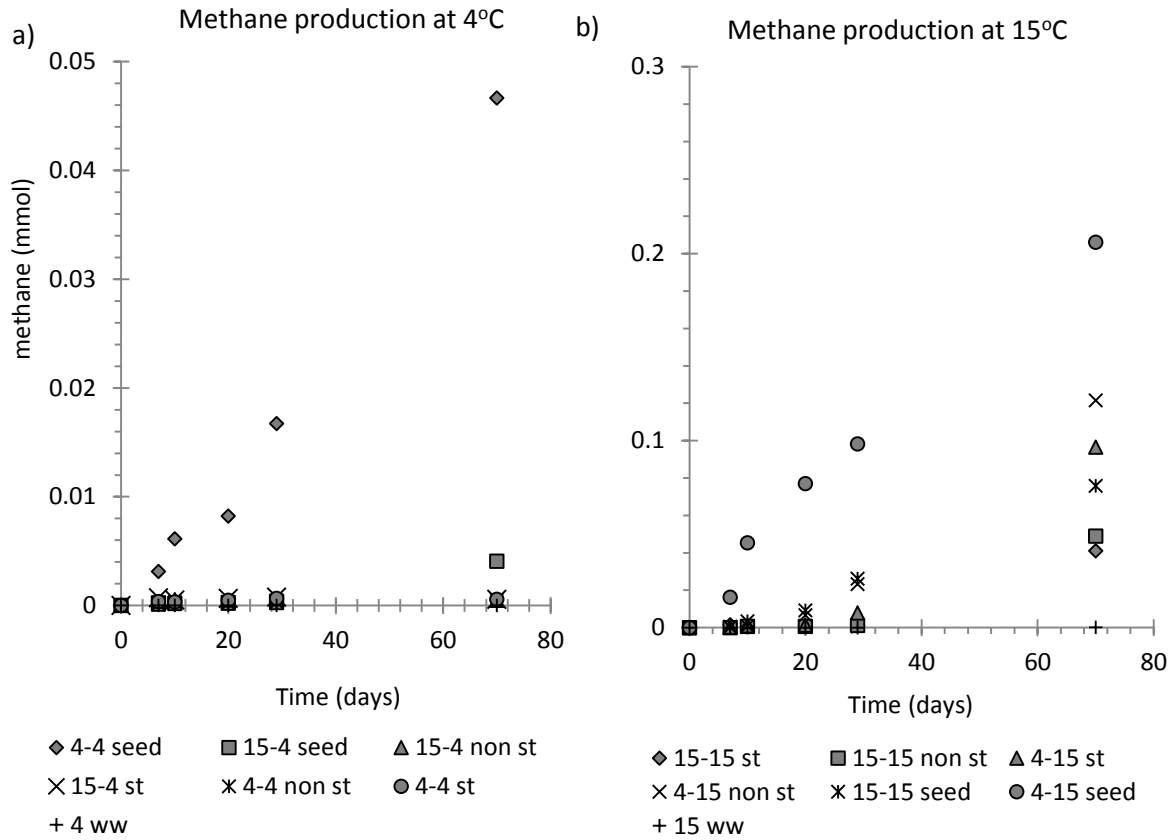
Figure 5.10 – Methane production versus time (a) at 4°C as average of duplicates (b) similarly at 15°C; labels represent acclimatization temperature to operational temperature (4-4, 4-15, 15-15, 15-4), st:seeded and fed with sterile WW, non st: seeded and fed with non-sterile WW, seed: seeded and unfed controls; WW: un-amended controls; all points represent the average of duplicate samples; average variation coefficient for 4-4seed, 15-4seed, 15-4non st, 15-4st, 4-4non st, 4-4st, 4ww is 0.37, 0.77, 0.36, 0.30, 0.32, 0.39, 0 respectively (plot a); 15-15st, 15-15non st, 4-15st, 4-15non st, 15-15seed, 4-15seed, 15ww is 0.55, 0.63, 0.31, 0.56, 0.31, 0.38, 0.57 respectively (plot b).

Slow methanogenic activity of the wastewater fed microcosms compared to the un-amended controls suggested that inhibition of the methanogenic population occurred originated from the substrate. Anion analysis showed increased levels of Cl$^-$ in the wastewater for the feeding day (day 1) compared to the next 3 weeks (day 7, 14, 21) (Table 5.3).

Table 5.3 - Concentration of Chloride ion (Cl$^-$) in the feed for day 1 (feeding the microcosms), days 7, 14 and 21 also presented for comparison purposes

Cl$^-$	day 1	day 7	day 14	day 21
Conc. (mg.L^{-1})	135,9	74,0	57,3	52,1

Other compounds (SO$_4^{-2}$, NO$_2^-$, NO$_3^-$Ca, Mg, K, Cd, Cr^{+-}, S, Ni, Al, Zn, Al, Fe, Pb, As, Si) were also measured but their concentration was consistent with low variation between day 1 (feeding) and days 7, 14, 21

The methanogenic groups that were identified through GenBank database were *Methanomicrobiales*, *Methanosarcinaceae* and *Methanosaetaceae*. Quantitative PCR for all samples showed that at all temperatures the dominant family is *Methanosaeta*ceae. The summation of all three is showed on Figure 5.11 for the first and final experimental day.

Methanogenic population
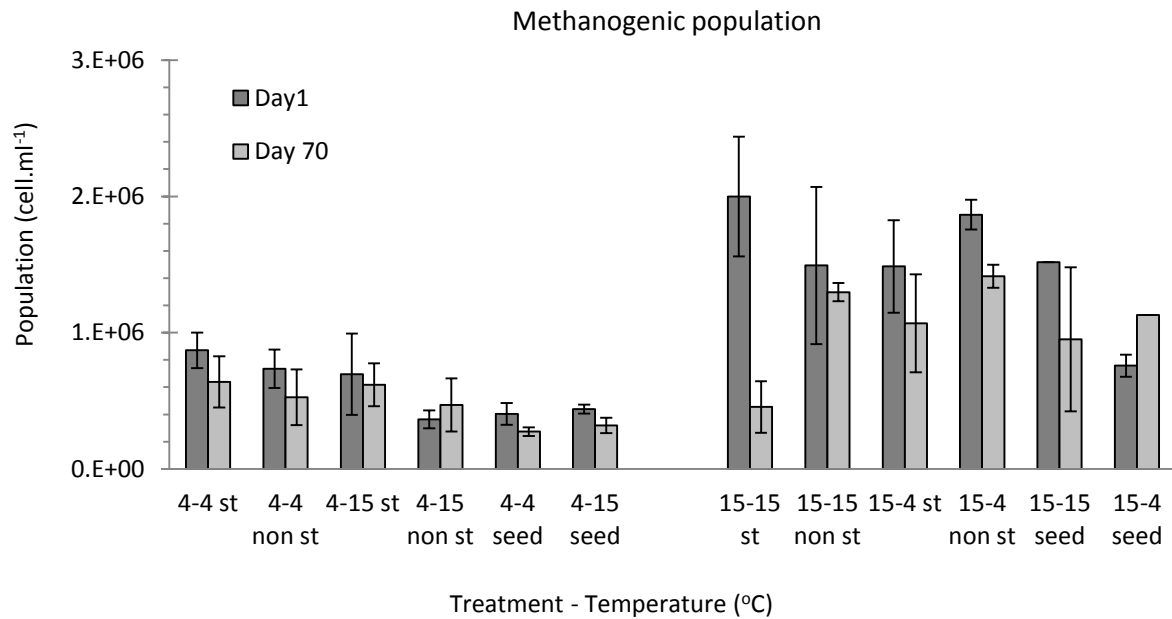
Figure 5.11 - Methanogenic populations in the microcosm at day 0 & 70 as asummary of the taxa detected most similar to Gen Bank database; particularly *Methanomicrobiales*, *Methanosarcina* and *Methanosaetaceae*; X axis: acclimatization temperature to operational temperature (4-4, 4-15, 15-15, 15-4), st:seeded and fed with sterile WW, non st: seeded and fed with non-sterile WW, seed: seeded and unfed controls; WW: un-amended controls; all points represent the average of duplicate samples, error bars indicate standard error (n=2)[4].

Cell counts showed that the bacterial population was approximately 1.25 times higher at 15 than at 4°C (Table 5.4), giving an extra advantage to the seed acclimatized to higher temperatures.

Table 5.4 - Bacterial population in the mixed liquor of the microcosms and in the wastewater feed at 4 and 15°C

Inoculum from 4°C	4-4 st	4-4 non st	4-15 st	4-15 non st	4-4 seed	4-15 seed
Cells.ml^{-1}	4,04 (0.28)	5.59 (0.56)	3.74 (0.46)	5.52 (0.39)	3.30 (0.15)	3.11 (0.27)
Inoculum from 15°C	15-15 st	15-15 non st	15-4 st	15-4 non st	15-15 seed	15-4 seed
Cells.ml^{-1}	4.16 (0.33)	6.61 (0.47)	4.1 (0.24)	5.76 (0.31)	4.61 (0.41)	4.25 (0.27)
Wastewater	4 WW	15 WW				
Cells.ml^{-1}	2.27 (0.34)	2.21 (0.26)				

Bacterial populations in the microcosm at day , measured from cell counts; Abbreviations: acclimatization temperature to operational temperature (4-4, 4-15, 15-15, 15-4), st:seeded and fed with sterile WW, non st: seeded and fed with non-sterile WW, seed: seeded and unfed controls; WW: un-amended controls; all values account for the average population calculated from duplicate microcosms, values in parenthesis indicate standard error (n=2)[4].

From the population and the methane production (Figure 5.10.a, b, 5.11) the activity was estimated and expressed per methanogenic cell (Figure 5.12) – specific activity. For the calculation the assumptions that were made were that all measured methanogenic population is active and that all methanogens produce methane at the same rate regardless taxonomy. The rates were calculated as an average of the cells between day 1 and 70. This resulted to a really high activity, especially for the un-amended controls.

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[4] The error bars were also calculated with the log-transformed data, however the back transformation of the results could not depict the variance of the population resulting to non-representative, really low values (e.g. SE of 1-5 cells.ml^{-1}). A different approach for error bars was the estimation of the 95% confidence level of the log transformed mean, however the back transformation resulted to extremely high mix and max bars due to the limited number of replication (duplicates, quadruplicates)

Figure 5.12 - Specific methanogenic activity at all conditions; X axis: acclimatization temperature to operational temperature (4-4, 4-15, 15-15, 15-4), st:seeded and fed with sterile WW, non st: seeded and fed with non-sterile WW, seed: seeded and unfed controls; WW: un-amended controls; all points represent the average of duplicate samples; calculated from days 1-70

Statistical community analysis revealed a clear differentiation for the bacterial (Figure 5.13.b, d), but not the archaeal communities. The switch in temperature combined with the increased competition from the wastewater cells seem to have an impact to the bacterial community (Figure 5.13.a, c) at such low seed:substrate ratio.

a)   b)

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
P a g e | 76

c)

d)

Figure 5.13 - a) Bacterial & b) Archaeal MDS plot day 70; c) and d) similarly plotted in dendrogram; labels correspond to the acclimatization temperature (1st) and operational temperature(2nd) (e.g.4-4, 4-15, 15-15, 15-4), st:seeded and fed with sterile WW, non st: seeded and fed with non-sterile WW, seed: seeded and unfed controls; WW: un-amended controls; all points represent the average of duplicate samples

5.2.5. Discussion

The temperature switch and the competition from the indigenous wastewater cells were able to affect the acclimatized at 15°C bacterial communities, although in higher abundance compared to at 4°C (Table 5.4) where the effect was less. This revealed that the 15°C acclimatized biomass, at the particular seed:substrate ratio (1:7) was not resilient to both factors. The competition caused a dynamic ecosystem (Pender 2004) that formed changes in the structure of the less sturdy community (acclimatized at 15°C) according to Figure 5.13.a, c. On the other hand, the biomass acclimatized at 4°C managed to retain its structure and performed better. This supports that the community ´shaped´ by the 4°C acclimation is a formed community, which once developed, is fixed. This renders very hard for the abundant species to go extinct as the probability for other cells (invaders) to hold a vacant position and colonize in the community is low due to their lack of adaptation at 4°C and their low population. This also suggests that that the acclimatized to lower temperature cells may be more persistent to various conditions and seem to be the key microorganisms that need to be present in biomasses that are exposed to low temperatures (< 15°C). Archaeal communities were not affected either by temperature or treatment, showing that the community is formed (Figure 5.13.b, d).

The bacterial community differentiation was not that evident to the previous trial (Chapter 5.1). I believe that the main reason was the seed:substrate ratio, which was twofold higher than the current one (1:3 and 1:7 for the

previous and current study respectively or 1:4 to 1:8 seed:total vol.). This (seed:substrate 1:3) formed a larger community that remained unaffected from the exogenous factors the time that the experiment lasted (96 days). For less dense communities though (seed:substrate 1:7) 70 days is an adequate time period to cause differentiations in the community structure. This observation agrees with Curtis and Sloan (2004) who pointed that the size of both the invaders and the established community are of major importance for the diversity preservation. This supports that for low temperature anaerobic wastewater treatment seed:substrate ratios lower than 1:3 are not recommended unless the seed includes resilient communities as those developed at 4°C acclimation.

From the cluster analysis it is shown that two major bacterial clusters were formed (Figure 5.13.a, c), mainly separated by acclimation (15°C and 4°C; similarity >30%). The exception was the acclimatized seeded microcosm that operates at 15°C with UV-sterilized WW, '15-15°C sterile' (R < 0.445, P = 0.009), which seem that remained unchanged retaining its original community structure (similarity >30%) compared to those inoculated with the same seed, operating under different conditions (i.e. '15-4°C sterile or non', '15-15°C seed' and '15-4°C seed') that had their community structure affected. This demonstrated that the autochthonous in wastewater bacteria, starvation and temperature switch may disturb the cells acclimatized to 15°C. Thus, it is possible that seeded biomass could be "overgrown" by potential presence of more tolerant bacteria in the wastewater. This could cause a loss of function and lead to treatment failure as regardless their tolerance, performance in AD-terms is not known.

From the lower temperature acclimation, the '4-15°C seed' microcosms were more similar to '15-15°C st' microcosms than to the '4-4°C seed'. This might have occurred due to regrowth of some of the organisms that were present in the original arctic seed and were in low populations after the prolonged exposure to the 4°C acclimation period. This supports that some of the cells might have been dormant and remained inactive without to fully deplete from the community matrix. No significant differentiation occurred between the 15-4°C (sterile or non) and the 15-15°C non-sterile.

The archaeal communities at both seed based clusters were of high similarity (≥70%) despite the conditions (Figure 5.13.b, d). This suggests that archaea were not affected by the indigenous in wastewater cells. WW communities were found different to all inoculated samples (similarity >20%, R = 1, P = 0.002). This depicts that the archaeal populations from WW cannot outcompete those habiting the seed. Temperature switch had a negligible impact to the populations from both seeds (4 and 15°C), obtaining a similarity of 80%. Describing the archaeal community as a dynamic ecosystem I conclude that the bio-reactor community structure acclimatized to low temperatures (<15°C) is hard to be challenged from the WW cell invasion or temperature differentiation. This indicates that the fixed and stable after years of acclimation archaeal community of the seed is hard to be outcompeted at these temperature from the low population of the wastewater-originated archaeal cells.

From the above, it is concluded that acclimation at 4°C results in a more stable bacterial community compared to at 15°C, as communities retain a high degree of similarity under different conditions. For archaea the diversity seems stable regardless the conditions (temperature, treatment).

The results are not in full agreement with the findings of the previous experiment (Chapter 5.1) where the bacterial communities of the seed remained stable and the archaeal communities were more prone to differentiation. Although the archaeal differentiation is not that intense between the current and previous chapter (5.1), the larger diversity in the bacterial community structure is obvious. This shows that the higher proportion of bacteria from wastewater in the mixed liquor may more actively compete with the seed-originated cells in the bacterial community when both exposed closer to the mesophilic range temperatures (15°C).

Presence of unusually high inhibitory compounds (Cl⁻) in the wastewater (Table 5.3) decelerated methanogenesis at all fed with wastewater microcosms. The inhibition is likely to have a chemical nature as it occurred only to the wastewater-fed microcosms, letting the un-amended controls perform normally. The responsible compound was possibly NaCl (as Cl⁻ found in high concentrations). This scenario is plausible due to the recent snow fall in the region, and excessive use of NaCl to tackle snow accumulation on the streets, which subsequently ended to the sewers. I believe that the remaining salt was dissolved in the wastewater and was driven to the wastewater treatment plant causing damage to the biomass. Inhibition from NaCl is common (Lefebvre et al 2007). The observed concentration in our study is relatively low however it is 100% and 200% higher with the observed concentration

of the next few weeks (Table 5.3). At 15°C cells could recover after 30 days, possibly due to higher growth rates and started performing normally after a while. Unfortunately at 4°C the recovery was slower and hardly noticable.

The methane production started rapidly at both temperatures for the unamended microcosms. This did not occur for the seeded and fed with sterile or non WW ones for the reason described above.

A comparison between the un-amended seeded microcosms (Figure 5.10.a, b) showed that the methane production at 15°C is greater than at 4°C supporting that also the volumetric rate at 15°C is higher. However, Figure 5.11 shows that the methanogenic population developed at 4°C was lower than at 15°C. Thus, a more representative approach would be the use of the specific methanogenic activity (Figure 5.12, $CH_4.cell^{-1}day^{-1}$), ideal for comparison of inoculum that differs in cell abundance (Figure 5.11). The specific activity per methanogenic cell revealed a different performing pattern (Figure 5.12.) with the cells acclimatized at 4°C operating better than the originally acclimatized at 15°C cells when both at 15°C. Less substrate (COD) at the 15°C-inoculum compared to at 4°C (Chapter 4.3) can be a plausible reason behind poor performance. The methane produced from the 'acclimatized at 15°C-working at 4°C' seed (15-4seed) was found lower than the methane formed from the acclimatized at '15°C-working at 15°C' one (15-15 seed). Additionally, the CH_4 production from both was found lower than the expected from the accumulated COD in their seed (from Chapter 4.3). This suggests that there was still organic material that could have been utilized. Thus, the cells from 15°C find it harder to utilize the specific substrate compared to those originated from lower temperatures for reasons that require further investigation. At 4°C it can be seen that in absence of other types of available substrates (carbohydrates and proteins) the cells may easier compromise and adapt to the insoluble substrate (lipids) that could not previously utilize.

The specific activities for the un-amended samples at the original acclimation temperatures (4-4°C seed and 15-15°C seed) were found significantly different (one way ANOVA, P-value: 0.05) with the one at 4°C operating better. This suggests that the initial Arctic inoculum (Batch 1, Chapter 4.1) contained psychrophilic (psychroactive) communities which were developed after prolonged acclimation (not observed on Chapter 4.2).

Temperature switch caused a shock to the un-amended samples acclimatized at 15°C that led to an activity decrease if not process failure. The shock was not apparent to the cells of the seed acclimatized at 4°C, operating at 15°C. This shows that cells acclimatized at 15°C may find it hard to cope with seasonal variation where those acclimatized at 4°C seem more capable to go on the process.

With regards to the microcosms fed with wastewater, unfortunately, those operating at 4°C (4-4°C 15-4°C sterile or non) could not properly recover from the inhibition; thus, it wouldn't be reliable to focus on them. With regards to those operating at 15°C, the pattern is similar to the un-amended controls. Specific activity showed that the seeded with biomass acclimatized at 4°C reactors perform at least 4 to 9 times (4-15°C sterile or non) higher than those seeded with 15°C acclimatized biomass when both at 15°C (one way ANOVA, P-value: 0.008). Between sterile or non wastewater fed reactors, the rates were higher for the latter as previously shown (Chapter 5.1). This occurs due to the supplementary bacterial cells present in wastewater that assist in hydrolysis of the insoluble material. As in Chapter 5.1 the cells from both origins co-operate and accelerate wastewater treatment.

Comparing the specific activity here with the activity presented on Chapter 4.2. (better representation at Chapter 5.3.) it is evident that after 5 batches the cells perform extremly higher, especially at 4°C. This reveals a potential of higher adaptation after at least 200 days since batch three. A second scenario would be a change in the predominance of the methanogenic population. In batch three the predominant methogenic families at 15°C were *Methanosaetaceae* and *Methanomicrobiales* where as at 4°C *Methanomicrobiales* was found to be the dominant one (Chapter 4.2). Currently, the most abundant family is *Methanosaetaceae* followed by *Methanosarcinacreae*. This suggests that these two communities are more active than *Methanomicrobiales*, a community that was outcompeted by these two after longer acclimation (since batch three). This scenario can be supported by the increasing population of *Methanosaetaceae* over the total methanogenic population compared to the first and third batch (data not shown). This also demonstrates that the pathway of Acetate to CH_4 might become predominant after long time of exposure to low temperatures. Further research is required to be certain of the above, as this result contradicts with previous findings (Chapter 4.1, 4.2).

In terms of wastewater engineering I suggest that digesters operating to low temperatures need to be inoculated with seed acclimatized at lower temperature, as the communities developed perform better regardless the substrate

(sterile or non). This points that the un-hydrolyzed material that accumulates during cold periods will be utilized at a higher rate when temperature will increase. The hypothesis is mainly supported from the un-amended controls as un-hydrolyzed material was the only substrate that could be utilized at a higher rate; *c.f.* the methanogenic activity of the '4-4°C seed' is greater than the '15-4°C seed'. The 9 fold higher specific activity of the '4-15°C non st.' compared to '15-15°C non st,' suggests that the method is promising for application in places where the warm period accounts for the 1/9 of the annual period.

5.2.6. Conclusion

The experiment suggests that acclimatization at 4°C results in a stable, sturdy community where hydrolysis limitation can be rapidly overcome during seasonal variation.

5.3. What are the conclusions combining chapter 4 & 5?

5.3.1. The volumetric rates

The cells that participate in the treatment process demonstrate high activity even after more than 800 days of exposure to low temperature. This supports that the organisms can in principle cope with the low temperatures and treat either raw or primary settled wastewater even at 4°C.

