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**Performances and microbial composition during
mesophilic and thermophilic anaerobic sludge digestion
processes**

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Contents

Preface and thesis outlines	4
Chapter 1. Anaerobic digestion of sludge: an overview	7
Chapter 2. Identification and role of proteolytic <i>Coprothermobacter</i> spp. in different thermophilic anaerobic systems: a review	26
Chapter 3. In situ identification of the syntrophic protein fermentative <i>Coprothermobacter</i> spp. involved in thermophilic anaerobic digestion process	45
Chapter 4. Evaluation of pretreatments effects on sludge floc structure	64
Chapter 5. Thermophilic anaerobic digestion of thermal pretreated sludge:role of microbial community structure and correlation with process performances	75
Chapter 6. Microbial diversity in innovative mesophilic/thermophilic temperature phased anaerobic digestion of sludge	104
Chapter 7. Efficacy of of the methanogenic biomass acclimation in batch mesophilic anaerobic digestion of ultrasound pretreated waste activated sludge	123
Concluding remarks	137
Future recommendations and research needs	138
Acknowledgements	139

Preface and thesis outlines

The anaerobic digestion (AD) of organic wastes still gathers a great interest due to the global needs for waste recycling and renewable energy production, in the form of biogas. The need of solutions for a sustainable sludge disposal is increasing and anaerobic sludge treatment can be used as a cost-effective strategy. In a worldwide perspective, anaerobic digestion of sewage sludge is far the most widespread use of anaerobic digestion. Anaerobic degradation of biological solids like waste activated sludge requires the hydrolysis of particulate matter to soluble substrates, rate-limiting step of the whole process. A pretreatment, mechanical or thermal, can enhance digestibility of sludge by disintegrating the biomass up to cell lysis, facilitating the release of organic matter in the aqueous phase. The performances of an anaerobic digestion process are primarily linked to the structure of the microbial community present in the system. However, the knowledge of the microbial community structure and function is still limited. This thesis reports the results of the microbiological characterization of the anaerobic mixed biomass selected in three different lab-scale anaerobic sludge treatments systems.

The research activities were conducted in the framework of the EU project “ROUTES” (Novel processing routes for effective sewage sludge management) aimed to discover new routes in wastewater and sludge treatment, in order to produce a clean and stabilized sludge suitable for a reuse in agriculture. Three different lab-scale anaerobic digestion processes were operated : a thermophilic anaerobic digestion (55 °C) of untreated and thermal pretreated WAS, an innovative two-phased anaerobic digestion (mesophilic/thermophilic) of untreated and ultrasound pretreated WAS, both in semi-continuous mode, and a batch mesophilic anaerobic digestion (37°C) of untreated and ultrasound pretreated WAS. In particular, the research was focused on three main tasks: i. evaluation of the impact of ultrasound and thermal hydrolysis pretreatment on the floc structure, microbial population and anaerobic digestion performances; ii. definition of the role of microbial population composition (i.e. hydrolytic bacteria, methanogens ecc.) in mesophilic and thermophilic anaerobic digestion processes; iii. improvement of the biomolecular tools to better understanding the overall composition and activity of the anaerobic microbial communities responsible of the processes under investigation in the project.

Chapter 1 gives an overview of the current knowledge on AD of sewage sludge and the ecophysiology of microorganisms during the process. In particular, the chapter is focused on the comparison between mesophilic and thermophilic microbial communities, their different metabolic behaviors and the several trophic webs that can be established at different temperatures. A summary of known key metabolic pathway and microorganisms in anaerobic digestion processes as well as

the main microbial syntrophic association reported in scientific literature is also given. Furthermore, chapter 1 discusses advantages and disadvantages of the various approaches described in literature in terms of methane production and pathogens removal.