From Chapters 4.2 and 5.1 it is concluded that temperature plays a key role in anaerobic wastewater treatment as it affects both hydrolysis and methanogenesis (Figure 5.14). Hydrolysis seems to be the rate limiting step at all temperatures regardless the treatment (sterile or non substrate) (Figure 5.14). Temperature switch from 15 to 4°C can potentially unbalance the microbial communities, as biomass pre-acclimatized at 15°C, at a seed:substrate ratio ≤1:7, finds it harder to retain its diversity stable (Figure 5.13.a) when temperature decreases in the presence of numerous from wastewater microbial populations. At larger seed:substrate ratios (≤1:3) the co-existence between the cells habiting the seed and those originated from wastewater is sufficient to boost hydrolysis even at 4°C and overcome the shock from temperature decrease (i.e.15 to 4°C non st WW, Figure 5.14).

Cells originally acclimatized at 4°C do methanize and hydrolyze at a higher rate compared to those operating at 4°C acclimatized at 15°C regardless the substrate (sterile or non), and the operational temperature (higher or lower) when both at 4°C (seeded at 4°C operating at 4°C (4°CMR) over those acclimatized at 15°C – operating at 4°C (4°C st WW, 4°C non st WW). This implies that the cells from 4°C are more tolerant to their acclimation temperature. Similarly, at 15°C the '15 non st WW' performs the highest, due to the mesophilic nature of the cells from WW that co-operate with the numerous microbial population of the seed. Expressing the rates per cell though renders the pattern unlikely to be kept the same.

5.3.2. The specific rates

After normalization of the methanogenic population (qPCR) based on the minimum DNA content (Chapter 4.2, 5.2, min: 2.8ng/μl) the specific activity was re-estimated (Figure 5.15). Normalization ensures that two or more samples with different DNA extraction efficiencies would become comparable after adjustment of the qPCR measured population based on a factor generated from the fraction between $DNA_{measured}$ and $DNA_{minimum-measured}$.

At 15°C after 2 batches (Chapters .4.4, 5.4.) the methanogenic performance was reduced by 2.5 times (15 MR and 15-15 st or non) possibly due to inhibition (Table 5.3), however the operation didn't stop. Comparing the 15 MR with the un-amended 15-15 makes clear that the performance after two batches increased (×2). This suggests that cells at 15°C may easily thrive to this temperature and adapt to the WW substrate (either easily degradable or un-hydrolyzed). Temperature decrease from 15 to 4°C (sterile or non) led to decelerated rates compared to those from 15-15 sterile or non WW fed microcosms (74-130 times for st and non st respectively). The same phenomenon occurred to the un-amended microcosms (un-amended 15-15, 15-4) at a lower level (20-folded decrease). Hence, temperature decrease from 15 significantly affects the performance of the cells as the reaction seems to stop (e.g. 15-4 seed), although substrate is present (supposing lipids mainly, evident from the activity from 15-15 seed).

At 4°C the specific activity after 2 batches (Chapters 4.2, 5.2) significantly increased (4MR to 4-4 seed). Unfortunately a comparison between 4 MR and 4-4st or non though would not be valid due to the chemical inhibition. When cells at 4°C were exposed to 15°C WW fed the performance increased of at least 4-9 times compared to those originally acclimatized at 15°C (4-15 st, non st - 15-15 st, non st). The phenomenon was

repeated for the un-amended 4-15 and 15-15 where the 1st achieved the highest specific activity at this study. This suggests that the microbial community developed at 4°C has a higher methanogenic capacity than the one originally developed at 15°C. In terms of wastewater treatment engineering this means that the conversion of COD:CH$_4$ would be far more accelerated. This manifests that seasonal variation would overcome the problem of limited hydrolysis and the substrate utilization rate would be higher if the acclimatized at lower temperature cells are predominant in the bioreactor. Finally, comparison of the original activity between the un-amended samples at 4 and 15°C (4-4seed – 15-15 seed) reveals the potential presence of a psychrophilic community at low temperatures (one way ANOVA, P-value: 0.05). The high performance of the acclimatized at 4°C when at 15°C community suggests that if such community it has either narrow temperature range and at 15°C different are more active, or that is cold adapted and can operate at both temperatures with an optimum closer to 15°C. WW sterilization decelerates methanogenesis as non-sterile samples produced higher amounts of CH$_4$. This is explained based on the lipolytic activity of raw wastewater (Chapter 6).
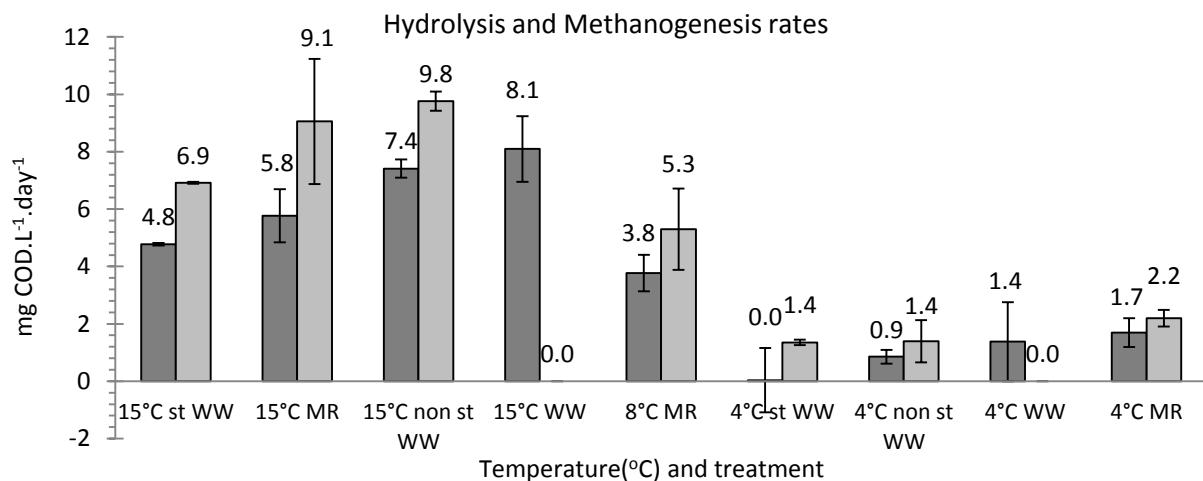
Figure 5.14 - Hydrolysis and Methanogenesis rates for 4, 8 15°C (Chapter 4.2), similarly for 15 to 4°C and 15 to 15°C either sterile or non (Chapter 5.1.); MR: original temperature (mother reactors); st WW: with sterile wastewater'; non st: non-sterile wastewater; WW stands for wastewater controls; 1, 3, 6, 7 double bars were all pre-acclimatized at 15°C – on the columns the temperature switch is being shown; error bars stand for standard error.
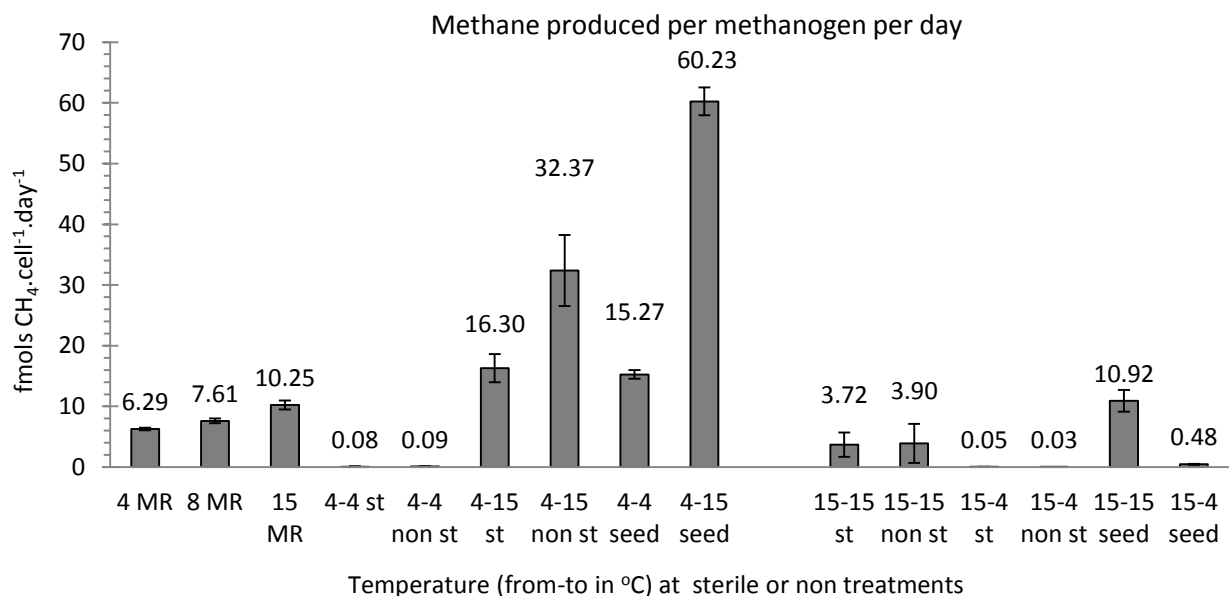
Figure 5.15 - Specific methanogenic activity rates for 4, 8 15°C (Chapter 4.2), similarly for 15 to 4°C or '4 to 15°C' either unamended controls (seed), sterile or non (Chapter 5.2.); MR: original temperature (mother reactors); st WW: with sterile wastewater'; non st: non-sterile wastewater; WW stands for wastewater controls; seed stands for un-fed controls (seed and DI water) – on the columns the temperature switch is being shown; all results previously normalized to a 2.8ngDNA/μl content.

Chapter 6: *Lipid degradation, lipases' kinetics at low temperature anaerobic treatment of domestic wastewater*
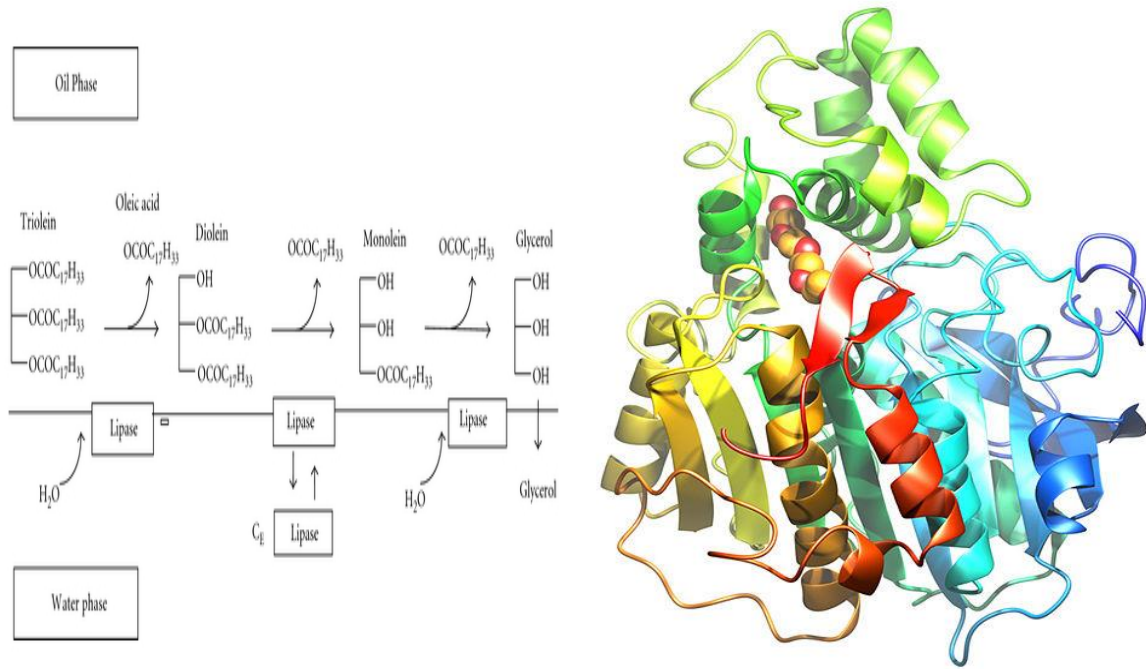
Figure 6.1 - Proposed mechanism of triolein hydrolysis from lipases of *Candida rugosa* in bi-phasic water-oil system. (Karigar and Rao 2011); Right: X-ray structure of *Candida Antarctica A* lipase in its closed state (ref: Proteopedia; active link: http://www.proteopedia.org/wiki/index.php/Lipase_from_Candida_ antarctica_in_ closed_state)

6.1. Low temperature lipolysis of domestic wastewater (4, 8 & 15ºC) - Lipases: kinetics and identification

6.2. Abstract

Previous studies enabled the hypothesis that lipids in anaerobic wastewater treatment accumulate the lower the temperature (15 to 4ºC). A lipase assay to enzymes that were extracted from seven anaerobic bio-reactors operating at 4, 8 and 15ºC confirmed the hypothesis as activity at 4ºC dropped close to 0. The main reason is likely to be the change in lipid structure in addition to deceleration to enzyme kinetics the lower the operational temperature. Incubation of the same enzymes (from 4, 8 and 15ºC) at 37ºC showed that the enzymatic activity was high at all temperatures. The specific activity based on samples' protein content was higher at 4ºC compared to 15ºC when both at 37ºC. This manifests that a biomass acclimatized to low temperatures produces more active enzymes than those secreted from the cells acclimatized to higher ones when operating at same temperatures. Kinetic analysis showed that temperature rise of 4 degrees (from 4 to 8ºC) is adequate to kick off the COD_{lipid}:CH_4 conversion and that the trigger for lipid hydrolysis lies in-between 4-8ºC. At a lower than this temperature either carbohydrates or proteins or both seem to be the only available substrates. Surprisingly, the anaerobic lipolytic activity in a bio-reactor operating at low temperatures is increased by the addition of indigenous from raw wastewater communities.

6.3. Introduction

This study gives an insight into the enzymes that are responsible for the domestic wastewater lipid degradation when these are exposed to low temperatures (4, 8 and 15ºC). It also helps the reader to clarify whether it is the presence or absence of the enzymes, or the kinetics that renders lipid degradation extremely slow the lower the temperature (Chapter 4.3). Estimation of the lipolytic activity of the cold adapted seed will potentially explain the reason of limited hydrolysis in anaerobic treatment at cold environments.

Previous experiments in low temperature anaerobic treatment showed that carbohydrates and proteins were efficiently degraded, where lipids accumulate (Chapter 4.3). A likely scenario could be that lipid structure tends to turn into a solid at low temperature (Neidleman 1987); however I believe that enzymes' kinetics' is also considered as a plausible reason. Lipid solubilisation is part of the hydrolysis process. Hydrolysis is the 1st step of anaerobic treatment and depends on extracellular mainly enzymes to create products that would be further uptaken by the acidogens and methanogens, transferring the electrons through metabolism, converting COD to CH_4 and CO_2 (biogas) (Kim et al 2012). Lipids are a major fraction of wastewater composition that may account for up to 41% of the domestic wastewater COD (Raunkjaer et al 1993); more if the substrate has a lipid based industrial origin (e.g. slaughterhouse, soap manufacturing etc.). Thus, it is of major importance to investigate the reasons that lead to limited lipid lysis.

Hydrolysis of lipids leads to LCFAs and VFAs formation, intermediates that are further used for methane production, the final product that represents the energy generation. The expected amount of energy from wastewater is estimated at 14.0kJ gCOD[-1] (Heidrich et al 2012). This shows that 41% of the WW COD might not be converted to energy and will be lost. Another drawback of limited hydrolysis is that the partly treated effluent that contains un-hydrolysed lipid COD would not be able to meet the discharge standards (UWWTD 91/271/EEC). Additionally, lipid accumulation is likely to cause formation of lipid agglomerates in the bioreactor that can potentially lead to biomass deterioration, washout (Uemura and Harada 2000, Elmitwalli et al 2000) and lead to treatment failure. Thus, lipases seem to hold a key position in the biological reaction as they are responsible of the hydrolysis of triacylglycerol and other lipids, important step in the COD to energy bio-conversion.

Lipases consist of a catalytic triad formed by Ser-His-Asp/Glu which is common for most serine-hydrolases (Cygler et al 2004). A specific feature for many of them is the alpha/beta-hydrolase fold consisting of a series of parallel β-sheets and a number of helices that flank the sheets on both sides (Ollis et al 1992, Schrag and Cygler 1997). The majority contains a lid that controls access of the substrate based on hydrophobicity as it is the main functional principle, responsible for substrate specificity. As described on Chapter 2.2.10, 2.2.6, and 2.2.7 various mechanisms have been naturally developed to render these enzymes able to sufficiently overcome the operational obstacles that may occur for anaerobic treatment at low temperatures (Chapter 2.2.3).

Activity based proteomics is the easiest approach to identify activity for specific proteins (e.g. lipases), as analysing the whole proteome is work intensive and time consuming.

Para-nitrophenyl palmitate (pNPP) assays, as described by Winkler and Stuckmann (1979) are commonly used to estimate the hydrolytic/esterolytic activity of lipases. The main protocol principle of the pNPP assay is the estimation of the para-nitrophenol (pNP) that is released following the 1:1 mole:mole reaction as a result of enzymatic hydrolysis of pNPP (Schmidinger et al 2006) (Figure 6.2.). The estimation is carried out at 410 nm whereas the activity is expressed in U (unit, required amount of enzymes for catalysis of 1μmol of *para*-nitrophenyl per minute). According to Gupta et al (2002) this protocol is commonly used for lipolytic research endeavors to estimate the esterolytic activity for both lipases and esterases. Other substrates of pNP such as pNP-caprylate, pNP-acetate, and pNP-valerate can also be used. The selection of –palmitate was based on its carbon number (C_{16}), is a compound that is more likely to be found accumulated in reactors with slow hydrolysis/fermentation compared to the other three. Additionally palmitate´s high melting temperature (63°C, Lide 2005) ensures that at 15°C most, if not all will have a solid nature. This would better represent the status of the reactors that have a high content of un-hydrolyzed lipids (Chapter 4.3) most likely in a solid phase.
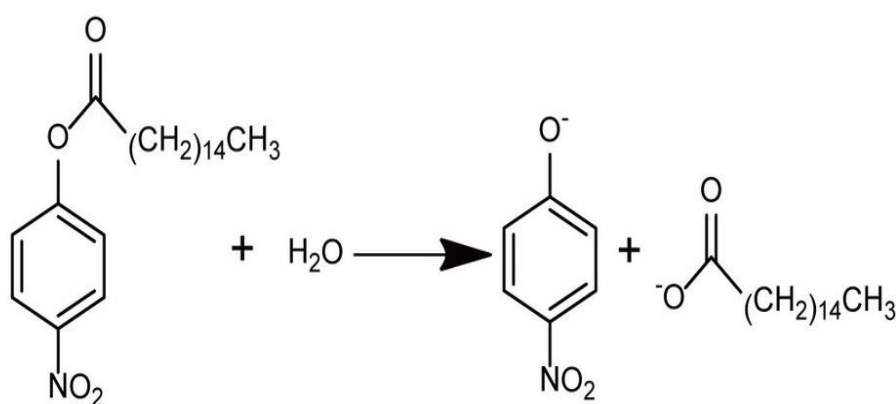
Figure 6.2- Hydrolysis of hydrophobic pNPP to amphipathic palmitate and p-nitrophenol (Sato et al 2013); for the lysis a lipase enzyme is required to be attached on the hydrophobic part of palmitate and subsequently break it down.

6.4. Materials and Methods

Reactors assembly; seven quick fit 1L glass batch reactors were used similarly to Chapter 4.1.3, set at 4, 8 & 15°C

Inoculum; laboratory-scale batch reactors were treating mainly raw, but also domestic WW and had been exposed to 4, 8 and 15°C for 3 years (same reactors as in Chapter 4). The selected seed:substrate ratio was 1:3 for all this period of time, the same ratio was followed for the particular study. These reactors were originally seeded with a mixture of putatively cold-adapted sediment from Lake Geneva and soils from Svalbard, in the high Arctic (same features to first, second, third and fifth batch, Chapter 4.1, 4.2, 4.3).

Wastewater as substrate; primary settled wastewater was collected from Tudhoe Treatment works at Spennymoor (UK) wastewater treatment plant (similar to fifth batch – Chapter 4.3). Where necessary UV-sterilization was undertaken as described previously (Chapter 5.2.1).

Re-feed and re-inoculation procedure was carried out as described on Chapter 4.1.3.

Methane; CH_4 was monitored in the gas phase as % by headspace volume, as described to Chapter 4.1.3.

VSS; Volatile Suspended Solids content was estimated gravimetrically based on APHA 2005 (as described on Chapter 4.1.3).

Enzyme extraction; the lipolytic activity protocol was based on Gessesse et al (2002). 10ml of mixed liquor was taken from the reactors. The pH was adjusted at 8.0 via addition of Trizma (Tris HCl) buffer (Sigma Aldrich, UK) achieving a final concentration of 10mM. Triton X-100 and EDTA solution was also added at the optimum concentration suggested (0.5 and 10mM respectively). For cell lysis a sonicator was used (Labsonic, UK), with maximum frequency of 13KHz. Sonication take place for 30minutes following a pace of 3minutes burst and 5

minutes of rest on ice. After lysis centrifugation took place for 10minutes at 4400rpm. The supernatant was further centrifuged at 13.000rpm for 10minutes. The 2nd supernatant was the sample for the assay application.

Enzyme assay; similarly, the assay protocol was based on Gessesse et al (2002) and Kim et al (2012). *P*-Nitrophenyl palmitate (pNPP) (Sigma Aldrich, UK) was selected as substrate. 20mM of stock were prepared in Isopropanol (Sigma Aldrich, UK). The solution was further diluted 1:20 in 20mM Trizma (Tris HCl, pH 8.0) Buffer (Sigma Aldrich, UK) containing 0.1% Gum Arabic and 0.4% Triton X-100 (Sigma Aldrich, UK).

Enzyme extract and substrate were set onto a plastic cuvette (VWR, UK) at a ratio 1:10. Samples were prepared in duplicates and incubated to the corresponding operational temperatures of 4, 8 and 15°C. Additional samples were set at 37°C. Unsterilized primary settled wastewater controls were also prepared to identify whether or not the activity is originated from the cells habiting the seed or from the indigenous in wastewater cells. An additional assay was carried out to raw wastewater samples. This WW controls were mainly added to support the finding from Chapters 5.1 and 5.2. Controls with only p-nitrophenyl palmitate were also prepared and tested at all temperatures.

The cuvettes were set into a spectrophotometer (Merck, UK), and the absorbance was measured at 410nm.The measurements were taking place every 20 minutes for the 1st hour and more sporadically after that. The correlation was estimated by the curve formed by known concentration of *p*-nitrophenol standards. The standards were tested at all operational temperature for time: 0 and after 30 minutes to examine whether there is a change in their structure of the compound after a certain time (calibration curves are shown on Appendix – Appendix 2, Figure a.2 a, b, c, d). As no differentiation occurred, the curves for time 0 were used. The results were expressed in Units (U) of lipase activity, which corresponds to the amount of enzyme that hydrolyses 1μmol of *para*-nitrophenyl (or produces 1 μmol of *para*-nitrophenol) per minute. For better interpretation the results were normalized based on protein content.

Protein assay; after cell lysis the protein content of the samples was estimated via the Bradford protocol (Brardford 1976), similarly to Chapter 4.1.3.

6.5. Results

The protein and mixed liquor VSS content depict the cellular differentiation amongst samples, which seems low when expressed in solids but considerable when in proteins (Figure 6.3.a, b). At both cases the samples that operate at 8°C seem to be the richer in cellular material.
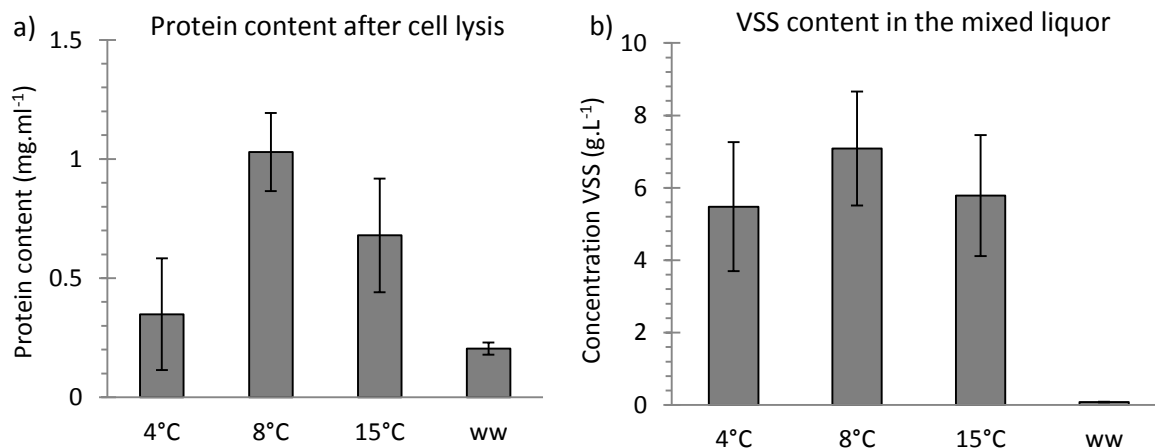
Figure 6.3 - a) Protein content of the reactor samples after cell lysis; b) VSS content of the reactors mixed liquor; error bars account for standard error, n = 2, 2, 3 for 4, 8 and 15°C respectively.

The CH$_4$ formation rate was related to temperature, with the highest amount appearing at 15°C (Figure 6.4). The differentiation between temperatures though (4, 8, 15°C) was not clear as rates were low and standard error especially at 15°C and 4°C was high.
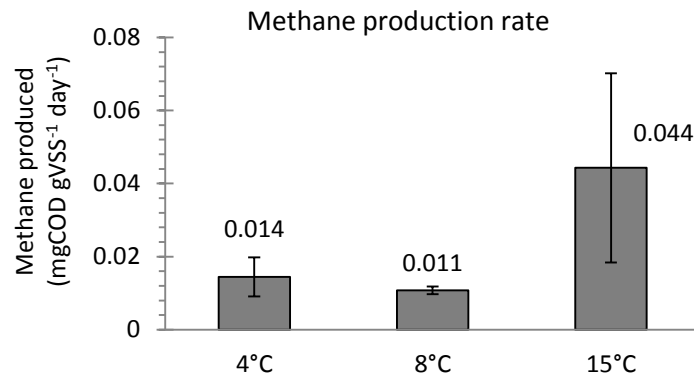
Figure 6.4 - Methane formation rate per day per gram of biomass as mixed liquor VSS inoculated in the reactors; error bars account for standard error, n = 2, 2, 3 for 4, 8 and 15°C respectively.

The measured lipase activity at all operating temperatures (and at 37°C) is shown on Figure 6.5.a (raw data on Appendix - Appendix 2, Table a.1, 2, calibration curves: Appendix 2 Figure a.2 a, b, c, d). Enzyme activity per ml of mixed liquor was found dependent on temperature. When the activity amongst all reactors was expressed per protein content and set at same conditions (37°C) the lipases at 4°C seemed to be more active than those from 8 and 15°C (Figure 6.5.b), or the proportion of the lipases per all proteins at 4°C is much higher than at 8 and 15°C. Plotting the activity after normalization (based on the minimum protein content (WW)) assists to the estimation of the lipases' lipolysis 'k' coefficient (Figure 6.5.c).

c)

Normalized lipase activity based on protein content

$$y = 0.0224e^{0.1076x}$$
$$R^2 = 0.9631$$

(y-axis: U ml^{-1} mixed liquor; x-axis: Temperature conditions)
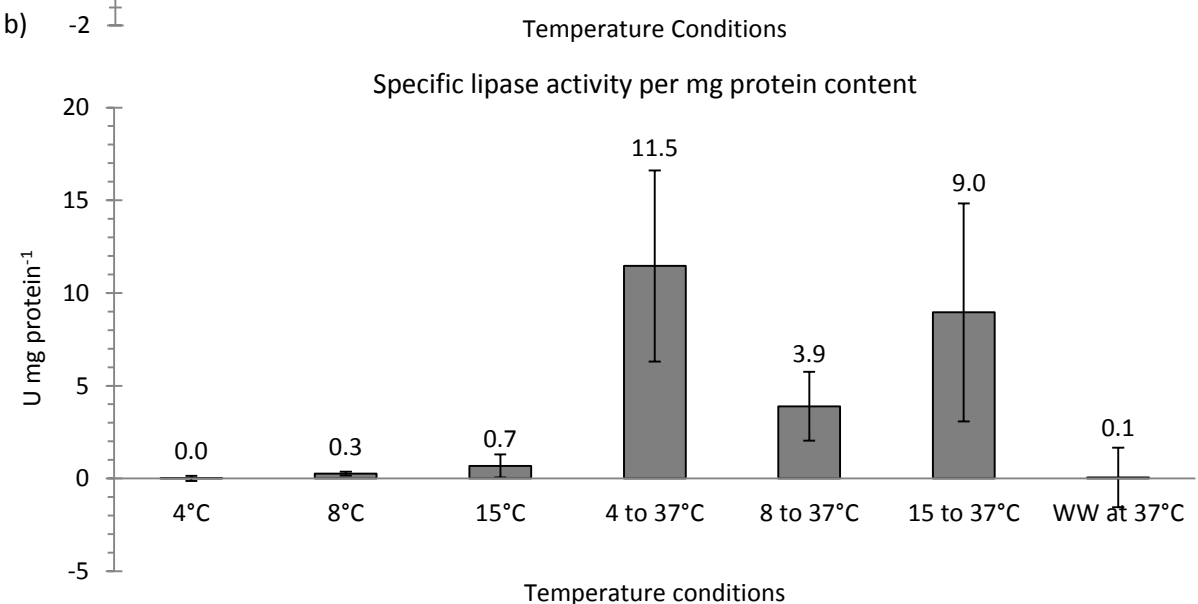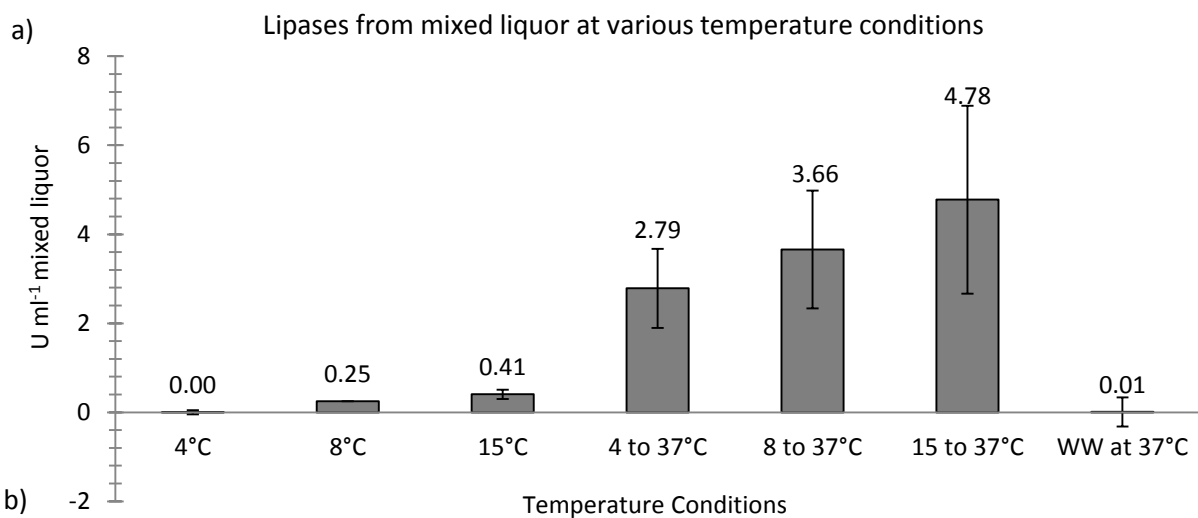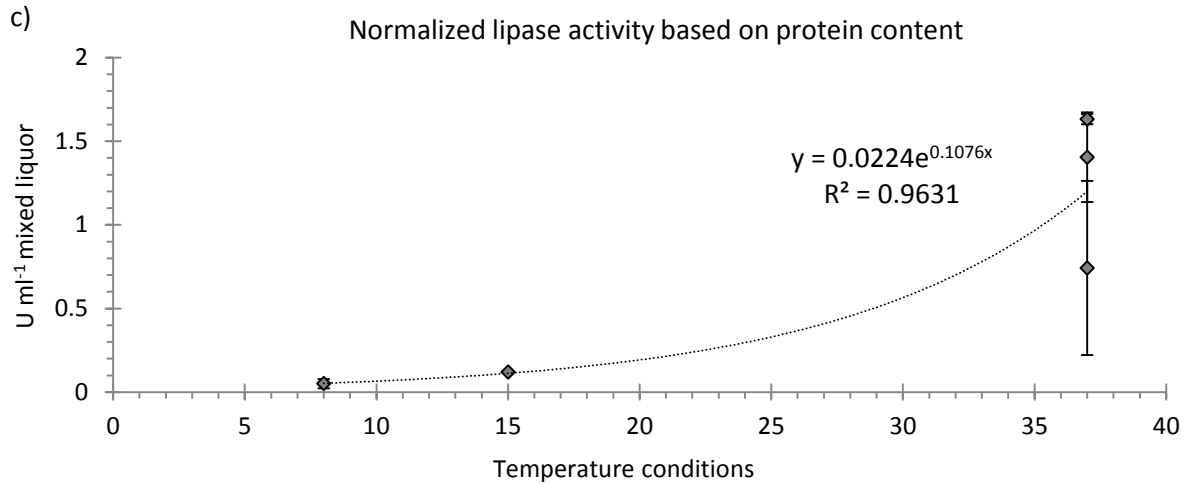
Figure 6.5 - a) Lipase activities 4, 8, 15 and 37°C after correlation with the calibration curve; b) similarly, expressed per gram of protein content; (c) normalized activity – kinetics representation (activity versus temperature); 4°C excluded (approx. 0.0), normalization to the minimum protein content to compare activities of different protein abundance: WW: 0.2 mg/ml, no activity detected for wastewater control at any of the operating temperatures; error bars stand for standard error

Combining the activity at 8 and 15°C (excluding 4°C, almost 0.0) with the activity of all operating temperatures at 37°C, plotting to an Arrhenius plot, the estimation of the developed lipolysis kinetics of the particular inoculum developed at the three temperatures (4, 8 and 15°C) can be defined (Figure 6.6.).

Lipase kinetics at all temperature samples

$$y = -10476x + 34.276$$
$$R^2 = 0.9998$$

$$y = -7923.6x + 25.288$$
$$R^2 = 0.9956$$

$$y = -9989.8x + 32.565$$
$$R^2 = 1$$

(y-axis: log (U.ml^{-1}); x-axis: 1/T (Temperature (K)))

Legend: 4°C; 8°C; 15°C; Linear (4°C); Linear (8°C); Linear (15°C)
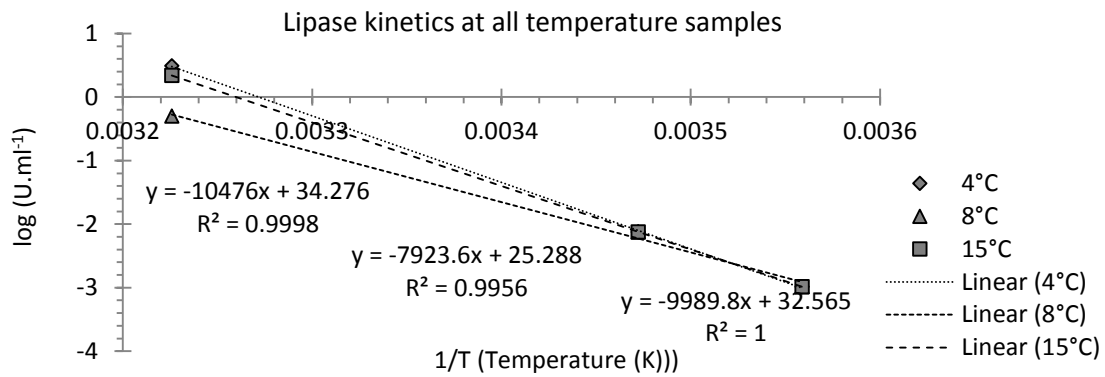
Figure 6.6 - K Arrhenius coefficient for all enzymes extracted at reactors operating at 8, 15 and 37°C after normalization to the minimum protein content, WW: 0.2 mg/ml; sample at 4°C was excluded (approx. = 0.0 U); trendline equations from top to bottom correspond to 4, 15 and 8°C.

Lipases activity combined with methane production data results to the identification of the relationship between lipolysis and methanogenesis as a function of temperature (Figure 6.7).

Relationship between lipid degradation and methane formation

$$y = 0.0799\ln(x) + 0.4013$$
$$R^2 = 0.9125$$

(y-axis: mmol p-NPP.gVSS^{-1}.min^{-1}; x-axis: mgCOD$_{CH4}$ .gVSS^{-1}.min^{-1})

Legend: 4°C; 8°C; 15°C
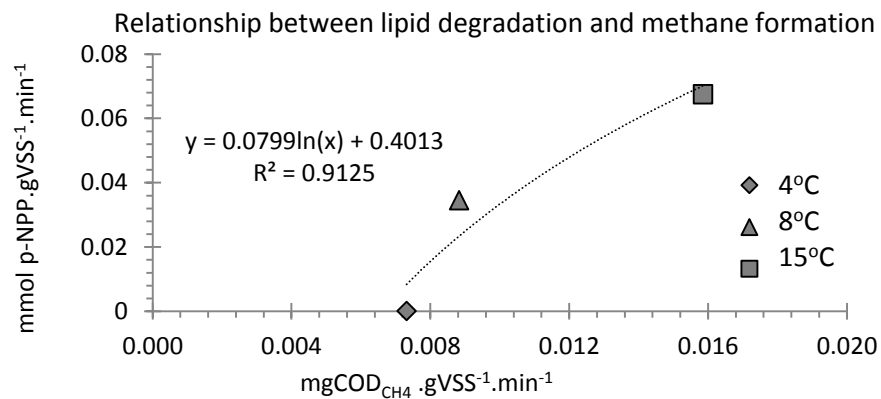
Figure 6.7 - Relationship between CH$_4$ formation and enzymatic lipolytic activity both developed from the seed at 4, 8, 15°C

For comparative purposes the activity was also expressed per gram VSS (U.gVSS^{-1} or µmol pNPP.gVSS^{-1}.min^{-1}) at 4, 8, 15 and 37ºC (Table 6.1) according to VSS content and the activity on Figure 6.3.b and 6.5.a respectively.

Table 6.1- Lipolysis activity per gram VSS of mixed liquor at all operational and incubation temperatures (4, 8, 15 and 37ºC)

Temperature (ºC)	4	8	15	4-37	8-37	15-37
U.gVSS-1	0,00	34,48	67,46	401,17	506,25	793,45

*4, 8, 15 correspond to the operational and incubation temperatures; 37ºC, 4-37, 8-37, 15-37 correspond to the operational and incubation temperature respectively e.g. 4-37 signifies operational temperature 4ºC, incubation for the essay 37ºC

Lipolytic activity in primary settled wastewater was negligible; however for raw wastewater the activity was similar to the one measured from thee seeded samples at their operating temperatures (Figure 6.8.a, b).
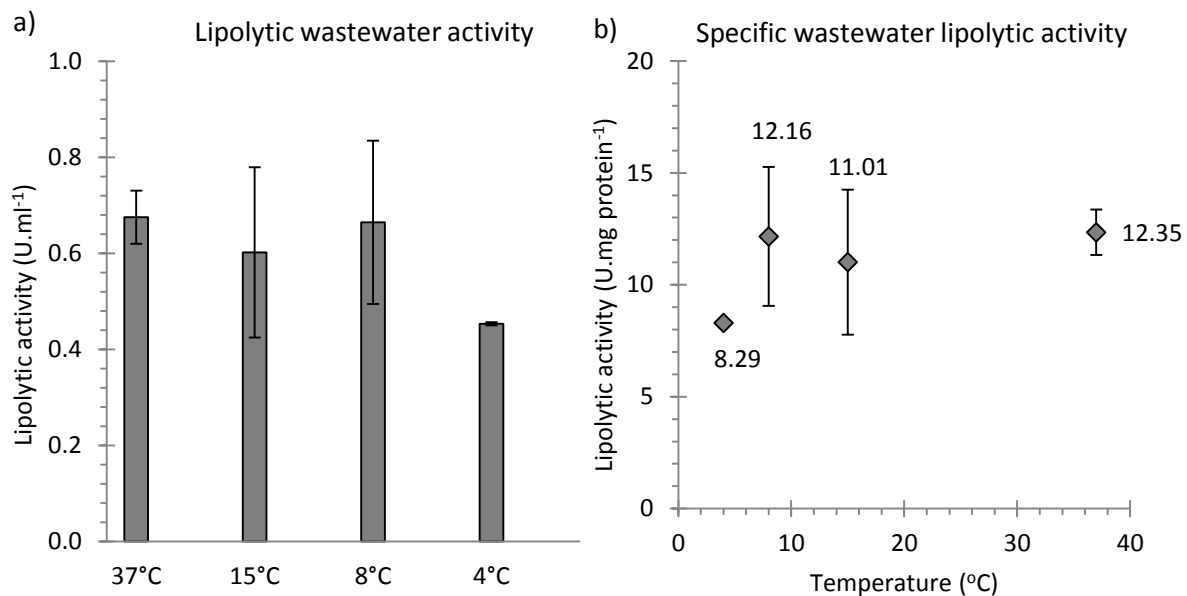
Figure 6.8 – Lipolytic activity from raw wastewater; a) activity at all operative temperatures plus at 37ºC for comparison with previous data; b) specific activity based on sample protein content; error bars stand for standard error, n 2 for all samples.

6.6. Discussion

Enzymatic activity was found present at all temperatures, 4, 8 and 15ºC (Figure 6.5.a, b, and c). The degradation pattern was found not canonical, following the 'activity-temperature' exponential trend, suggested from Burgess and Pletschke (2008), which indicates that for every 10ºC raise the activity becomes double-folded. This shows that lipid degradation is dependent to temperature, which affects either enzyme kinetics or substrate properties or both. Activity at 4ºC was found close to 0; however I believe that there is activity but too low to detect. This is supported by the presence of activity at all three temperatures when at 37ºC. This demonstrates that for a bio-engineered lipolysis approach at 4ºC a numerous population of cells is required to achieve a good performance as a summary of cellular excreted enzymes.

Apart from the scenario of activity-temperature dependence, a different explanation for the enzyme deceleration would be the likelihood of lipid structure differentiation at different temperatures (solidification - crystallization) (Neidleman 1987). As lipids solidify the lower the temperatures lipases find it hard to penetrate the external lipid surface that works as a barrier. In the case of palmitate this is very likely. P-nitrophenyl palmitate is an ideal substrate due to its high melting point (63ºC) in addition to its very slow solubility (0.000145 mg.L^{-1} at 25ºC). The effect of solubity was excluded by adding only p-NPP controls. Furthermore, p-NPP is a saturated fatty acid. Saturation is a factor that decelerates lipolysis as the absence of at least one double hydrogen bond renders the movement of the lipase within the lipid molecule difficult. This obstacle is increased at low temperatures considering that saturated fats solidify at much lower temperatures compared to the unsaturated ones. These values would ensure guaranty that the specific substrate would remain solid during experimentation retaining Alteration

of the substrate´s structure/nature can be supported from the raw data collected (Appendix - Appendix 2 Figure a.2 a, b, c, d), as samples were incubated to the corresponding temperatures (4, 8, 15 and 37°C) the starting optical density immediately increased the lower temperatures (value after 20 minutes incubations: approx. 1.04, 0.64, 0.61 and 0.27 at 4, 8, 15 and 37°C respectively). This clearly supports change in lipid structure when this is exposed to different temperatures. This may affect the enzyme activity as lipases may not be able to act equally in different lipid surfaces. In such case it is important to keep the substrate in a form that would be hard to solidify. One of the recommended options to tackle solidification would be the addition of 0.1-1% ($^v/_v$) detergent (Lee et al 2001). The detergents though need to be friendly to the microbial communities to avoid disturbance of the population. Additionally, prior application the detergent kinetics needs to be examined to low temperature to determine if it is active or not at such conditions.