The **Chapter 2** is focused on *Coprothermobacter spp.*, thermophilic bacteria with known proteolytic activity. Members of this genus are capable to ferment proteins and to grow well in presence of peptides. *Coprothermobacter spp.* were identified in several studies focused on the analysis of microbial community structure selected in anaerobic thermophilic systems. This microorganism has attracted the attention of researchers for its potential applications in high temperature environments, because only a few proteolytic anaerobic thermophiles have been characterized so far. The presence of these bacteria in different anaerobic systems and the relationship with the availability of the substrates, as well as the possible improvement of the overall process due to the establishment of syntrophic associations between them and hydrogenotrophic methanogens are discussed in this chapter.

The design and the evaluation of a FISH probe targeting the genus *Coprothermobacter* is reported in **Chapter 3**. The newly designed CTH485 probe and helper probes hCTH429 and hCTH439 were optimized for the analysis of thermophilic anaerobic sludge samples by fluorescence in situ hybridization. In situ probing revealed that thermo-adaptive mechanisms shaping the 16S rRNA can affect the in situ identification of thermophilic microorganisms, despite do not adversely impact on the analysis. The novel developed FISH probe extends the possibility to study the widespread thermophilic syntrophic interaction of *Coprothermobacter spp.* with hydrogenotrophic methanogenic archaea, whose establishment is a great benefit for the whole anaerobic system.

Chapter 4 reports the evaluation of pretreatment effects of ultrasounds and thermal hydrolysis on sludge floc structure. These pretreatments were applied to improve the following hydrolysis phase during the anaerobic digestion process. Assessment of the pretreatment effect on sludge flocs was done by dimensional, chemical and microbiological analysis. Despite microscopic analysis showed that ultrasound pretreatment caused a slight disintegration of flocs, not observed after thermal hydrolysis, chemical and microbiological analysis highlighted that the latter had a higher solubilization efficiency in terms of organic matter release and cell lysis.

Chapter 5 describes the structure and dynamics of the microbial communities selected in two semi-continuous thermophilic (55°C) anaerobic digesters fed with untreated and thermal pretreated sludge, during 250 days divided in three experimental phases characterized by different organic loading rate (OLR). Thermal pretreatment of sludge permitted to obtain higher biogas gain due to the availability of readily biodegradable organic matter, and in particular led to a crucial improvement in transformation of the complex organic substrate into simple protein and peptides,

and this outcome favored the growth and dominance of the hydrogen producing bacteria *Coprothermobacter*, with a consequent marked increase of methane production by hydrogenotrophic archaea *Methanothermobacter thermoautotrophicus*.

The new approach of an innovative TPAD system is discussed in **Chapter 6**. This experimental setup was based on the idea to sub-divide the anaerobic digestion process into three different stages: 1) an ultrasounds pretreatment (20 kHz) to improve hydrolysis, 2) a short mesophilic stage (37°C) to improve volatile fatty acids formation and 3) a final thermophilic stage (55°C) to convert these intermediates into methane and contemporarily assure the complete hygienization of the digested sludge. Focus of this study was the characterization of the microbial communities growing in this innovative system, fed by real waste activated sludge, either untreated or pretreated by ultrasounds. Analysis of biodiversity of mesophilic and thermophilic community showed that thermophilic anaerobic biomass may be more susceptible to operative changes and less prompt to adapting to operative variations. Mesophilic microbial community quickly changed in response to variations in HRT and OLR, switching from methane to VFA production. The start-up microbial population of thermophilic inoculum slowly changed in response to substrate availability, highlighting the importance of a longer acclimation step for thermophilic microbial communities.

The importance of acclimation of inoculum was detailed during a mesophilic batch anaerobic digestion, carried out for 20 days using two different anaerobic inocula. Each inoculum was fed either with untreated or ultrasound pretreated (20 kHz) waste activated sludge. This experimental step is described in **Chapter 7**. The presence of well-acclimated archaeal community resulted in good methane yield, especially for the reactor fed with ultrasound pretreated sludge. FISH analysis showed that the simultaneous presence of the aggregated forms of the two acetotrophic archaea *Methanosarcina* and *Methanosaeta* was linked with better performances of the systems.