The lipolysis capacity of the lipases fell close to 0, if not stop at 4°C incubation (Figure 6.5.a, b); however methane was formed (Figure 6.4., 6.7). This suggests that either readymade compounds (VFAs) were present or hydrolysis of proteins and carbohydrates occurs at 4°C. The 1st scenario is less likely as no VFAs were detected in the feed. The ion exchange chromatography detection limit of 0.8ppm for VFA, combined with the small amounts of methane produced cannot drive to a clear conclusion. Previous studies showed that carbohydrates and proteins don't accumulate (Chapter 4.3) at 4°C; this suggests that the 2nd scenario is more plausible. The combined lipid degradation activity and methane production at 8°C suggest that temperature increase of 4 degrees is sufficient to convert lipids to methane. Consequently, the trigger for lipid hydrolysis and its conversion to methane lays in-between 4-8°C, whereas carbohydrates and proteins seem to be the only available substrate at 4°C for the specific inoculum. This drives to the conclusion that introduction of energy for temperature elevation needs to be taken into consideration only when temperature drops lower than 8°C. This conclusion is promising considering that most anaerobic digesters operate to temperature >35°C, a fact that results in increased maintenance costs, high carbon footprint and unsustainability.

Incubation of all lipases at 37°C clearly answers whether it is the enzymes themselves or their absence the 'Achilles's heel' for anaerobic treatment at low temperatures. Obviously the lipases are present (Figure 6.5.a, b, c) at all temperatures; their activity varies based on temperature. Thus, bacterial organisms that produce lipases do exist at low temperature and are capable to produce extracellular enzymes.

From a comparison between the activities of the reactors per gVSS at 37°C (Table 6.1) with similar studies it is concluded that the lipolysis activity of the particular psychrophilic/cold-adapted inoculum is very promising. Kim et al (2012) estimated the activity of the lipases from a mesophilic (35°C) and a thermophilic (55°C) anaerobic sequence batch reactor equal to 60 and 65 U.gVSS^{-1} respectively when at 37°C. These values are 6.2, 8.4 and 13.2 times lower than the activities that were estimated at 37°C incubation from the lipases produced from the 4, 8 and 15°C inoculum respectively Actually the activities after incubation of the inoculum at the operational temperature, 8°C and 15°C, found to be two times lower and equal to Kim et al (2012) ´s activity per gram VSS. Additionally Gessesse et al (2003) who optimized this method measuring the activity from activated sludge samples measured an activity of approximately 300U.gVSS^{-1} at 37°C, close to the one valued in this study when the 8°C inoculum was incubated at 37°C but less than the 15°C when at 37°C.

The amount of proteins was quantified (Figure 6.3.a) as an indicative value of the enzymes present; however it does not necessarily correspond to the lipase amount, as not all proteins are lipases. Though, it is the most suitable quantification approach as it is the closest parameter to enumerate the enzymes. . Although the results were also correlated with the VSS content (Figure 6.3.b) the approach would be perilous and was used only comparison purposes with similar studies. The nature of the seed (sediment) is rich in plant material or other inadequate for the process bacterial cells (e.g. *Diatoms*) that would result to underestimation of the seed's lipolytic ability

Expressing the activity, based on the protein content did not result to a noteworthy difference at the operational temperatures (Figure 6.5.b). This means that the limitation of lipid degradation cannot be tackled by the seed:substrate ratio increase unless high volume of seed would be introduced as specific activity at 4°C is negligible. Incubation at 37°C showed a different picture. Even though growth rates and cellular populations might be low at 4°C, 'specific activity' was slightly higher than at 15°C when both samples were incubated at 37°C. This suggests that although lipolysis is limited at low temperatures, the enzymes are present, able to hydrolyze substrate faster when temperature raises (Figure 6.5.b, 6.6). The higher specific lipolytic activity at 4°C suggests that a bio-

reactor that operates at low temperatures should be seeded with an inoculum strictly acclimatized to low temperatures, preferably at 4ºC. This would ensure apart from a higher lipid degradation rate, higher methane production activity and a more stable microbial community (Chapter 5.1, 5.2.). In other words, I recommend that the start-up of bio-reactors that operate to ambient-low temperature conditions need to take place during the winter period.

Wastewater lipolytic activity varied according to wastewater process origin. Primary settled wastewater showed no activity at the operational temperatures. On the other hand, for raw wastewater the activity was similar to the reactors' samples at 4, 8 and 15ºC (Figure 6.5.a and 6.8.a respectively). Thus, the lipolytic activity in a bio-reactor would be promoted by the addition of the indigenous from wastewater communities. This was also shown on Chapter 5.1, 5.2, as non-sterile microcosms had the higher hydrolytic rates that resulted to higher methane production rates. Raw wastewater activity was also detected at 4ºC, lower than at 8 and 15ºC, but slightly higher from the seeded samples.

Methanogenesis occurred faster and at higher rates (Figure 6.4) the higher the temperature. This was supported by the lipolytic activity (Figure 6.7), pointing a strong relationship amongst these 2 processes. This makes sense as lipids account for an important COD fraction (Raunkjaer et al 1993).

Although the gas volume was relatively low due to the low wastewater COD content, methanogenesis did not stop. This shows that even a small amount of carbon is enough for the cells to kick off. I tend to believe that the introduction of generally low in COD wastewater allows only the highly active cells to stay alive or 'awake' regardless the starvation, ready to metabolise any type of available carbon source.

In terms of wastewater engineering the study suggests that the problem of lipid accumulation can be tackled by the daily day-night cycle, as a 4-degree temperature increase is adequate to trigger the reaction. At extreme latitudes though, such increase is not always likely. In such cases the accumulation can be tackled by the seasonal variation, where lipids that accumulate during an extended cold period would be utilized during a warmer one (as generally shown on Chapter 5.2). As it would be worthless to increase the seed: substrate ratio due to the reasons described earlier (activity approx. 0.0 t 4ºC), the most applicable engineered solution would be an enzymatic pool for pre- or post-treatment. High amounts of purified enzymes would be introduced to the system to increase the lipolytic capacity and lead to higher COD:CH$_4$ conversion. This would require a 2-phased reactor configuration with a minimum operational temperature at 8ºC. In terms of applicability, I recommend encapsulation of enzymes in beads and set in a reactor with a membrane barrier.

6.7. Conclusion

Lipid degradation rate reduces the lower the temperature gets due to lipase activity kinetics in addition with changes in lipid structure.

Enzymes are present at all temperatures ≥4ºC, those that are produced from the cells acclimatized at 4ºC have higher degradation capacity than those generated from the cells acclimatized at higher temperatures when both at warm conditions. This suggests that inoculation of a bio-reactor with low temperature acclimatized biomass is highly desired.

Indigenous raw wastewater cells also produce enzymes able to hydrolyze lipids at low temperatures. The cells from primary settled wastewater though were generally found of negligible lipolysis capacity, possibly due to low population as showed from VSS and protein content.

Lipolysis is limited at 4ºC however carbohydrates and proteins seem easier to be hydrolysed. A 4-degree temperature increase (from 4-8ºC) is adequate to trigger the COD$_{lipid}$:CH$_4$ conversion.

The daily day-night cycle in addition with the seasonal variation would efficiently reduce the accumulation of lipids.

Chapter 7: *Engineering low temperature anaerobic treatment of domestic wastewater - scale up*

Figure 7.1 - Construction site works of an anaerobic digester at Kansas (ethanol plant with start-up planned soon); Dec. 2012 (Ethanol Produce Magazine, Dec. 2012).

7.1. From lab to treatment plant, scaling up via optimizing HRT in a digester operating at 4ºC≤T≥15ºC - volume, HRT & cost.

7.2. Abstract

The transition of a research study from a bio-reactor phase to a treatment plant is challenging as variety of parameters need to be considered. An anaerobic treatment simulation was carried out based on the effect of temperature to specific methanogenic activity. A 36-months temperature dataset from Newcastle upon Tyne (UK) region was introduced to adjust the specific methanogenic activity from 4 to 15ºC, and achieve wastewater treatment for a hypothetical municipality of 20.000 equivalents persons. Safety factors inspired from the $COD_{removed}$:$COD_{methanized}$ were included. The results showed that low temperature anaerobic treatment is feasible with this specific inoculum, when the methanogenic population will reach the 1.52×10^9 cells.ml^{-1}, at an average HRT that lies in-between 8.8 and 13.5 hours and corresponds to a volume of 745 to 1125 m^3. The results also showed that there is a significant relationship between previous and current's month temperature, and subsequently HRT. Finally, it was shown that the capital cost for the particular HRTs and volumes does not decisively vary and further details need to be considered for scale-up applications.

7.3. Introduction

A successful experimental scale up declares whether an experimental study was productive or not. In terms of wastewater treatment this is highly important as scaling up from a bio-reactor to a treatment plant requires a massive investment of time and money.

Full scale anaerobic wastewater treatment plants at mesophilic and thermophilic conditions are generally designed based on Monod kinetics. These equations were introduced in the 60s to describe the cellular metabolism for wastewater treatment engineering. At lower temperature though, it is not certain whether the kinetics would follow these equations. Likelihood of substrate´s physical state changes (Neidleman 1987), variation of media density (Veeken and Hamelers 1999) and differentiation in bacterial-archaeal diversity render Monod kinetics an oversimplified way to scale up low temperature anaerobic wastewater treatment. Furthermore, the introduction of VSS in the calculations (Monod) would possibly lead to biomass underestimation, due to the biomass nature (sediments contain plant material that result to excess VSS which might mislead the design) and the presence of insoluble COD (as VSS, Chapter 4.1). This could potentially cause treatment failure; consequently VSS need to be avoided. Thus, there is a need of a different approach in sizing and scaling up a treatment plant operating at low temperatures.

I recommend the introduction of specific methanogenic rates for HRT and size calculation as methanogens are the fundamental cells, which finalize the process, converting all intermediates to CH$_4$, I believe that specific methanogenic rates are the key to accurately evaluate the capacity of the seed, and subsequently the size of the treatment vessel. As cellular activity changes according to temperature, 'reactor volume-performance-behavior' would follow the same pattern. Thus, sizing via specific rates following the temperature patterns would include the effect of ambient temperature variation to the biomass as an in principle fundamental design parameter. This would lead to a treatment plant that includes the only un-controlled parameter that dictates the treatment performance in the design, temperature.

To achieve complete treatment the HRT needs to be accurately estimated. A mistaken estimation would lead to treatment plant flooding or biomass starvation and decay. Hence optimizations are required.

I believe that there is a relationship between the previous and current month's temperature, considering that there is a 'smooth' climate transition between cold and warm periods during the year time. An equation that connects these two would minimize the HRT errors from a mistaken weather forecast as it would include a known temperature value. This correlation between temperatures would promote more accurate HRT adjustments in anaerobic wastewater treatment plants operating at low temperatures and would lead to a more reliable and efficient treatment.

 In this study 3 approaches are presented for HRT evaluation. The actual HRT, based on the cellular performance as a function of a 3-year temperature data-set, the optimized one, by increasing the polynomial trend line fit of the actual HRT versus time and the 'forecasted' one, where the HRT would be estimated including the previous and

current month's temperature. The last would assist the operator engineers to adjust the HRT accurately, minimizing the effect of a false weather forecast by including the previous month's temperature.

Finally, as HRT dictates the reactor volume, it potentially affects the cost. Capital cost estimation was carried out for the 3 HRT-volume approaches.

7.4. Materials and Methods

For applicability purposes 7 assumptions were made.

Assumptions:

1. 2010-2012 temperature data were collected for Newcastle upon Tyne region (Figure 7.3.) (Metoffice)
2. The methanogenic population of the seed (seed:substrate, 1:7) assumed for the study as 7.6×10^8 methanogenic cells per ml (or 1.52×10^8 for a $\times 2$ biomass increase, 1:3 seed:substrate from 1:7, or 1:4 seed:total vol. to 1:8 seed:total vol.).
3. All decimal places at temperatures are excluded (<0.5 rounds down, >0.5 rounds up), (concept 3 below was carried out with both actual and adjusted T values).
4. Temperatures lower than 4 were set as 4°C; similarly, temperatures higher than 15 were set as 15°C (concept 3 below was carried out with both actual and adjusted T values).
5. Maximum population served by the WWTP = 20.000 persons
6. Organic load per person = 55g COD.person^{-1}.day^{-1}; hydraulic load = 100L.person^{-1}day^{-1}
7. 50% of the raw influent is removed from preliminary treatment in terms of COD; 125 mgCOD.L^{-1} is the acceptable discharge level according to UWWTD (91/271/EEC) and the desired treatment level.

The ratio seed:substrate was selected as 1:3. From the previous 2 studies (Chapter 5.1 and 5.2) it was showed that this ratio is efficient in COD:CH$_4$ conversion and ensures predominance and preservation of the microbial diversity regardless temperature or treatment (non sterile wastewater) conditions. The calculations were carried out using the data from a 1:7 seed:substrate approach (based on Chapter 5.2) and were converted to a 1:3 ratio as the latter ratio guaranteed the predominance of the arctic inoculum cells over the wastewater originated ones. The acclimation of biomass at 4°C rather than at 15°C is highly recommended (Chapter 5.2), hence a start-up over the winter is highly desired.

The HRTs and reactor size estimation is based on the maximum specific rates observed (Figure 7.2.). Activity from 4, 15°C and the temperature switches from higher to lower range and vice versa were included. Linear interpolation was applied for the values in-between (Eq. 17). At this point an exponential approach could have been more representative; however as there is no 3rd value in-between, the linear approach is the only option.

$$y = y_1 + (x - x_1) \times \left(\frac{y_2 - y_1}{x_2 - x_1} \right) \qquad Eq.\ 17$$
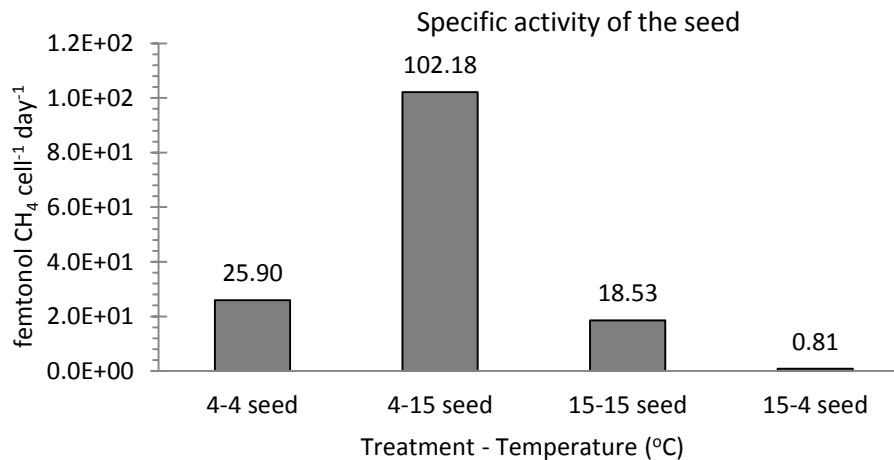
Figure 7.2 - Specific methanogenic activity from seeded samples with un-hydrolyzed wastewater material; the numbers show acclimation temperature -operational temperature (reproduced from Chapter 5.2, Figure 5.12).

Concept 1: Sizing a reactor

- The specific rates (fmols $CH_4.cell^{-1}.day^{-1}$) were converted to accommodated OLR ($kgCOD.m^{-3}.day^{-1}$)
- The wastewater based on the municipal population was converted to concentration ($kgCOD.m^{-3}$)
- From the cellular methanogenic capacity and the concentration that needs to be treated the HRT was estimated; the introduction of a factor inspired from Chapter 4.2 as $COD_{removed}:COD_{methanized}$ (Figure 7.4.) was introduced to include the possibly accumulated fraction of un-hydrolyzed material (as COD)
- The HRT was expressed in hours based on a 1:7 seed:substrate ratio (as specific rates were estimated at the particular ratio (Figure 5.12)) and then divided by a factor of 2 to represent a 1:3 ratio.
- HRT × Q (flow) results to the required tank volume; choosing a circular digester the 'h' height and 'd' diameter were selected.
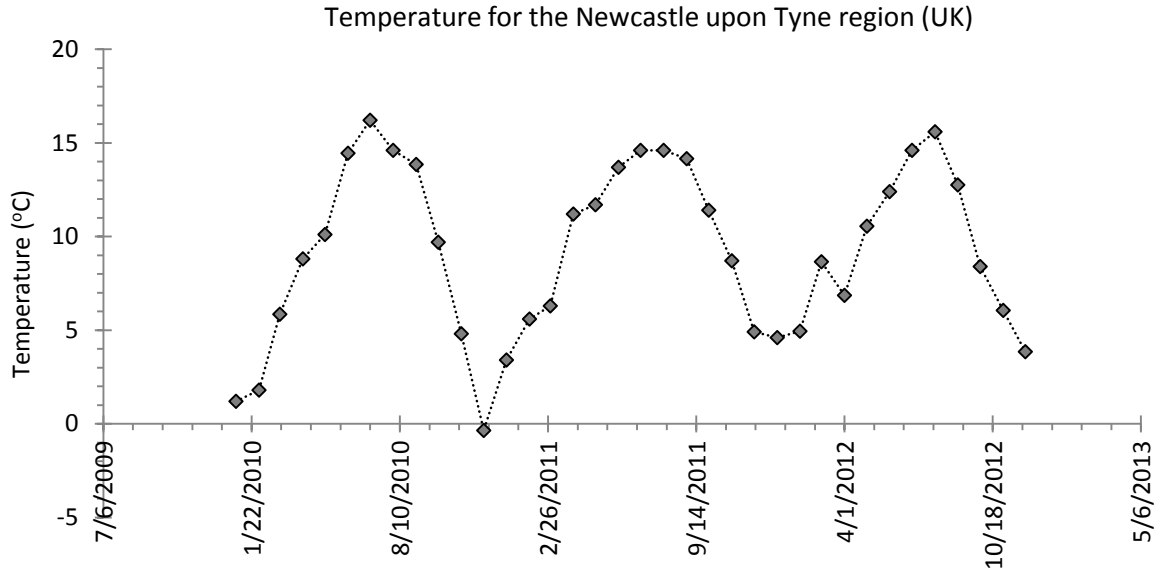
Figure 7.3 – Newcastle upon Tyne temperature for the period 2010-2012 (Metoffice).
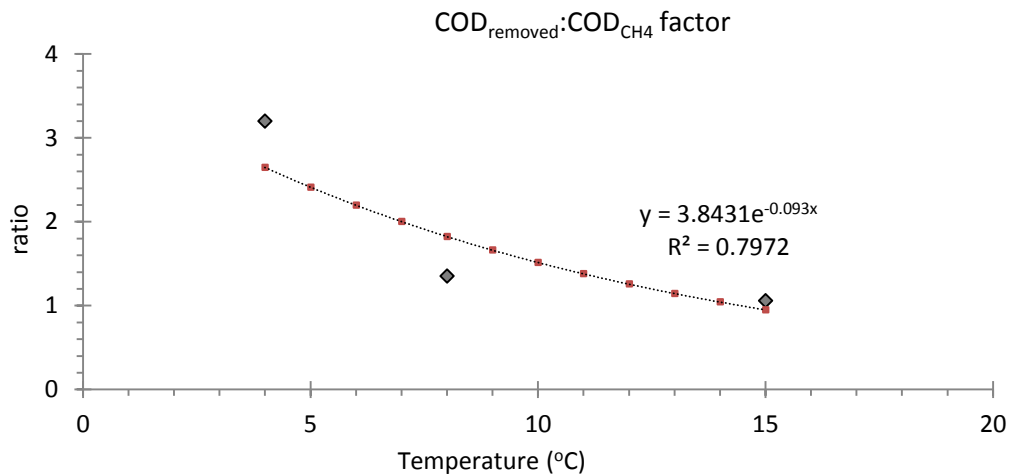
Figure 7.4 – ratio COD removed to COD methanized (reproduced from Chapter 4.2) versus temperature

From Figure 7.4, solving for 'y' the safety factor values for all temperatures can be estimated (Table 7.1):

Table 7.1 – Safety factor as a fraction of COD methanized to total COD removed

T (°C)	COD $_{removed}$: COD $_{methanized}$
4	2.65
5	2.41
6	2.12
7	2.00
8	1.83
9	1.66
10	1.52
11	1.38
12	1.26
13	1.15
14	1.05
15	0.95

Concept 2: Optimizing HRT

On excel a polynomial trend line cannot exceed the 6th degree; to improve fit a multivariate regression is required. This would increase the R^2 and the trend line fit between the data points at a level that will not affect the Diagnostics tests. In terms of wastewater engineering this would provide with a more accurate estimation of the average HRT. In practice this would create a model that can accurately describe the manipulation of the methanogenesis from wastewater performance according to the regional temperature pattern.

The regression analysis was carried out on Excel 2010 using the Analysis Tool Pak add-on. The HRT values were logarithmically transformed and the calendar dates were expressed numerically as in sequence (i.e. January 2010 = 1, February 2010 = 2…36). This step is mandatory as regression analysis strictly assesses numerical data. The time values were further transformed using the *Eq.*18 and formed a matrix of [8x36] (Appendix - Appendix 3, Table a.3 blue cells). No specific mathematical background lies behind this option other than the need of a transformation that would 'lift' the regression (Figure 7.5). Other transformations were also tested with no significant fit (square roots, squares, etc.).

$$x_1 = 1^1$$

$$x_2 = 1^2$$

$$x_3 = 1^3$$

$$x_4 = 1^4 \qquad \text{Eq. 18}$$

$$x_5 = 1^5$$

$$x_6 = 1^6$$

$$x_7 = 1^7$$

$$x_8 = 1^8$$

Where 1 = the numerical value of the month 1, 2, 3... ..36, and superscript is the polynomial degree (1, 2... 8).

The regression amongst the 2 matrixes Ln(HRT) and Time (transformed) provided with an equation of 8 coefficients and an intercept 'c'. This formed a table of [1x8]. The 8th degree was chosen as the regression achieved the maximum R^2, without failing the diagnostics tests (heteroscedasticity, autocorrelation and multicollinearity). Trials with more/less than 8 coefficients were also analyzed but were rejected due to lower validity with regards to the statistical diagnostics tests compared to the 8th degree polynomial.

All 8 coefficients were tested for statistical significance prior use (ANOVA). Apart from diagnostic tests Akaike criterion (AIC) tests were also carried out, showing also that the 8th degree polynomial is the most suitable regression to describe the HRT based on temperature relationship.

The new trendline was designed (Ln(HRT) on Y axis versus time)) using the appropriate x (transformed time 1, 2, ..8) and y (coefficients 1, 2, ..8) in the equation and the function MMULT (matrix multiplication) of excel, adding the intercept 'c'. Setting the new values on the natural logarithm led to the estimation of the new optimized average HRT.

Concept 3: Predicting HRT & decision making

To define the relationship between the current and previous month´s temperature and HRT a new regression was carried out and studied, combining the Ln(HRT) from 'concept 2' and the 2 temperatures (current 'T$_{(t)}$' and previous 'T$_{(t-1)}$') for all months excluding the 1st. The 'T$_{(t)}$' plays the role of the one given from a hypothetical weather forecast. The regression was carried out based on both actual and adjusted temperatures.

Using Excel 2010 and the Analysis Tool Pak add-on, the new model equation based on the parameters above was designed. Statistical tests (ANOVA and Diagnostics (heteroscedasticity, multicollinearity and autocorrelation)) investigated the coefficient validity and their statistical significance. The results showed that high validity is ensured for the two coefficients of the equation that correlates the current temperature (T$_{(t)}$) with the previous month's one (T$_{(t-1)}$) and the constant 'c' (for both actual and adjusted temperatures).

Multiplication of the coefficients by the corresponding temperatures (a$_1$ x T$_{(t)}$ and a$_2$ x T$_{(t-1)}$, (*Eq.*19)) leads to an estimation of a new HRT, which is correlated with the previous month's T. The new model proves that there is a significant relationship between previous 'T$_{(t-1)}$' and current 'T$_{(t)}$' temperature, and subsequently between their corresponding HRTs.

$$\textbf{Ln(HRT}_{\textbf{forecasted}}) = a_o + a_1\ T_{(t)} + a_2\ T_{(t-1)} \quad \textit{Eq. 19}$$

Where a$_o$ is the constant ´c´, a$_1$ and a$_2$ are the 1st and the 2nd coefficient, t is time and T accounts for the temperature.

The forecasted approach was carried out with both the adjusted (assumption 3, 4) and actual temperature values with no significant difference observed in terms of HRT; this shows that actual and adjusted temperatures are highly correlated (tan φ: 0.982).

An interesting approach would also be the introduction of the rate of change of temperature. This would assist in the projection of the future temperature by us; however this is not the purpose of this study and not much analysis was carried out on that.

Concept 4: Capital cost

The total capital cost (C$_{TC}$) of a digester is highly related to the reactor volume (*Eq.* 20) (Brown 2003).

$$C_{TC} = M \times V_D^{SF} \qquad \textit{Eq. 20}$$

Where V$_D$ is the digester volume, SF is the scaling factor and M is the multiplier based on the type of unit operation (i.e. Brown 2003). For anaerobic digestion though such values are unavailable. AgSTAR (2009) provided with cost related graphs for plug flow digesters (at 2005-2008); thus the equation below (*Eq.* 21) can deliver a rough estimation of the cost.

$$C_{TC} = 617 \times S_{OP} + 566.000 \qquad \textit{Eq. 21}$$

Where S$_{OP}$ is the operational size that can be estimated from the equation below (*Eq.*22). Changing the potential methane (PCB) and expected energy from methane (ED) according to the domestic wastewater characteristics we would have a representative of the cost of a digester treating domestic wastewater equation (*Eq.*22).

$$V = S_{OP} \times \left(b \times \frac{HRT \ \times PCB \ \times f_{CH\$}}{ED \ \times f_{BD} \times TS} \right) \qquad \textit{Eq. 22}$$

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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Where V is the reactor volume, S_{OP} as above, beta (b) is a lumped unit conversion (numerical value is 0.0377) (Faulhaber et al 2012), HRT is the hydraulic retention time (calculated above), PCB is the potential of methane per person ($0.020625 m^3$/person/day for 55gCOD/person/day), f_{CH4} is the percentage of methane in the biogas composition equal to 90% (from Chapter 4.2), ED is equal to the energy expected equal to 14.0 MJ/kgCOD (Heidrich et al 2011), f_{BD}, TS are the total solids equal to 0.03kg/L (Chapter 6, estimated for the VSS needs).

Solving $Eq.22$ for S_{OP} and using the $Eq.21$ the C_{TC} can be estimated.

The same can be carried out for the HRT_{actual}, $HRT_{non-optimized}$ and the $HRT_{forecasted}$ and end up with a comparative cost study with the only parameter that changes be the Volume based on the HRT.

The price needs to be corrected to 2013 US Dollars ($) (8.9% reduction based on U.S. Inflation 2013).

As the treatment plant is not initially planned to produce biogas for electricity purposes a 36% correction (reduction) in the total cost price is recommended (USDA 2008).

7.5. Results

Concept 1

The collected temperature data (assumption 1) were rounded and transformed (all $4^oC < T < 15^oC$) according to assumption 3, 4; the temperature time series is presented on Figure 7.5.a. Combining the methanogenic population (assumption 2), with the temperature pattern (switch up or down or stable) and the activity from Figure 7.2, the specific activity for 7.6×10^8 cells per ml per day at all temperatures was estimated and presented on Figure 7.5.b. The activity was further converted into the OLR that can be accommodated. From the population, the organic/hydraulic load per person and the pre-treatment efficiency (Assumption 5, 6, 7) the concentration that needs to be treated estimated at 0.15 kgCOD.m^{-3}. From the comparison between the OLR that can be accommodated and the concentration that needs to be treated, including the safety factor values (Table 7.1) the treatment HRT is estimated (Figure 7.5c for a seed:substrate ratio of 1:7 and 1:3). All steps are shown on Appendix – Appendix 3, Table a.3.

The HRTs from Figure 7.5c has an average of 27.0 and 13.5 hours for the 1:7 and 1:3 seed:substrate ratios respectively. From the population (assumption 5) a flow of 2.000 m^3.day^{-1} enters the WW treatment plant; as $V = Q \times t$, solving for V (m^3) a volume of 2245.8 and 1122.9 m^3 for 1:7 and 1:3 seed:substrate ratios respectively is required. Selecting the 1:3 ratio, the proposed dimensions for a cylindrical digester are shown on Table 7.2:

Adjusted Temperature for the Newcastle upon Tyne region (UK)

Specific activity as a function of temperature

HRT from OLR as a function of temperature

$$y = 5E\text{-}14x^6 - 1E\text{-}08x^5 + 0.0013x^4 - 72.079x^3 + 2E\text{+}06x^2 - 4E\text{+}10x + 2E\text{+}14$$
$$R^2 = 0.4893$$

$$y = 3E\text{-}14x^6 - 7E\text{-}09x^5 + 0.0007x^4 - 36.039x^3 + 1E\text{+}06x^2 - 2E\text{+}10x + 1E\text{+}14$$
$$R^2 = 0.4893$$
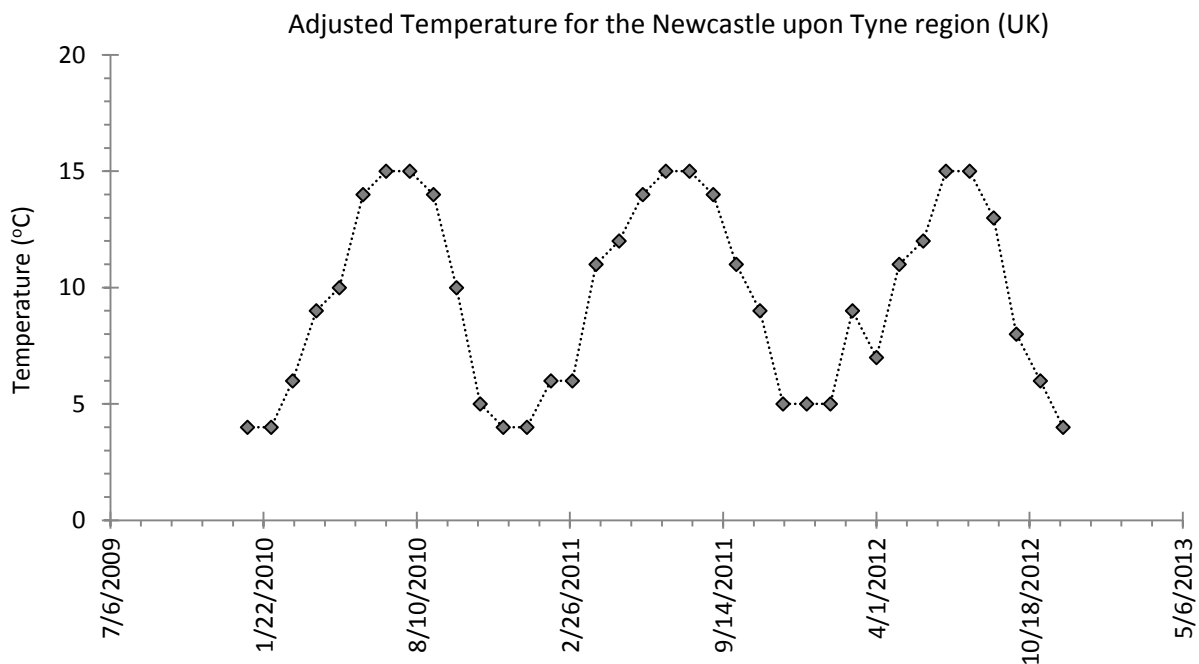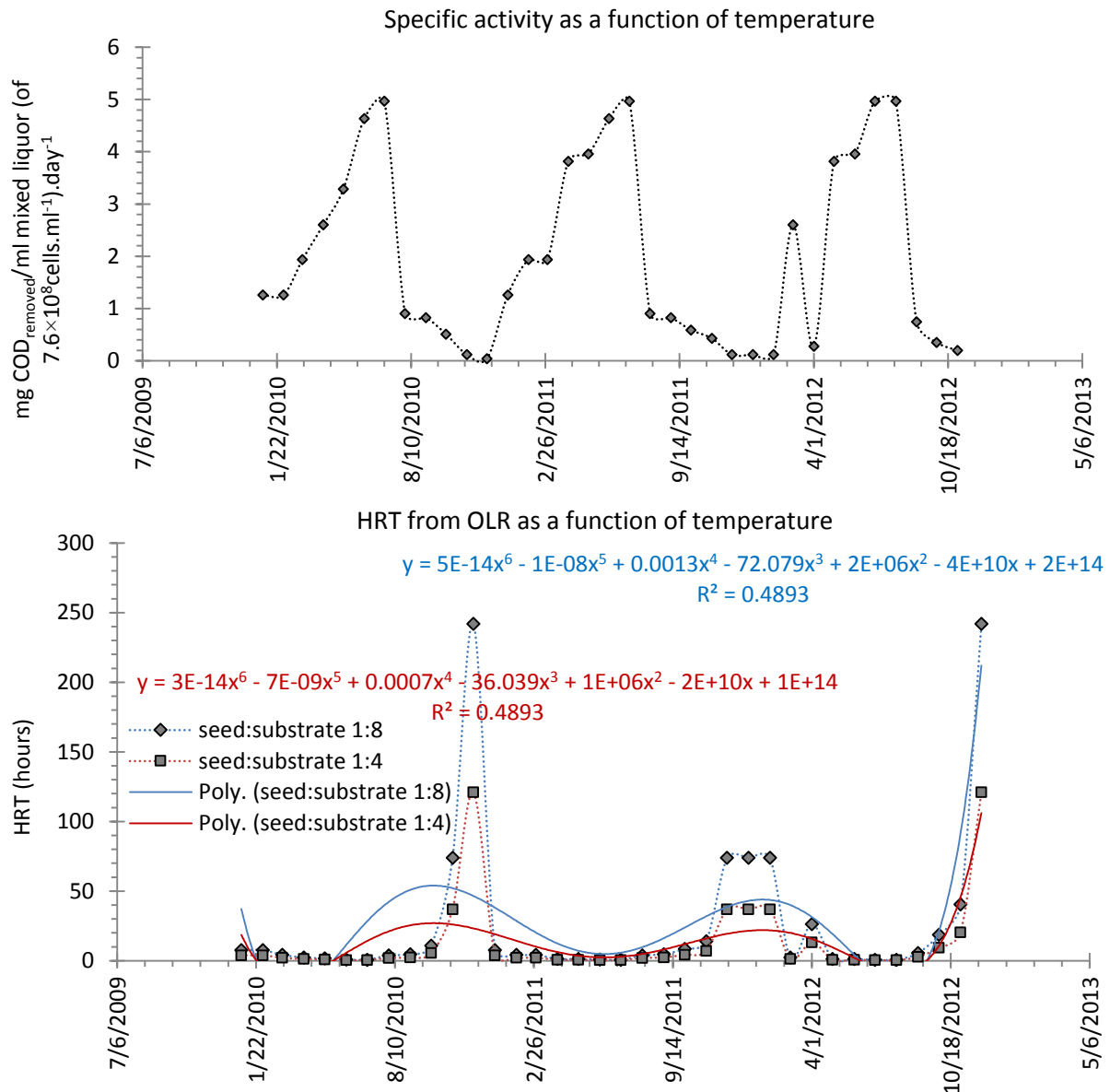
Figure 7.5 – a) Adjusted temperature, rounded and transformed (all 4ºC < T < 15ºC) (assumption 3, 4) as function of time; b) Specific activity as a function of temperature trend (up, down, stable) for the desired methanogenic population (assumption 2) and Figure 7.5a); c) estimated HRT for 1:3 and 1:7 seed:substrate ratios – trend line based on 6th degree polynomial.

Table 7.2 – Digester design parameters

Parameters	Value
h (m)	8.30
d (m)	4.10
ratio h:d	2.02
Area (m^2)	13.20
total vol. (m^3)	1135.70

Concept 2

After the estimation of a general HRT and the selection of the 1:3 ratio, an optimized regression fit between HRT and time would provide with a more accurate value in terms of HRT and tank volume.

The HRT values from 'concept 1' were logarithmically transformed; the independent variable (calendar months) was numerically expressed as 1, 2, 3.. .36. After selection of an 8 degree polynomial the values of time were

I n v e s t i g a t i n g t h e t r u e l i m i t s o f a n a e r o b i c t r e a t m e n t o f w a s t e w a t e r a t
l o w t e m p e r a t u r e u s i n g a c o l d a d a p t e d i n o c u l u m
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further transformed based on *Eq.* 18. This formed a new matrix of [8x36]. A new regression between ln(HRT) [1x8] and the transformed time [8x36] (Appendix - Appendix 3, Table a.4 blue cells for transformed time, red cells for LnHRT) provides with an equation of 8 coefficients, or a table [1x8] and an intercept. The new regression is shown below (*Eq.* 23, Table 7.3).

Diagnostics tests for heteroscedasticity, multicollinearity and autocorrelation showed no systematic error (high R^2 adjusted), no linear relationship and generally no relationship between the trendline's residuals respectively (autocorrelation, heteroscedasticity, multicollinearity). This proves that the equation should not be optimized further than the 8-degree polynomial (autocorrelation = 1.36). The 8 degree option ensures that the AIC criterion value is the minimum from all other options of coefficients' numbers, a fact that also supports the model-equation validity. An ANOVA test (Appendix - Appendix 3, Table a.6) also proved the statistical significance of the equation (Table 7.3). Its significance is also supported from the high R^2 and R^2 adjusted (Appendix - Appendix 3, Table a.5), the 2nd supports that its validity is unlikely to change with addition of more variables-predictors.

Regression equation (*Eq.* 23):

$$Y(t) = -3933c + 8.539t_1^1 - 4.282t_2^2 + 0.893t_3^3 - 0.094t_4^4 + 0.0054t_5^5 - 0.0002t_6^6 + 2.91E\text{-}06t_7^7 - 2.00E\text{-}08t_8^8$$

$$R^2 = 0.7951 - R^2 \text{ adjusted} = 0.7345$$

Table 7.3 – Equation coefficients with corresponding statistical validity results (from ANOVA)

	Coefficient*	Std. Error	t-Statistic	Prob.
c	-3.9327	2.5550	-1.5392	0.135394622
t1	8.5389	2.7709	3.0816	0.004699298
t2	-4.2820	1.0131	-4.2268	0.000242294
t3	0.8930	0.1750	5.1032	2.30828E-05
t4	-0.0939	0.0164	-5.7264	4.36393E-06
t5	0.0054	8.8090E-04	6.1480	1.43383E-06
6	-0.0002	2.7102E-05	-6.4153	7.13698E-07
t7	2.9123E-06	4.4355E-07	6.5659	4.83221E-07
t8	-1.9831E-08	2.9917E-09	-6.6287	4.11033E-07

*The coefficients correspond to the *Eq.* 23 intercept and coefficients, c, t_1, t_2 t_3, t_4, t_5, t_6 ,t_7, t_8
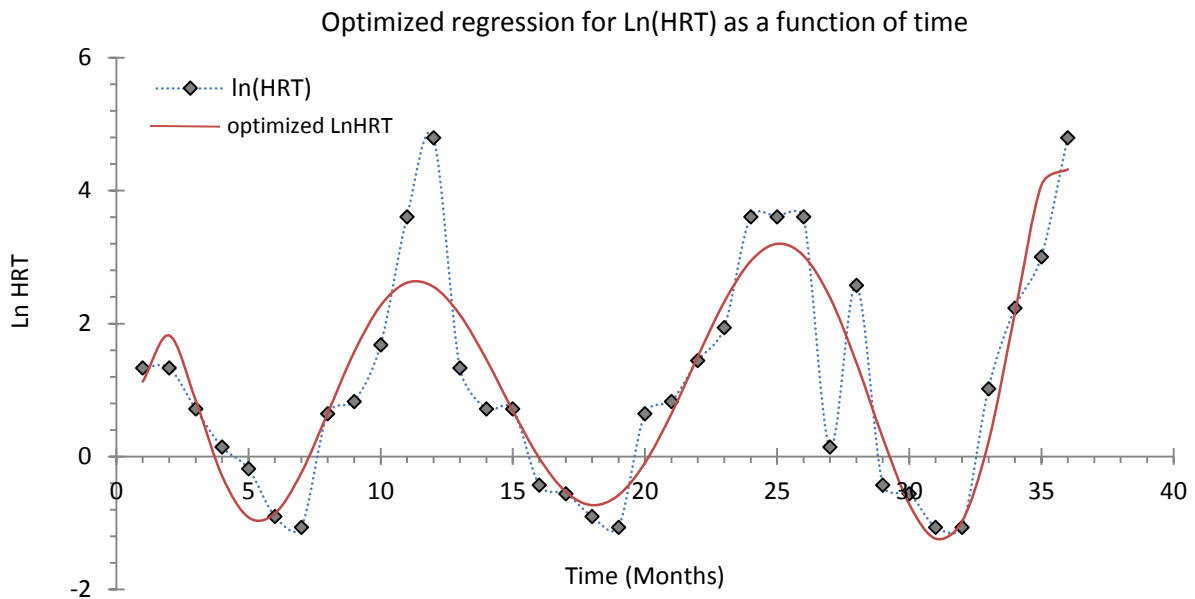
Figure 7.6 – Logarithmically transformed HRT as a function of time and its new optimized polynomial trendline (8th degree) (*Eq.* 23).

Solving *Eq.* 23 using the transformed time data results to a new update 8th degree polynomial trendline (Figure 7.6). On Excel this is feasible using the MMULT function between the matrix of the coefficients and the one of the transformed time, adding the intercept 'c'.

The new values from the multiplication were set on the natural logarithm to convert them to HRT (hours). From the new values the average optimized HRT was estimated, $HRT_{optimized}$: 8.88 hours.

Knowing the flow (Q) and HRT (t) the volume of the digester was estimated as $740.1m^3$; in detail (Table 7.4).

Table 7.4 – Optimized digester design parameters

Parameters	Value
h (m)	7.4
d (m)	3.80
h:d	1.95
Area (m^2)	11.34
Volume (m^3)	747.54

Concept 3

To build a relationship between $HRT-T_t$ and $HRT-T_{(t-1)}$ a new model is required to involve both temperatures. Applying regression between actual T_t, T_{t-1} and Ln(HRT) the equation below (*Eq.*24) is formed. LnHRT accounts for the Ln transformed values of HRT that were estimated on concept 1. The X, Y for the regression is showed on Appendix – Appendix 3, Table a.7 (blue for X, red for Y).

$$Y_{(HRT)} = 3.392a_o - 0.564T_t + 0.320T_{(t-1)} \ (Eq. \ 24)$$

With an $R^2 = 0.849 - R^2$ adjusted $= 0.840$

Where a_o is the constant ´c´, T_t is the coefficient for current temperature, and $T_{(t-1)}$ is the coefficient for the previous month's one

The equation is significantly valid as it implies to all diagnostic tests (heteroscedasticity, autocorrelation and multicollinearity), with high R^2, R^2 adjusted and statistically significant coefficients based on their p-values (>95% validity) (Table 7.5) (Appendix – Appendix 3, Table a.9, 10). Akaike criterion (AIC) was also found <1.0.