Finally, the results obtained in the different chapters are discussed. In general, we can confirm that the investigated sludge pretreatments had an overall positive effect on AD performances. During thermophilic anaerobic digestion, syntrophic protein fermentation was the main metabolic pathway, due to high protein hydrolysis. In the two-stage mesophilic/thermophilic process the significant impact of the microbial biodiversity on the process evolvement was highlighted. In the mesophilic batch digestion the acetate conversion into methane was improved in system with well-acclimated microbial community. Overall, understanding of the microbial composition and dynamics is crucial to improve different operational parameters and the reactors performances. Further research is needed to elucidate the functional role of novel microbial groups involved in syntrophic associations, especially during thermophilic process, to complete knowledge on trophic relationship between bacteria and archaea.

Chapter 1

Anaerobic digestion of sludge: an overview

Gagliano M.C.

1. Wastewater treatment plants (WWTPs): background

Advanced biological methods commonly applied in municipal WWTP convert a substantial part (about 50%) of the wastewater pollution into sludge (Jenicek et al., 2012). Sewage sludge is regarded as the residue produced by the wastewater treatment process, during which liquids and solids are being separated. Liquids are discharged to aqueous environment while solids are removed for further treatment and final disposal (Fityli and Zabaniotou, 2008).

A conventional municipal WWTP produces two different types of sludge. Primary sludge settles from the primary clarifiers and is formed by particles with higher density than water and eventually by chemicals added to the wastewater as conditioners to enhance settleability. Secondary or waste activated sludge (WAS) settles in the secondary clarifiers after the oxidation tank and is a “biological” sludge formed principally by biomass flocs (Chang et al., 2001; Metcalf and Eddy 2003).

In the past 20 years there was an increase in wastewater treatment plants (WWTP) and a consequent growth in sludge treatment (De la Rubia et al., 2013). Of the constituents removed by effluent treatment, sludge is by far the largest in volume, therefore its handling methods and disposal techniques are a matter of great concern (Fityli and Zabaniotou, 2008). The sludge is widely considered as undesired byproduct of wastewater treatment, and its disposal represent up to 50% of the current operating costs of a WWTP (Appels et al., 2008). Latest information on disposal and recovery of sludge in Europe indicated that 45% was recycled to land (largely in agriculture), 18% landfilled, 17% incinerated and around 1% is disposed off to surface water (despite this being prohibited since 1 January 1999) (European commission, 2004). Recent objective of European Union is to prepare sludge for agricultural utilization by transforming it in a very clean and stabilized product (Braguglia et al., 2011).

Before disposal, municipal sludge must undergo some treatment in order to reduce its associated volumes and to eliminate the bacteria, viruses and organic pollutants (Appels et al., 2008; Fityli and Zabaniotou, 2008). Examples of effective sludge treatment processes used in Europe and their description are listed in Table 1. Anaerobic digestion will be extensively described in the next section. In all WWTPs, treatment concerns mixtures of primary and secondary sludges. Secondary sludge alone is particularly troublesome to stabilize and to dewater, because of the particulate organic matter (mainly flocs and biomass) hardly to biodegrade, and the high amount of bound water. However, from a disposal point of view secondary sludge is more suitable for agricultural utilization, because much more rich in nutrients and poor in organic micropollutants with respect to

primary sludge. Therefore, a separation between the two sludge streams could be a sustainable solution from an ecological point of view.

Table 1- Examples of effective sludge treatment processes used in Europe (Fityli and Zabaniotou, 2008).

Process	Description
Sludge pasteurization	Min. of 20 min at 70 °C or min. of 4 h at 55 °C, followed in all cases by primary mesophilic anaerobic digestion
Mesophilic anaerobic digestion	Mean retention period of at least 12 or 24 days primary digestion in temperature range 35 ± 3 or 25 ± 3 °C, respectively, followed by a stage providing a mean retention period or at least 14 days
Thermophilic aerobic digestion	Mean retention period of at 7 days digestion. All sludge to be subject to a minimum of 55 °C for a period of maturation adequate to ensure that compost reaction process is substantially complete
Composting (windrows and aerated piles)	The compost must be maintained at 40 °C at least 5 days and for 4 hours during this period at a minimum of 55 °C within the body of the pile followed by a period of maturation adequate to ensure that the compost reaction process is substantially complete
Lime stabilization of liquid sludge	Addition of lime to raise pH to greater than 12.0 and sufficient to ensure that the pH is not less than 12 for a minimum period of 2 h. The sludge can then be used directly
Liquid storage	Storage of retreated liquid sludge for a minimum period of 3 months
Dewatering and storage	Conditioning of untreated sludge with lime followed by dewatering and storage of the cake for a minimum period of 3 months. Storage for a period of 14 days if sludge has been subject to primary mesophilic anaerobic digestion