Table 7.5 – Equation coefficients with their corresponding statistical validity (from ANOVA)

	Coefficient	Std. Error	t-Statistic	Prob.
a_o	3.392605	0.285423	11.886233	2.816E-13
T_t	-0.563846	0.044609	-12.639625	5.5126E-14
$T_{(t-1)}$	0.320419	0.043455	7.373416	2.1880E-08

Combining the current and previous month's temperature with the equation coefficients based on the significant relationship between 'T_t', 'T_{t-1}' the forecasted HRT can be estimated (Figure 7.7, 7.8).

The regression procedure was repeated for the HRT forecast based on the adjusted temperature data (Appendix – Appendix 3, Table a.8). The new coefficients were really close to those estimated from the actual temperature. The new regression is shown below (*Eq.* 25). ANOVA test for the new coefficients is shown on Appendix – Appendix 3, Table a.11, 12.

$$Y_{(HRT)}´ = 3.633a_0 - 0.5819_t + 0.317_{(t-1)} \ (Eq. \ 26)$$

With an $R^2 = 0.78 - R^2$ adjusted $= 0.766$

Using the natural logarithm the forecasted LnHRT can be converted to HRT (hours). This was carried out for both actual and adjusted temperature. The graph with the actual and forecasted HRT is shown on Figure 7.8. On the figure the optimized HRT versus time from concept 2 was also included.
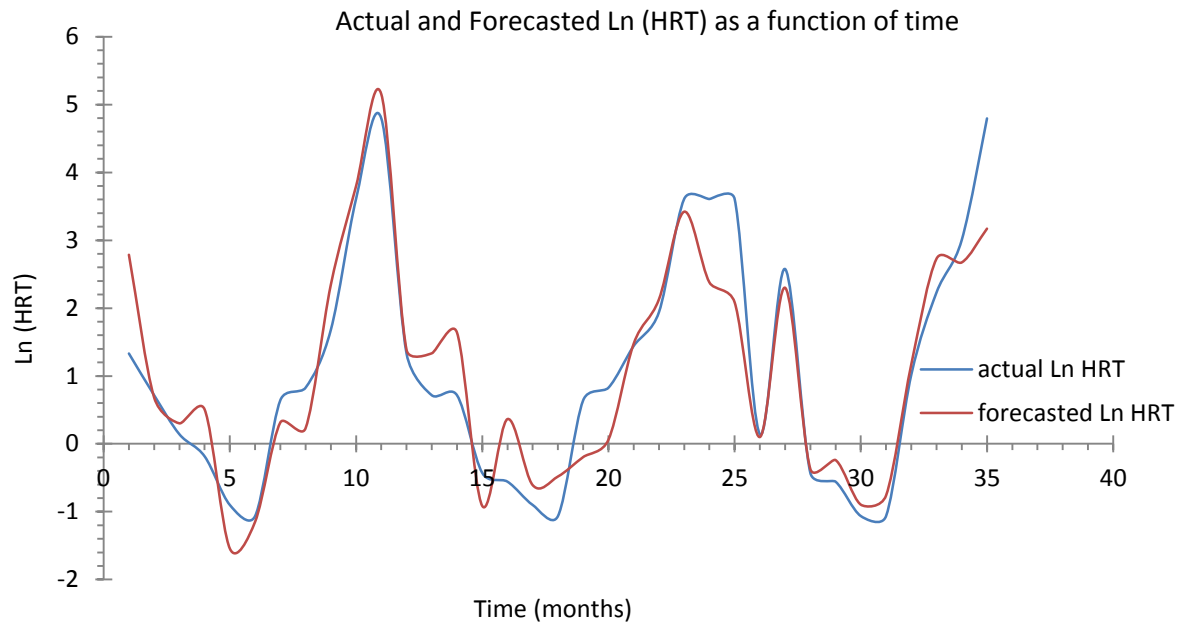
Figure 7.7 – Actual and forecasted Ln(HRT) as a function of time (regression based on the actual temperatures).
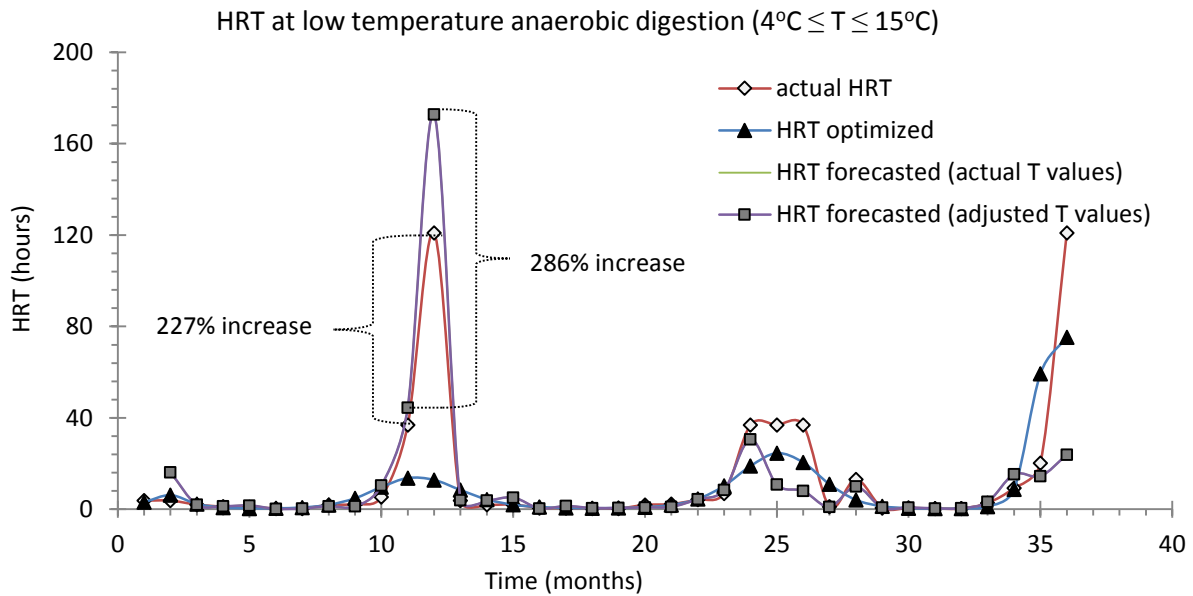
Figure 7.8 – Forecasted (for both adjusted and actual temperature), optimized and actual HRT (defined on concept 1) as a function of time; adjusted and actual lines are overlapping due to the high correlation that renders them almost identical.

Designing a reactor based on the forecasted HRT (actual T) may also be an engineering approach based on the average forecasted HRT_{actual} value (Table 7.6). The use of the $T_{adjusted}$ leads to a minor volume increase of 3m³.

Table 7.6 – Forecasted digester design parameters

Parameters	Value
h (m)	7.80
d (m)	4.00
h:d	1.95
Area (m²)	12.57
Volume (m³)	967.40

A different approach to forecast HRT is by predicting the temperature with no need of a weather forecast. A rough estimation can be carried out based on the rate of change.

The rate of change of the HRT of the 36 forecasted HRTs is estimated as shown on *Eq.*26.

$$\text{Average } ((\text{HRT}_{(t)} - \text{HRT}_{(t-1)}) / \text{HRT}_{(t-1)}) = +0.06342 \ (Eq.\ 26),$$

or 6.34% with 34.3% standard deviation - high variations such as those for months 3, 12, 13 were excluded due to extreme fluctuations (Stdev > 3.0 sigma needs to be excluded Pukelsheim 1994).

Thus, the HRT can be in theory estimated by combining the previous month's temperature plus 'Average $\Delta\text{HRT}_t/\text{HRT}_{t-1}$'. A more accurate approach would also be the introduction of the T-statistic value (1.96) as the variance of the fore coming months is unknown; however, this would create an even higher standard deviation and would risk the applicability of the equation. Its use might be applicable if more values will be assessed to lower the deviation.

As an overall, the validity and description capacity can be increased the more the time passes as more temperature data would be added and result to an improved description of the temperature phenomena over time. This suggests that the model would have the capacity to minimize HRT errors from weather forecasts by including the previous month's temperature. More monthly temperature data is likely to also provide with a more accurate rate of change that would assist in HRT forecasting based on past detailed treatment plant's behaviour. This approach seems to be a useful tool for wastewater treatment plant operators that need to predict temperature conditions, to boost anaerobic treatment capacity at low temperature conditions (i.e. 4°C≤T≤15°C). The approach can not only be applicable in a monthly basis as the rationale is the same even for shorter time periods (weekly or daily average). I believe that a monthly basis is a more representative approach as average temperature variations are more distinguishable compared to those occurring for shorter period of time, and subsequently are enough to cause evident changes in the specific cellular activity.

Concept 4

The optimum solution, the optimized HRT (HRT = 8.82 hours and Volume = 737.4 m³) accounts for a cost that can be estimated by *Eq.* 22, solving for S_{OP} with the parameters as described at Chapter 7.4-concept 4

S_{OP} = 7502.8 as operational size

From *Eq.* 21 solving for C_{TC}

C_{TC} = 5.194.478,2 US Dollars ($)

Due to inflation from 2009 the dollar price reduced by 8.9%.

Thus C_{TC} 2013 = 5.657.117 US Dollars ($)

As the digester is not going to be used for electricity production purposes a reduction of a 36% of the capital cost is acquired:

→ C_{TC} for optimized HRT = **3.672.471 US Dollars ($)**

Similarly for actual HRT (average HRT = 13.5 hours and Volume = 1135.7 m³) the capital cost is:

→ C_{TC} for actual HRT = **3.628.174 US Dollars ($)**

For the forecasted HRT with adjusted or actual Temperature (average HRT = 11.5 hours and Volume = 967.4 m³) the capital cost:

→ C_{TC} for forecasted HRT = **3.651.088 US Dollars ($).**

7.6. Discussion

The 1ˢᵗ Concept shows that it is feasible to estimate an operational HRT for the treatment of a hypothetical municipality based on specific cellular activity. All the expected HRTs are relatively low even though the operation is at low temperature. Exception is the one that was estimated from the transition of higher temperatures to 4°C (up to 121 hours, Appendix – Appendix 3, Table a.3). This indicates the size of the shock that cells

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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experience when an acclimatized at relatively high temperature (15°C) seed transits to an extremely low one (4°C). This also depicts the challenge that previous treatment endeavors met after short term acclimation of mesophilic biomasses to low temperatures (e.g. Bowen et al 2014). The shock can be avoided if the seed was cold-adapted or psychrophilic (e.g. activity of the acclimatized and operative at 4°C). Another benefit of low temperature acclimation is the high methanogenic capacity of the seed with regards to specific activity. Hence, extremely low temperatures (4°C) might be useful for the development of a sturdy community (as discussed on Chapter 5.2), however it might be unattainable as an operational temperature for anaerobic wastewater treatment unless a biomass highly active at such conditions is introduced (e.g. activity 4-4°C, 4-15°C, Chapter 5.2). An average tank volume of 1135.7m^3 is realistic, fact that manifests that LTAD with a proper inoculum may be feasible with no need of high HRT and large tank volumes; however, the development of an active methanogenic community equal to 1.52×10^9 cells.ml^{-1} seems to be the greatest challenge.

The regression from concept 1 between the HRT versus time (Figure 7.5.c) appears with a maximum fit of a 6th degree polynomial and an R^2 of 0.4819. The descriptive capacity of the trendline is poor. Practically, this might lead to operational errors as the regression cannot exactly describe the relationship between the temperature trend and HRT. Hence, further optimization should be carried out via regression analysis. Testing for the best fit from a 7th degree polynomial up to a 20th it was concluded that the 8th degree is the optimum. Higher or lower degrees polynomials couldn´t pass the diagnostics tests (i.e. 8th degree for autocorrelation p = 0.0136, close to the limit). Thus, the optimum degree is the 8th with an R^2 of 0.7951 and an R^2 adjusted equal to 0.7345, increasing the descriptive capacity between HRT and temperature by 49% (Figure 7.6). The high R^2 adjusted value supports that the new regression is unlikely to change regardless if new variables enter the model. This automatically renders the coefficients highly significant. The significance of the new polynomial is also supported by all p-values for the individual coefficients from the ANOVA test. Values close to ≈ 0.000 and a t-statistic >|1.96| demonstrate validity higher than 95%. Another evidence that supports the significance of the coefficients of the equation is the Significance F value, which is really small (1.6×10^{-7}) (R^2 and ANOVA on Appendix – Appendix 3, Table a.4, 5). The optimized regression results to an average HRT that reduces the tank volume by 37% compared to the estimated HRT from the actual values. The smoothness of the curves excludes all peak points and results to an average HRT of 8.82 hours. This value is satisfactory as it is usually workable at mesophilic or thermophilic treatment plants where introduction of energy is mandatory. Thus, a sophisticated design may ensure good performance, similar to conventional wastewater treatment plants even at a temperature range between 4 and 15°C using the specific inoculum. This approach combined with an active at low temperatures seed would easily promote sustainability, not only due to its treatment potential but also based on its easy application (relatively low HRT) and the net energy consumption and neutral carbon footprint (or at least a minimum amount to prevent frost, temperatures ≤ 5°C are not recommended).

Adjusted and actual temperatures showed to be highly correlated (tan = 0.982). This suggests that the assumptions 3, 4 (Temperature round up-down; 4°C < T < 15°C) cannot have a significantly affect the operational HRT. Hence, HRT, volume and cost based on the 1st temperature approach (actual) should be similar to the 2nd (showed on concept 3). The introduction of the 'COD$_{removed}$:COD$_{methane}$' factor plays key role to the treatment efficiency as the lower the temperature the more the un-hydrolyzed material remaining in the tank (shown on Chapter 4.2). Insoluble COD may cause treatment inhibition due to toxicity, biomass degradation or reactor failure if accumulation will reach a certain level. Including this important for the operation parameter excludes one of the factors that usually lead to treatment plants´ failure. Additionally it is a factor that is based on a sophisticated rationale compared to many of the formulas in wastewater engineering that introduce empirical parameters that were observed a few decades ago and their applicability is under question. Apart from this operational factor, no other safety factor was introduced. I believe that in terms of organic treatment this factor is efficient; however in case of high hydraulic fluctuations a 2nd one might need to be taken into consideration.

The model that includes the previous month's actual temperature to estimate HRT (Concept 3) demonstrated high descriptive capacity comparing with the actual HRT (Figure 7.7, 7.8). An additional regression using the adjusted temperature values also showed high accuracy in forecasting HRT. The strong descriptive capacity is supported by the R^2 and R^2 adjusted with values of 0.849 and 0.84 for the regression with the actual temperatures and 0.78, 0.76 for the one based on the adjusted temperature (Appendix – Appendix 3, Table a.9, 11). Comparing these two it seems that the one with the actual temperatures is more significant not only due to the slightly highest R^2, R^2

adjusted but also from the F value that indicates higher validity ($6.7 \times 10^{14} > 3 \times 10^{11}$ for actual and adjusted respectively) based on the ANOVA test (Appendix – Appendix 3, Table a.10, 12).

The HRT$_{(Tt, t-1)}$ model for the HRT estimation including T_t and T_{t-1} revealed that there is a strong relationship between the previous and current month's temperature and subsequently between previous and current month's HRT. The results seem to support my hypothesis that there should be a smooth transition from a warm period to a cold one and vice versa. The new regression appeared to have statistically significant coefficients, with p-values low enough to support a statistical confidence level, passed the diagnostics tests and had a t-statistic >|1.96|. This equation can be a tool to minimize the effect of weather forecast errors to HRT adjustments. HRT is a fundamental wastewater treatment parameter, which ensures treatment reliability; higher HRT values lead to biomass starvation and lower lead to insufficient treatment. Thus, accuracy is mandatory, especially at treatment plants operating at low temperatures, as small temperature fluctuations result to tremendous operation differences (e.g. 1 degree difference between 4 and 5°C leads to massive HRT differentiation). This phenomenon was also evident as expected to the concept 1 and possibly implies for the limit of LTAD in terms of applicability. Generally though, this equation was based on the temperature data that were gained for the Nort-East British region, hence, it is under question whether it can be applicable where the temperature pattern differs. Nonetheless the rational should be the same and following the methodology am convinced that a similar model can be built.

A different approach to forecast HRT is the introduction of the rate of change of HRT to indicate that the current temperature is equal to the previous one differentiated according to the rate. The rate of change was calculated from the sequential differences between the 36 temperature values, however the not so great amount of data combined with their high variance led to high standard deviation. To nullify prediction errors the 1.96 value of t-statistic can be included. This would achieve higher predictive capacity but less accuracy as it will increase the standard deviation. Hence, a model development with more month by month data is likely to boost accuracy and minimize the standard deviation. Thus, it is the engineer's responsibility whether or not to use the rate of change according to the level of accuracy that is required. For the current study I believe that the rate of change cannot lead to a reliable prediction due to the high standard deviation that may reduce the accuracy of the HRT forecast.

As showed on Figure 7.8 the HRT estimation or projection (Concept 1, 3) generally demonstrated a smooth HRT evolution between temperatures except when the transition is from 5 to 4°C, where high HRT values were observed. Specifically, for this transition the retention time increases at least 3 times. Thus, 1 degree of temperature differentiation at the lowest temperature range requires at least 3 times higher reactor volume. In terms of finance and management analysis I believe that a WWTP that operates at 4°C as minimum temperature is not attainable and in terms of applicability it might need to be considered that the temperature limit for anaerobic wastewater treatment lies at 5°C. Maybe a 3rd or more specific activity data points between 4-15°C would have given us a different pattern by revealing a smoothest transition in the adaptation of cells when the temperatures decreases. In general though, the increase of the volume at this level suggests that in terms of applicability the operation of a digester at 4°C is financially forbidden as it would require the use of a massive land investment. Thus, I say that the limit of low temperature anaerobic digestion lies at 5°C.

The 3rd Concept resulted to 35 HRT values (excluding the 1st as there is no previous value) with an average HRT of 11.5 hours to efficiently remove the wastewater COD at low temperature (<15°C). This HRT reduces the tank volume by 15%, compared to the volume that was estimated based on the actual temperature values (Concept 1) and increases it by 23% compared to the optimized one (Concept 2). The difference in terms of volume is important as volume may cause implications, affect the cost (mainly maintenance), the construction area, the construction risk assessment and subsequently the decision making process.

These 3 approaches provided with 3 HRT values, the actual, the forecasted and the optimized. HRT dictates the tank volume capacity, which subsequently affects other parameters in an anaerobic treatment plant. Using the Eq.21, 22 for the 3 HRTs to estimate cost I concluded that the prices do not importantly differ compared to the volume alterations. This happens due to the relationship between the denominator and the numerator (HRT, V). The linear relationship amongst them leads to a standard capital cost with low fluctuations based on the building details. In practice this means that a tank size after a certain point does not significantly alternates the cost. Thus, in terms of application, capital cost cannot point which HRT is the most cost-efficient. Therefore, it is up to the

I n v e s t i g a t i n g t h e t r u e l i m i t s o f a n a e r o b i c t r e a t m e n t o f w a s t e w a t e r a t
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engineer to choose the desired retention time based on secondary factors (i.e. number of reactors, maintenance cost, field management etc.) rather than the overall cost itself.

7.7. Conclusions

The limit of temperature for anaerobic wastewater treatment with regards to applicability lies at 5°C.

An anaerobic treatment reactor with an HRT in-between 8.8 and 13.5 hours, and volume of 745 to 1125 m^3 is sufficient to cope with the WW treatment needs of a municipality of 20.000 equivalent persons, using the particular inocula at a 1:3 seed:substrate ratio, operating at low temperatures (4-15°C).

There is a significant statistical relationship between previous and current month's temperature, subsequently previous and current's month HRT.

HRT estimation via the use of the previous month's temperature leads to a more reliable treatment performance as a known parameter (previous month's temperature) is included.

Detailed analysis of the relationship between HRT and time (or corresponding temperature) leads to a retention time optimization and tank volume minimization.

Reactor scale up at this level (745-1125 m^3) requires a total capital cost of 3.645.514±8852 US Dollars ($). The choice of the desired HRT-Volume should further include other parameters as the cost itself cannot result to a clear decision due to the relationship between the HRT and the tank volume and their position in the particular equation (denumerator and numerator respectively).

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t   l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m

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Chapter 8: *General conclusions & future work*

Figure 8.1 - Image of transparent water movement.

Figure 8.1 - Image of transparent water movement.

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t
l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m
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8.1. General conclusions and recommendations

The specific cold-adapted inoculum efficiently treats anaerobically domestic wastewater at 4, 8 and 15°C (batch 2, 3, Sub-culture 1), based on UWWTD (91/271/EEC) for COD standards.

The start-up of methanogenesis from UV sterile raw wastewater as substrate was highly related to temperature and required 40 to 140 days at 15°C and 4, 8°C respectively (batch 1). The inoculum showed high adaptive capacity to the particular complex substrate at low temperatures as the rates of CH_4 production between the first and the second batch increased up to 15 times at 4°C. Soluble and total COD reduction was also higher on the second batch than at the first. The amount of CH_4 between 15°C and 4°C was only two-fold higher for the 1st after three batches. This was the minimum difference that was observed during the batch studies. Batch 3 revealed that anaerobic raw wastewater treatment at low temperature (4°C to 15°C) is feasible (tCOD). Thus, the selected biomass manages to cope with low temperature conditions and after 56 days of biological reaction the effluent at 4, 8 and 15°C meets the UWWTD (91/271/EEC) COD standards for discharge on a water surface.

COD to CH_4 conversion reached the 80% at 4°C when fed with non-sterile substrate, using an inoculum acclimatized at 15°C (sub-culture 1). The performance was increased than when the wastewater was sterile due to the symbiosis phenomenon between the indigenous bacterial communities from wastewater with the cells originated from the seed; the 1st assisted to hydrolysis of insoluble organic material and the 2nd biomethanized it. For the particular experiment, a 1:3 seed:substrate ratio was used (similar to batch studies, Chapter 4). No significant difference occurred to bacterial and archaeal diversity from the ´invasion´ of the microbial communities habiting the wastewater. Thus, sterilization as a pre-treatment step is not required. It can only be applied when the communities of the seed are outnumbered and an advantage to these cells is necessary (e.g. seed:substrate <1:3).

Comparing the behavior of the communities based on acclimation temperature (4, 15°C) and how they behave to temperature differentiation, it was shown that acclimatization at 4°C results in a stable, sturdy community where hydrolysis limitation can be rapidly overcome during seasonal variation (sub-culture 2).

Hydrolysis limitation was evident from the 1st experimental days, as the VSS:TSS ratio increased the lower the temperature (second batch). Additionally, sCOD and VFA peaks after 200 days at low temperature (4°C) for batch 1 revealed slow hydrolysis. The third batch and sub-culture 2 proved that hydrolysis is indeed the limiting step and gave an insight on how slower it occurs compared to methanogenesis. The limitation becomes less evident the lower the temperature as methane production rate was also decreasing. Batch 3 also indicated the discrepancy between $COD_{removed}$ and COD_{CH4} and proved that un-hydrolysed material remains in the bio-reactor. A safety factor for design purposes was enabled from this fact. This disagreement confirmed that a digester works partially as a clarifier the lower the temperature gets; thus, larger volume at lower temperatures is required to accommodate higher wastewater volumes due to higher retention time.

Limited hydrolysis was caused mainly by accumulation of lipids in the mixed liquor (fifth batch, chapter 4.3). Proteins and carbohydrates seem to be easier bio-methanized, where lipid accumulation seems to have a strong relationship with poor COD:methane conversion at low temperature. Investigation of the enzymes responsible for lipid degradation (lipases) showed that the limitation occurs due to the kinetics of lipase activity in addition to changes in the lipid structure as enzymes were present at all temperatures ≥4°C (batch 8). Lipases that were produced from cells acclimatized at 4°C had a higher degradation capacity than those excreted from cells acclimatized at higher temperatures when all were exposed to the same temperature (37°C). This suggests that inoculation of a bio-reactor for wastewater treatment purposes with low temperature acclimatized biomass is highly desired. Indigenous raw wastewater cells also produce enzymes able to solubilize lipids at low temperatures. The cells from primary settled though were inactive. Lipolysis was limited at 4°C however carbohydrates and proteins are easier to be hydrolysed as methane occurred at 0.0 lipolytic activity. A 4-degree temperature increase (from 4-8°C) is adequate to trigger the COD_{lipid}:CH_4 conversion (batch 8, chapter 6) revealing that the limit of LTAD lies in-between 4 and 8°C. Practically this suggests that the daily day-night cycle in addition to the seasonal variation would efficiently reduce the accumulation of lipids.

Change of the substrate from raw to primary settled wastewater showed that the 2nd can be successfully treated in 15 days, in terms of COD, according to UWWTD (91/271/EEC) standards at all temperature (4, 8 and 15°C). The accelerated performance occurred due to the substrate's higher biodegradable fraction, which boosted treatability.

The 'k´ COD removal coefficients for all temperatures were higher than those for raw wastewater. Higher biodegradability assisted hydrolysis and led to quantitatively and qualitatively richer intermediates´ formation.

Molecular analysis showed from the early experimental days (batch 1) the capacity for hydrogenotrophic methanogenesis at low temperature. *Methanomicrobiales* was the predominant taxon at all operational temperatures (4, 8 and 15ºC). However, there was a high likelihood of inactive archaeal population present in the seed, due to a disagreement between FISH and qPCR archaea enumeration, with the 2nd indicating higher numbers. By the end of the first batch the effect of temperature to archaeal communities was appeared in terms of band intensity mainly. At batch 3 enumeration confirmed that hydrogenotrophs find lower temperature more optimum than acetotrophs. However, Acetotrophs (*Methanosaetaceae*) seemed more consistent, as their population slightly changed amongst early and final experimental days (batch 1, 3). The hypothesis was confirmed by the 2nd sub-culture, which showed predominance of *Methanosaetaceae* at all temperatures, and specific methanogenic rates massively higher than before (batch 3). Further analysis (454 sequencing) might shed more light to the identification of the predominance.

Bacterial differences were not significantly observed amongst temperatures. The main conclusion was that the use of an 1:7 seed:substrate ratio is not recommended, especially for seeds acclimatized at 15ºC as bacterial diversity is likely to change when the inoculum is exposed to lower temperature.

Specific rates for hydrolysis and methanogenesis for raw wastewater at 4, 8 and 15ºC were calculated so they can be further used as fundamental parameters for applied engineering purposes (batch 3). Additionally, specific methanogenic rates for primary settled wastewater for 4, 15ºC and for temperature alterations (4 to 15ºC and vice versa) were calculated (sub-culture 2). The activity for primary settled WW was higher than for raw. This possibly happened due easier to biodegrade COD, longer cellular adaptation and depletion of the inactive population that might have been previously involved to the specific activity estimations.

A simulation showed that an anaerobic treatment reactor with an HRT between 8.8 and 13.5 hours, and volume of 745 to 1125 m^3 is adequate to cope with the needs of a municipality of 20.000 equivalent persons (Conc. of 0.15 kgCOD.m^{-3}), operating at temperatures between 4-15ºC, using the particular inocula at an 1:3 seed:substrate ratio (Chapter 7). This would require a total capital cost of 3.645.514±8852 US Dollars ($). The choice of the desired HRT-Volume should include more parameters (i.e. number of digesters, landscape etc.) as the cost itself cannot result to a decisive conclusion. The same study showed that there is a significant statistical relationship between the previous and current month's temperature, therefore previous and current month's HRT. Additionally it was shown that a more detailed description of the relationship between the HRT and temperature leads to a retention time optimization and tank volume minimization. Finally it showed that the limit of the anaerobic treatment of domestic wastewater at low temperature in terms of applicability lies at 5ºC.

8.2. Future work

I strongly recommend the future researchers and their endeavours to focus their research interests on the topics below:

8.2.1. Lipases identification

Chapter 6 showed that lipases hold a key position in the limited lipid hydrolysis. The low content of lipases/proteins within the samples which were prepared for the activity proteomics did not lead to any fruitful conclusions (all 1D SDS PAGE gels were either faded or blank and for that reason they were not included in this Thesis). Further investigation on this field would help in the identification (SDS-PAGE – MOLDI-TOF) of the lipases excreted at 4ºC or not present at 15ºC and vice versa. This would assist in the identification of the enzymes operating maximally when exposed to high temperatures after acclimation to lower ones. Further research on this field might have an application not only to the wastewater treatment industry but also in numerous other processes and products (detergents, biodegradability processes, bioremediation, cleansing products etc.)

8.2.2. Scale up to a pilot plant

A reactor scale-up based on the specific activity from Chapters 4 and 7 would give a new direction in anaerobic wastewater LTAD designation. A successful scale up would bring the endeavour closer to real life application,

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setting under examination its sustainable character at actual temperature conditions. Additionally, it would prove that designing in the basis of specific activity is attainable and it would examine how reliable it is compared to the up to now design approaches. My proposed setup is a granular anaerobic MBR reactor to ensure that biomass is retained within the reactor body as growth might be slow enough to compensate in the case of washout. Other configurations may also be applicable. However the presence of a membrane seems to be of highest importance due to its major advantage of active biomass preservation (as showed on Chapter 5)

8.2.3. Reactor configuration (compartments)

The need of a comparative study between a 1-phased and a 2-phased (or more) configuration should also be considered. Based on the findings from Chapter 4.3 and 6 lipids accumulation occurs. This might be of high risk for the biomass which should be isolated from the potentially acidified accumulated substrate. An assessment of more than one phase reactors would demonstrate which configuration is optimum by comparing the required size to both the net energy produced and the COD removal efficiency that is achieved.

Other configurations such as steps of post- pre- treatment using manufactured enzymes may also be included to promote engineered hydrolysis. The enzymes can be immobilized onto or into beads depending on the substrate to be hydrolysed. The results would show whether such an approach is viable, efficient and adequate to accelerate treatability taking into account that a small temperature increase (from 4 to 8ºC) is satisfactory to trigger overall wastewater hydrolysis (Chapter 6).

8.2.4. Further addition of HRT(T_t-T_{t-1}) data, model optimization and validation.

The enrichment of the HRT - T_t-T_{t-1} model with more temperature data would promote the model accuracy (R^2, R^2 adjusted). It would also lead to the development of a temperature predictive model, and subsequently HRT prediction, for the specific region based on the rate of change. The scaling up of such an anaerobic treatment system operating in the specific temperature conditions (4-15ºC) would enhance model validation and contribute to the accuracy and reliability of the model.

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10. Appendix

10.1. Appendix 1

a)
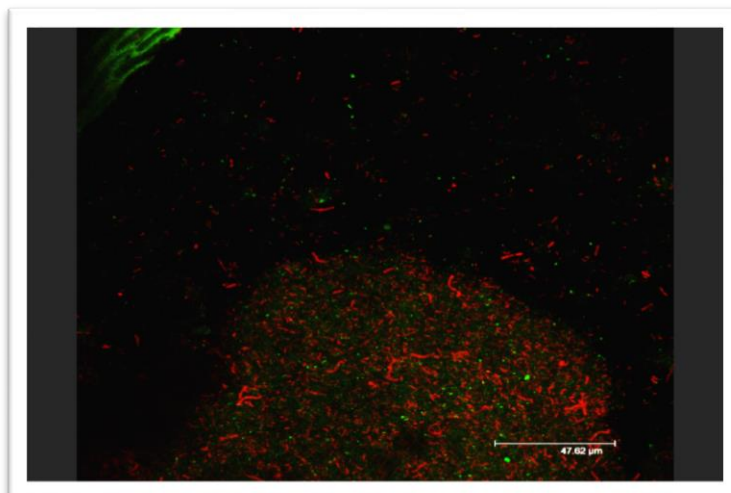
b)
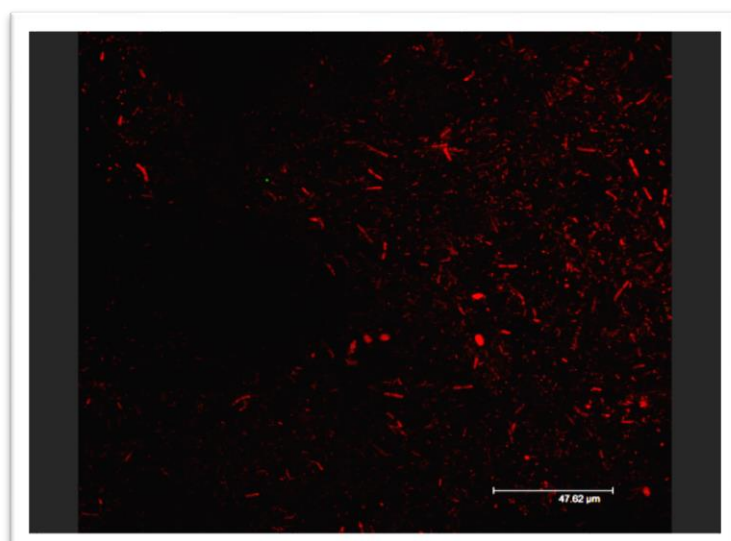
c)
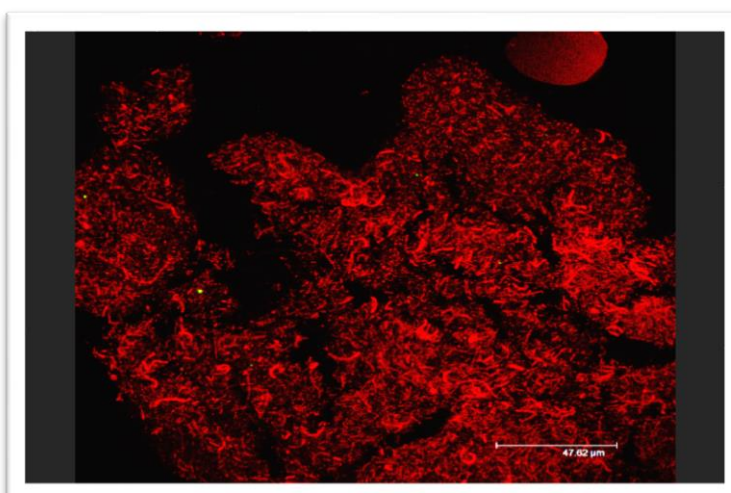
Figure a.1 - FISH images from day 102 of the 3rd Batch; the images account for Bacterial (red) and Archaeal (green) populations that were developed in the 1L bioreactors at 4 (a), 8 (b) and 15°C (c), inoculated with the specific cold adapted inocula (described on the Materials and Methods, Chapter 4.2).

I n v e s t i g a t i n g t h e t r u e l i m i t s o f a n a e r o b i c t r e a t m e n t o f w a s t e w a t e r a t
l o w t e m p e r a t u r e u s i n g a c o l d a d a p t e d i n o c u l u m
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10.2. Appendix 2

Table a.1 - Raw data of the measured O.D. via spectrophotometry at 410nm; the samples account for the lipases activity; R1,2 were operatig at 4ºC, R5, 6 at 8ºC and R5, 7, 8 at 15ºC. WW accounts for only wastewater control at all temperatures and pNPP accounts for only p-pintrophenyl palmitate control at similar conditions.

Time (min.)	0	20	70	110	150	195	230	275	320
R1	0.714	1.046	1.06	1.119	1.146	1.189	1.234	1.225	1.274
R1	0.711	1.042	1.072	1.121	1.147	1.191	1.232	1.232	1.279
R2	0.659	0.962	0.993	1.038	1.067	1.106	1.142	1.152	1.204
R2	0.653	0.962	0.994	1.033	1.072	1.112	1.145	1.153	1.2
R3	0.67	0.637	0.652	0.675	0.692	0.711	0.737	0.741	0.769
R3	0.668	0.636	0.65	0.672	0.69	0.711	0.738	0.739	0.765
R5	0.667	0.651	0.665	0.68	0.692	0.709	0.727	0.726	0.744
R5	0.666	0.644	0.66	0.677	0.691	0.708	0.725	0.726	0.748
R6	0.707	0.687	0.71	0.731	0.746	0.779	0.796	0.801	0.827
R6	0.708	0.687	0.711	0.732	0.753	0.775	0.795	0.792	0.835
R7	0.657	0.611	0.627	0.638	0.65	0.673	0.694	0.691	0.725
R7	0.659	0.615	0.622	0.631	0.648	0.674	0.694	0.7	0.73
R8	0.692	0.669	0.704	0.745	0.784	0.836	0.871	0.904	0.952
R8	0.688	0.671	0.708	0.75	0.789	0.843	0.879	0.912	0.943
Ww 4	0.608	0.928	0.97	1.005	1.035	1.076	1.103	1.084	1.159
Ww 8	0.608	0.588	0.595	0.599	0.609	0.635	0.647	0.645	0.665
Ww 15	0.609	0.567	0.565	0.561	0.565	0.579	0.589	0.581	0.592
pNPP 4	0.683	1.058	1.155	1.155	1.196	1.236	1.246	1.236	1.295
pNPP 8	0.677	0.668	0.688	0.688	0.701	0.733	0.746	0.747	0.759
pNPP 15	0.679	0.644	0.656	0.656	0.664	0.678	0.684	0.682	0.69

Table a.2 - Similar to Table a.1 for all samples after set to an experimental temperature of 37ºC.

Time (min.)	0	20	40	70	100	130	160	190
R1	0.562	0.388	0.291	0.296	0.378	0.463	0.55	0.637
R1	0.581	0.409	0.326	0.327	0.411	0.503	0.597	0.689
R2	0.546	0.412	0.33	0.281	0.307	0.362	0.421	0.482
R2	0.538	0.41	0.324	0.278	0.309	0.372	0.433	0.491
R3	0.597	0.519	0.511	0.595	0.774	0.957	1.139	1.312
R3	0.583	0.513	0.506	0.609	0.807	0.995	1.179	1.354
R5	0.517	0.344	0.275	0.276	0.336	0.409	0.48	0.557
R5	0.508	0.345	0.276	0.317	0.379	0.444	0.519	0.6
R6	0.567	0.441	0.386	0.427	0.55	0.684	0.809	0.947
R6	0.557	0.424	0.383	0.431	0.558	0.693	0.827	0.966
R7	0.547	0.414	0.384	0.459	0.611	0.7755	0.887	1.028
R7	0.532	0.397	0.385	0.471	0.633	0.776	0.918	1.143
R8	0.484	0.289	0.191	0.154	0.17	0.194	0.226	0.253
R8	0.477	0.288	0.191	0.154	0.169	0.197	0.226	0.26
ww	0.45	0.278	0.175	0.124	0.132	0.151	0.177	0.199
ww	0.459	0.271	0.172	0.169	0.134	0.152	0.173	0.201
pNPP	0.588	0.44	0.336	0.231	0.18	0.178	0.184	0.193
pNPP	0.578	0.447	0.32	0.225	0.17	0.17	0.182	0.191
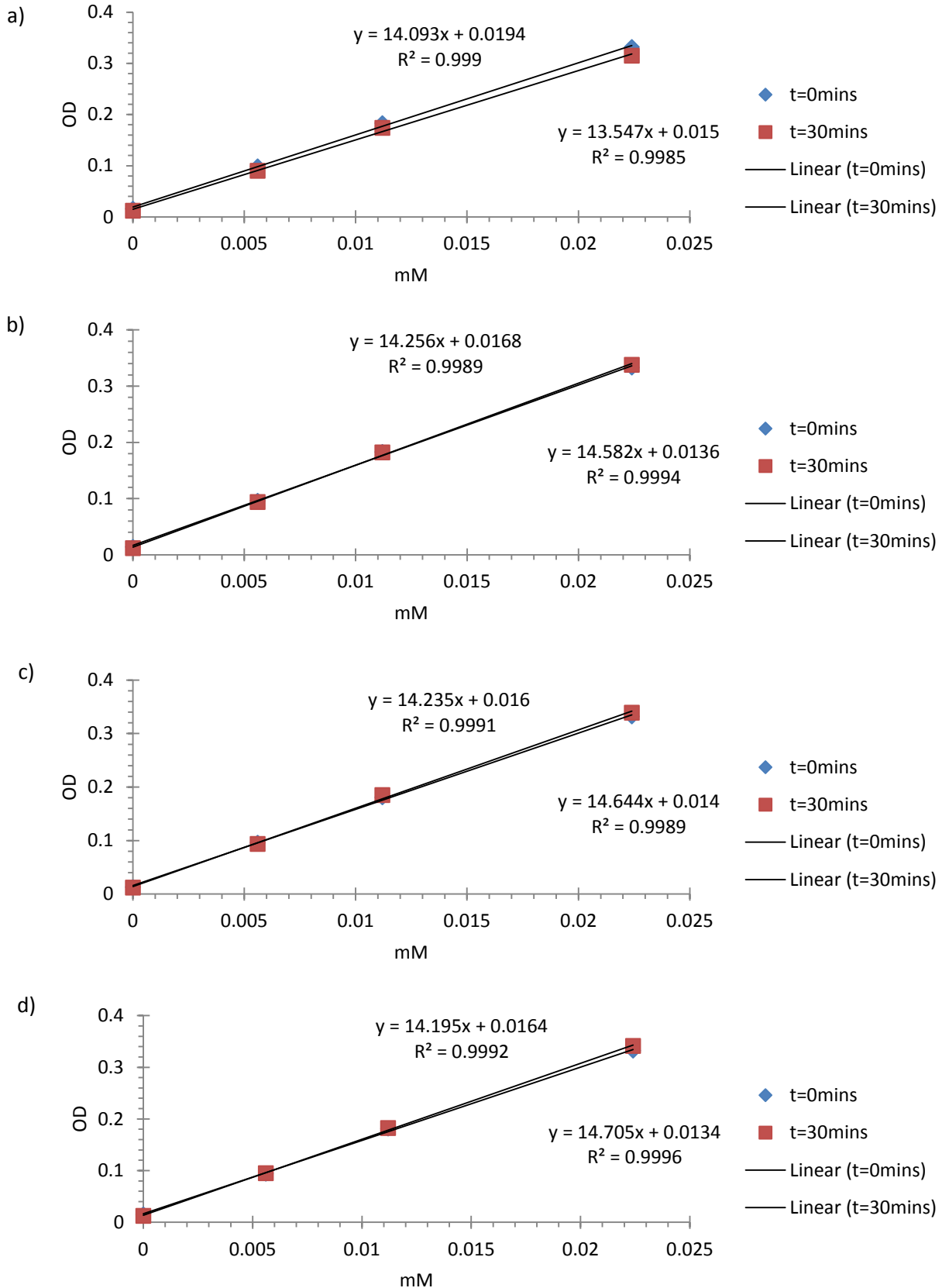
Figure a.2 - Calibration curves implemented with known amounts of p-nitrophenol at all operational temperatures (a, b, c, d for 4, 8, 15 and 37ºC respectively). Trials were also carried out 30 minutes after application of the standards in the cuvette to examine weather there is any differentiation over time.

I n v e s t i g a t i n g   t h e   t r u e   l i m i t s   o f   a n a e r o b i c   t r e a t m e n t   o f   w a s t e w a t e r   a t   l o w   t e m p e r a t u r e   u s i n g   a   c o l d   a d a p t e d   i n o c u l u m

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10.3. Appendix 3

Table a.3 - Digester's HRT estimation on the basis of specific activity; in red-green the specific activities' evolution based on temperature variation and the amount of OLR (blue) that can be acommodated with this rate at the corresponding temperature for a population of 7.6×10^8 methanogenic cells. The safety factor inspired from Chapter 4.4 was included to the HRT calculations as a multiplication factor for the COD_{inflow} concentration. Red and green cells account for increasing and decreasing temperature trends respectively.