2. Anaerobic digestion: a sustainable solution for sludge disposal and energy production

As described above, the management of sewage sludge in an economically and environmentally acceptable manner is one of the critical issues facing modern society, due to the increase in sludge production, as a result of the implementation of the EU Directive 1991/271 (Braguglia et al., 2011). The global energy demand is growing rapidly, and about 88% of this demand is met at present time by fossil fuels. Scenarios have shown that the energy demand will increase during this century by a factor of two or three. Our societies are also generating an increasing quantity of organic wastes, such as industrial, municipal and agricultural ones. In this context, energy production from wastes, residues, and energy crops will play a vital role in future (Weiland et al., 2010).

AD is the most common treatment technique for sludge stabilization due to the reduction of sludge volume, destruction of pathogenic organisms, stabilization of the sludge and production of an energy-rich biogas (Gupta et al., 2012). For these reasons, anaerobic sludge digestion optimizes WWTP costs and is considered a major and essential part of a modern WWTP (Appels et al., 2008).

In a worldwide perspective, anaerobic digestion of sewage sludge is far the most widespread use of anaerobic digestion (Ahring et al., 2002).

AD is a complex process which requires strict anaerobic conditions to proceed, and depends on the coordinated activity of a complex microbial association to transform organic material into mostly CO₂ and methane. However, four major steps can be distinguished (Fig.1): in the first hydrolysis step, both solubilisation of insoluble particulate matter and biological decomposition of organic polymers take place. Acidogenesis and acetogenesis follow in the second and third step and results in the generation of a wide variety of fermentation endproducts (VFAs, H₂), which are transformed into methane by methanogenic community.

Organic polymeric material cannot be utilized by microorganisms unless it is broken down to soluble compounds (usually mono- or dimers) which can then pass the cell membrane. Therefore, solubilization of particulate matter is the first step in the anaerobic degradation of complex polymeric organics like sewage sludge (Pavlostathis and Giraldo-Gomez, 1991). For this reason, before AD process, sewage sludge can undergo to a disintegration pretreatment in order to enhance its digestibility. This feature will be extensively discussed in Chapter 4.