Month	date	Average T	Adjusted T	trend	fmol/cell/day sp. rates	gCOD/cell/day	gCOD/7.6×10⁸ cells/ml/day 760000000	mg COD/ml/day	OLR (kg COD/m³/day)	HRT (days)	seed:substrate 1:7 hrs	seed:substrate 1:3 hrs
1	01/01/2010	1.2	4	n/a	25.9	1.66E-12	0.00125984	1.25984	1.25984	0.32	7.6	3.8
2	01/02/2010	1.8	4	up	25.9	1.66E-12	0.00125984	1.25984	1.25984	0.32	7.6	3.8
3	01/03/2010	5.85	6	up	39.77	2.55E-12	0.001934432	1.934432	1.934432	0.17	4.1	2.0
4	01/04/2010	8.8	9	up	53.47	3.42E-12	0.002600723	2.6007232	2.6007232	0.10	2.3	1.2
5	01/05/2010	10.1	10	up	67.51	4.32E-12	0.003283622	3.2836224	3.2836224	0.07	1.7	0.8
6	01/06/2010	14.45	14	up	95.25	6.10E-12	0.004632813	4.6328128	4.6328128	0.03	0.8	0.4
7	01/07/2010	16.2	15	up	102.18	6.54E-12	0.004970112	4.970112	4.970112	0.03	0.7	0.3
8	01/08/2010	14.6	15	up	18.53	1.19E-12	0.000901248	0.901248	0.901248	0.16	3.8	1.9
9	01/09/2010	13.85	14	down	16.92	1.08E-12	0.000822899	0.8228992	0.8228992	0.19	4.6	2.3
10	01/10/2010	9.7	10	down	10.48	6.70E-13	0.00050951	0.5095104	0.5095104	0.45	10.7	5.4
11	01/11/2010	4.8	5	down	2.42	1.55E-13	0.000117772	0.11777152	0.11777152	3.07	73.8	36.9
12	01/12/2010	-0.35	4	down	0.81	5.19E-14	0.000039424	0.039424	0.039424	10.08	241.9	121.0
1	01/01/2011	3.4	4	down	25.9	1.66E-12	0.00125984	1.25984	1.25984	0.32	7.6	3.8
2	01/02/2011	5.6	6	up	39.77	2.55E-12	0.001934432	1.934432	1.934432	0.17	4.1	2.0
3	01/03/2011	6.3	6	up	39.77	2.55E-12	0.001934432	1.934432	1.934432	0.17	4.1	2.0
4	01/04/2011	11.2	11	up	78.5	5.02E-12	0.003818092	3.81809216	3.81809216	0.05	1.3	0.7
5	01/05/2011	11.7	12	up	81.38	5.21E-12	0.003958214	3.9582144	3.9582144	0.05	1.1	0.6
6	01/06/2011	13.7	14	up	95.25	6.10E-12	0.004632813	4.6328128	4.6328128	0.03	0.8	0.4
7	01/07/2011	14.6	15	up	102.18	6.54E-12	0.004970112	4.970112	4.970112	0.03	0.7	0.3
8	01/08/2011	14.6	15	up	18.53	1.19E-12	0.000901248	0.901248	0.901248	0.16	3.8	1.9
9	01/09/2011	14.15	14	down	16.92	1.08E-12	0.000822899	0.8228992	0.8228992	0.19	4.6	2.3

10	01/10/2011	11.4	11	down	12.09	7.73E-13	0.000587853	0.5878528	0.5878528	0.35	8.5	4.2
11	01/11/2011	8.7	9	down	8.86	5.67E-13	0.000431162	0.4311616	0.4311616	0.58	13.9	6.9
12	01/12/2011	4.9	5	down	2.42	1.55E-13	0.000117772	0.11777152	0.11777152	3.07	73.8	36.9
1	01/01/2012	4.6	5	down	2.42	1.55E-13	0.000117772	0.11777152	0.11777152	3.07	73.8	36.9
2	01/02/2012	4.95	5	down	2.42	1.55E-13	0.000117772	0.11777152	0.11777152	3.07	73.8	36.9
3	01/03/2012	8.65	9	up	53.47	3.42E-12	0.002600723	2.6007232	2.6007232	0.10	2.3	1.2
4	01/04/2012	6.85	7	down	5.64	3.61E-13	0.000274464	0.274464	0.274464	1.10	26.3	13.1
5	01/05/2012	10.55	11	up	78.5	5.02E-12	0.003818092	3.81809216	3.81809216	0.05	1.3	0.7
6	01/06/2012	12.4	12	up	81.38	5.21E-12	0.003958214	3.9582144	3.9582144	0.05	1.1	0.6
7	01/07/2012	14.6	15	up	102.18	6.54E-12	0.004970112	4.970112	4.970112	0.03	0.7	0.3
8	01/08/2012	15.6	15	up	102.18	6.54E-12	0.004970112	4.970112	4.970112	0.03	0.7	0.3
9	01/09/2012	12.75	13	down	15.31	9.80E-13	0.00074455	0.7445504	0.7445504	0.23	5.5	2.8
10	01/10/2012	8.4	8	down	7.25	4.64E-13	0.000352813	0.3528128	0.3528128	0.78	18.6	9.3
11	01/11/2012	6.05	6	down	4.03	2.58E-13	0.000196115	0.1961152	0.1961152	1.68	40.4	20.2
12	01/12/2012	3.85	4	down	0.81	5.19E-14	0.000039424	0.039424	0.039424	10.08	241.9	121.0

Table a.4 – This table presents the time in months (1-36), the transformed HRT (red), the transformed time (blue), the lnHRT after MMULT function, and the actual HRT after exp(LnHRT); in blue and red the two components of the regression, for Y axis: ln(HRT) and X axis: transformed time; the 'trendline (ln(HRT))' accounts to the multiplication of the transformed time (blue) with the coefficients.

t (months)	HRT	ln(HRT)	1	2	3	4	5	6	7	8	trendline (ln (HRT'))	HRT (hours)
1	3.785134	1.331081	1	1	1	1	1	1	1	1	1.128489633	3.09098445
2	3.785134	1.331081	2	4	8	16	32	64	128	256	1.82117169	6.17909419
3	2.046748	0.716252	3	9	27	81	243	729	2187	6561	0.847148531	2.33298492
4	1.151743	0.141276	4	16	64	256	1024	4096	16384	65536	-0.29271849	0.74623218
5	0.831203	-0.18488	5	25	125	625	3125	15625	78125	390625	-0.915134056	0.40046294
6	0.406123	-0.9011	6	36	216	1296	7776	46656	279936	1679616	-0.855963196	0.42487376
7	0.344943	-1.06438	7	49	343	2401	16807	117649	823543	5764801	-0.244896152	0.78278583
8	1.902256	0.643041	8	64	512	4096	32768	262144	2097152	16777216	0.657896133	1.93072607
9	2.286421	0.826988	9	81	729	6561	59049	531441	4782969	43046721	1.573396261	4.82300058

10	5.356823	1.678371	10	100	1000	10000	100000	1000000	10000000	100000000	2.273145154	9.70989194
11	36.89495	3.608075	11	121	1331	14641	161051	1771561	19487171	214358881	2.615098519	13.6685629
12	120.9584	4.795447	12	144	1728	20736	248832	2985984	35831808	429981696	2.55381664	12.8560773
13	3.785134	1.331081	13	169	2197	28561	371293	4826809	62748517	815730721	2.132069505	8.43229945
14	2.046748	0.716252	14	196	2744	38416	537824	7529536	1.05E+08	1475789056	1.460139702	4.30656112
15	2.046748	0.716252	15	225	3375	50625	759375	11390625	1.71E+08	2562890625	0.688305957	1.99034095
16	0.651365	-0.42868	16	256	4096	65536	1048576	16777216	2.68E+08	4294967296	-0.022809427	0.97744874
17	0.572509	-0.55773	17	289	4913	83521	1419857	24137569	4.1E+08	6975757441	-0.530145547	0.58851931
18	0.406123	-0.9011	18	324	5832	104976	1889568	34012224	6.12E+08	11019960576	-0.731276173	0.48129438
19	0.344943	-1.06438	19	361	6859	130321	2476099	47045881	8.94E+08	16983563041	-0.580366815	0.55969303
20	1.902256	0.643041	20	400	8000	160000	3200000	64000000	1.28E+09	25600000000	-0.095530636	0.90889052
21	2.286421	0.826988	21	441	9261	194481	4084101	85766121	1.8E+09	37822859361	0.64310213	1.90237315
22	4.230604	1.442345	22	484	10648	234256	5153632	1.13E+08	2.49E+09	54875873536	1.504487033	4.50184374
23	6.947197	1.938338	23	529	12167	279841	6436343	1.48E+08	3.4E+09	78310985281	2.326026705	10.2371853
24	36.89495	3.608075	24	576	13824	331776	7962624	1.91E+08	4.59E+09	1.10075E+11	2.939477216	18.90596
25	36.89495	3.608075	25	625	15625	390625	9765625	2.44E+08	6.1E+09	1.52588E+11	3.200886493	24.5542877
26	36.89495	3.608075	26	676	17576	456976	11881376	3.09E+08	8.03E+09	2.08827E+11	3.021252228	20.5169675
27	1.151743	0.141276	27	729	19683	531441	14348907	3.87E+08	1.05E+10	2.8243E+11	2.393787142	10.9549033
28	13.14447	2.576001	28	784	21952	614656	17210368	4.82E+08	1.35E+10	3.77802E+11	1.412879846	4.10776812
29	0.651365	-0.42868	29	841	24389	707281	20511149	5.95E+08	1.72E+10	5.00246E+11	0.279039992	1.32186021
30	0.572509	-0.55773	30	900	27000	810000	24300000	7.29E+08	2.19E+10	6.561E+11	-0.716683191	0.4883694
31	0.344943	-1.06438	31	961	29791	923521	28629151	8.88E+08	2.75E+10	8.52891E+11	-1.236119478	0.29050936
32	0.344943	-1.06438	32	1024	32768	1048576	33554432	1.07E+09	3.44E+10	1.09951E+12	-0.973343398	0.37781773
33	2.773308	1.020041	33	1089	35937	1185921	39135393	1.29E+09	4.26E+10	1.41E+12	0.221103173	1.24745213
34	9.3174	2.231884	34	1156	39304	1336336	45435424	1.54E+09	5.25E+10	1.79E+12	2.16142234	8.68347974
35	20.18862	3.005119	35	1225	42875	1500625	52521875	1.84E+09	6.43E+10	2.25E+12	4.082696621	59.305178
36	120.9584	4.795447	36	1296	46656	1679616	60466176	2.18E+09	7.84E+10	2.82E+12	4.320642061	75.2369195

Table a.5 - Regression Statistics, descriptive capacity of the model equation – coefficients and intercept (regression optimization, polynomial 8th degree)

Regression Statistics	
Multiple R	0.891730061
R Square	0.795182502
Adjusted R Square	0.734495836
Standard Error	0.880888084
Observations	36

Table a.6 - ANOVA test for the model validity (regression optimization, polynomial 8th degree)

	df	SS	MS	F	Significance F			
Regression	8	81.34015451	10.16751931	13.10308	1.66E-07			
Residual	27	20.95102303	0.775963816					
Total	35	102.2911775						
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	-3.932680814	2.555018853	-1.539198354	0.135395	-9.17515	1.309785	-9.17515	1.30978484
X Variable 1	8.538866017	2.770900805	3.081620967	0.004699	2.853447	14.22428	2.853447	14.22428485
X Variable 2	-4.282035074	1.013066357	-4.226806117	0.000242	-6.36068	-2.20339	-6.36068	-2.203394608
X Variable 3	0.892974925	0.174983907	5.103183135	2.31E-05	0.533938	1.252012	0.533938	1.252012247
X Variable 4	-0.093880221	0.016394397	-5.726360164	4.36E-06	-0.12752	-0.06024	-0.12752	-0.060241697
X Variable 5	0.005415777	0.000880903	6.147981391	1.43E-06	0.003608	0.007223	0.003608	0.007223242
X Variable 6	-0.00017387	2.71024E-05	-6.415301874	7.14E-07	-0.00023	-0.00012	-0.00023	-0.00011826
X Variable 7	2.91232E-06	4.4355E-07	6.565930614	4.83E-07	2E-06	3.82E-06	2E-06	3.8224E-06
X Variable 8	-1.9831E-08	2.99168E-09	-6.628696126	4.11E-07	-2.6E-08	-1.4E-08	-2.6E-08	-1.36925E-08

Table a.7 - HRT, LnHRT, actual ambient temperature (t, t-1) for each month; additionally, the Y and X axis values that were involved for the regression analysis (blue and red values respectively), the new trendline´s data points (as lnHRT and HRT forecasted), the monthly rate of change*.

| Month | actual Ln HRT | | | | forecasted Ln HRT | Forecasted HRT | Rate of Change |
	HRT	Ln HRT	Temperature (t, t-1)				
1	3.785134	1.331081	1.2				
2	3.785134	1.331081	1.8	1.2	2.786097	16.21761	0.5
3	2.046748	0.716252	5.85	1.8	0.68239	1.978601	
4	1.151743	0.141276	8.8	5.85	0.302094	1.352689	0.5042735
5	0.831203	-0.18488	10.1	8.8	0.506061	1.658744	0.14772727
6	0.406123	-0.9011	14.45	10.1	-1.54445	0.21343	0.43069307
7	0.344943	-1.06438	16.2	14.45	-1.14908	0.316929	0.12110727
8	1.902256	0.643041	14.6	16.2	0.315607	1.371091	-0.0987654
9	2.286421	0.826988	13.85	14.6	0.230434	1.259146	-0.0513699
10	5.356823	1.678371	9.7	13.85	2.342979	10.41221	-0.299639
11	36.89495	3.608075	4.8	9.7	3.7964	44.54056	-0.5051546
12	120.9584	4.795447	-0.35	4.8	5.152344	172.8361	
13	3.785134	1.331081	3.4	-0.35	1.385186	3.995571	
14	2.046748	0.716252	5.6	3.4	1.334236	3.797095	0.64705882
15	2.046748	0.716252	6.3	5.6	1.63906	5.150328	0.125
16	0.651365	-0.42868	11.2	6.3	-0.91443	0.400744	0.77777778
17	0.572509	-0.55773	11.7	11.2	0.364651	1.440012	0.04464286
18	0.406123	-0.9011	13.7	11.7	-0.60926	0.543751	0.17094017
19	0.344943	-1.06438	14.6	13.7	-0.48155	0.617827	0.06569343
20	1.902256	0.643041	14.6	14.6	-0.19457	0.823188	1.2167E-16
21	2.286421	0.826988	14.15	14.6	0.060432	1.062295	-0.0308219
22	4.230604	1.442345	11.4	14.15	1.475294	4.37232	-0.1943463
23	6.947197	1.938338	8.7	11.4	2.128441	8.401761	-0.2368421
24	36.89495	3.608075	4.9	8.7	3.420872	30.59608	-0.4367816
25	36.89495	3.608075	4.6	4.9	2.379201	10.79627	-0.0612245
26	36.89495	3.608075	4.95	4.6	2.085207	8.046257	0.07608696
27	1.151743	0.141276	8.65	4.95	0.10012	1.105304	0.74747475
28	13.14447	2.576001	6.85	8.65	2.299917	9.973357	-0.2080925
29	0.651365	-0.42868	10.55	6.85	-0.37072	0.690236	0.54014599
30	0.572509	-0.55773	12.4	10.55	-0.23928	0.787195	0.17535545
31	0.344943	-1.06438	14.6	12.4	-0.89607	0.408172	0.17741935
32	0.344943	-1.06438	15.6	14.6	-0.76124	0.467085	0.06849315
33	2.773308	1.020041	12.75	15.6	1.172635	3.230493	-0.1826923
34	9.3174	2.231884	8.4	12.75	2.728906	15.31612	-0.3411765
35	20.18862	3.005119	6.05	8.4	2.67354	14.49118	-0.2797619
36	120.9584	4.795447	3.85	6.05	3.170896	23.82882	-0.3636364
37			4.094184439		2.331028	10.28852	
			Average:			11.49996	0.06342453

*The values that are missing account for those with a standard deviation higher than 3 sigma (Anderson et al 2011).

Table a.8 - Similarly with Table a.5 but with adjusted ambient temperatures (red) based on Consumption 3, 4, chapter 7.2. NO rate of change was included in this option.

		actual Ln HRT			forecasted Ln HRT, HRT	
	HRT	Ln HRT	Temperature (t, t-1)			
1	3.785134	1.331081	4			
2	3.785134	1.331081	4	4	2.57517	16.21761
3	2.046748	0.716252	6	4	1.411202	1.978601
4	1.151743	0.141276	9	6	0.29993	1.352689
5	0.831203	-0.18488	10	9	0.669965	1.658744
6	0.406123	-0.9011	14	10	-1.34063	0.21343
7	0.344943	-1.06438	15	14	-0.65326	0.316929
8	1.902256	0.643041	15	15	-0.33592	1.371091
9	2.286421	0.826988	14	15	0.246069	1.259146
10	5.356823	1.678371	10	14	2.256665	10.41221
11	36.89495	3.608075	5	10	3.897225	44.54056
12	120.9584	4.795447	4	5	2.89251	172.8361
13	3.785134	1.331081	4	4	2.57517	3.995571
14	2.046748	0.716252	6	4	1.411202	3.797095
15	2.046748	0.716252	6	6	2.045882	5.150328
16	0.651365	-0.42868	11	6	-0.86404	0.400744
17	0.572509	-0.55773	12	11	0.140677	1.440012
18	0.406123	-0.9011	14	12	-0.70595	0.543751
19	0.344943	-1.06438	15	14	-0.65326	0.617827
20	1.902256	0.643041	15	15	-0.33592	0.823188
21	2.286421	0.826988	14	15	0.246069	1.062295
22	4.230604	1.442345	11	14	1.674681	4.37232
23	6.947197	1.938338	9	11	1.886629	8.401761
24	36.89495	3.608075	5	9	3.579885	30.59608
25	36.89495	3.608075	5	5	2.310526	10.79627
26	36.89495	3.608075	5	5	2.310526	8.046257
27	1.151743	0.141276	9	5	-0.01741	1.105304
28	13.14447	2.576001	7	9	2.415917	9.973357
29	0.651365	-0.42868	11	7	-0.5467	0.690236
30	0.572509	-0.55773	12	11	0.140677	0.787195
31	0.344943	-1.06438	15	12	-1.28793	0.408172
32	0.344943	-1.06438	15	15	-0.33592	0.467085
33	2.773308	1.020041	13	15	0.828053	3.230493
34	9.3174	2.231884	8	13	3.103293	15.31612
35	20.18862	3.005119	6	8	2.680561	14.49118
36	120.9584	4.795447	4	6	3.20985	23.82882

Table a.9 - Regression Statistics, descriptive capacity of the model equation - coefficients and intercept (regression for forecast with actual temperature values)

Regression Statistics	
Multiple R	0.921841864
R Square	0.849792422
Adjusted R Square	0.840404449
Standard Error	0.692720169
Observations	35

Table a.10 - ANOVA test for the model validity (regression for forecast with actual temperature values)

	df	SS	MS	F	Significance F
Regression	2	86.87336702	43.43668351	90.51925974	6.71536E-14
Residual	32	15.35555943	0.479861232		
Total	34	102.2289265			

	Coefficients	Standard Error	t Stat	P-value
Intercept	3.392605111	0.285423069	11.88623304	2.81695E-13
X Variable 1	-0.56384662	0.044609439	-12.63962593	5.51264E-14
X Variable 2	0.320419182	0.043455998	7.373416768	2.18804E-08

Table a.11 - Regression Statistics, descriptive capacity of the model equation - coefficients and intercept (regressikon for forecast with adjusted values)

Regression Statistics	
Multiple R	0.883475718
R Square	0.780529344
Adjusted R Square	0.766812428
Standard Error	0.83733673
Observations	35

**Table a.12** - ANOVA test for the model validity (regression fpr forecast with adjusted values)

	df	SS	MS	F	Significance F
Regression	2	79.79267687	39.89633844	56.90268	2.9E-11
Residual	32	22.43624958	0.701132799		
Total	34	102.2289265			

	Coefficients	Standard Error	t Stat	P-value
Intercept	3.633746488	0.388885349	9.344004598	1.16E-10
X Variable 1	-0.581983994	0.059244896	-9.82336083	3.5E-11
X Variable 2	0.31733987	0.059244896	5.356408551	7.04E-06

Curriculum Vitae

I was born in Greece on the 17th of August 1982. I have obtained my bachelor degree in civil engineering at 2008 at the Technological Educational Institute of Thessaloniki. I have obtained my masters' and Ph.D. degree in environmental engineering at 2010 and 2015 respectively at Newcastle University.

I have gained a broad working experience in several aspects including: freelancing at my family's fruit production & manufacturing business and involvement in engineering projects such as the construction of water and wastewater treatment plant in Greece. Finally I gained the fundamental academic experience as a tutor and demonstrator at Newcastle University (Civil and Environmental Engineering Dpt.), a role in parallel with my Ph.D. research work.
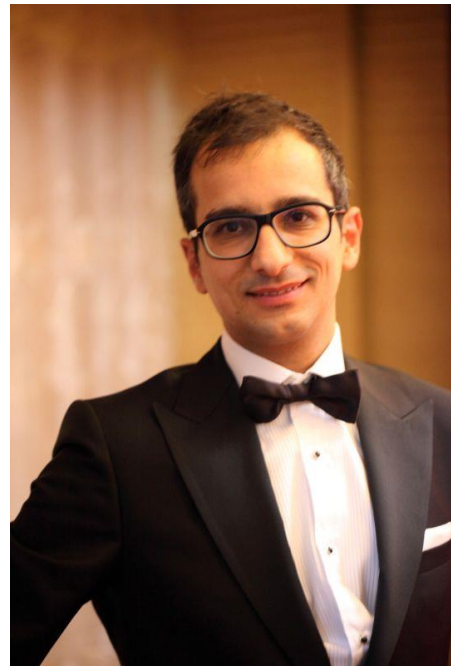
For the past months I have been working in Newcastle University (UK) as a Marie Curie Research Fellow in AD-WINE project, which is a European Commission collaboration of prestigious European research universities and institutes (Newcastle University (UK), ISAH (DE), CRAB (ITA), IFV (FR)), hosted at the facilities of a design and construction engineering company, AEMA (SP).