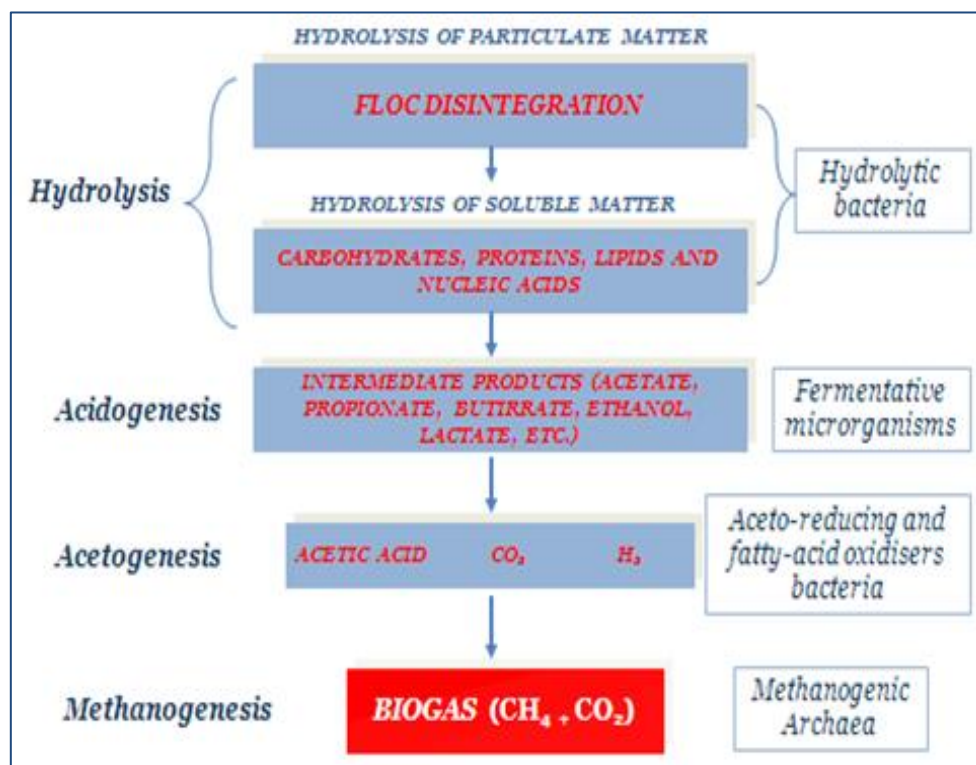


Figure 1 – Main steps of the anaerobic digestion process.

2.1 Biogas production and utilization

The potential of using biogas as energy source is widely recognised. Biogas is a versatile renewable energy source, which can be used for replacement of fossil fuels in power and heat production, and it can be used also as gaseous vehicle fuel. Methane-rich biogas (biomethane) can replace also natural gas as a feedstock for producing chemicals and materials (Weiland et al., 2010).

Biogas is primarily composed of methane and carbon dioxide, contains smaller amounts of hydrogen sulfide and ammonia, and is saturated with water vapor (Weiland et al., 2010). Biogas constituents are listed in Table 1.

Table 1 – Biogas composition (Al Saedi et al., 2009)

Compound	Chemical symbol	Content (Vol.-%)
Methane	CH ₄	50-75
Carbon dioxide	CO ₂	25-45
Water vapour	H ₂ O	2 (20°C) -7 (40°C)
Oxygen	O ₂	<2
Nitrogen	N ₂	<2
Ammonia	NH ₃	<1
Hydrogen	H ₂	<1
Hydrogen sulphide	H ₂ S	<1

The composition and properties of biogas varies to some degree depending on feedstock types, digestion systems, temperature, retention time etc. The theoretical gas yield varies with the content of carbohydrates, proteins, and fats (Tab.2). The energy content of biogas from AD is chemically bounded to methane (Al Saedi et al., 2009).

Table 2 – Maximal methane yields and theoretical methane content (Weiland, 2010)

Substrate	Biogas (Nm ³ /t TS)	CH ₄ (%)	CO ₂ (%)
Carbohydrates ^a	790–800	50	50
Raw protein	700	70–71	29–30
Raw fat	1,200–1,250	67–68	32–33
Lignin	0	0	0

^a Only polymers from hexoses, not inulins and single hexoses

Without further treatment, biogas can only be used at the place of production, and an enhanced potential of use can only be achieved after removing the CO₂ and contaminants (Appels et al.,

2008). CO₂ is a non-combustible gas, sulphur compounds are corrosive and reactive, combustion of ammonia lead to NO_x formation, etc. For this reason, there are several technologies for biogas purification, as water scrubbing, chemical absorption, cryogenic separation, bio-filter, etc. (Zhao et al., 2010).

In particular, biogas must be desulfurized and dried before utilization to prevent damage of the gas utilization units (Weiland et al., 2010). There are four basic ways of biogas utilization: production of heat and steam, electricity generation/co-generation, use as vehicle fuel, and (possibly) production of chemicals (Appels et al., 2008). These utilisations are governed by national frameworks.

Two main biogas recovery techniques used in the European Union are electricity and heat production, and a third emerging recovery technique is the biomethane injection into the natural gas network (EurObserv'er, 2013). In the main producer country, Germany, the 19.4 TWh of electricity generated in 2011 from biogas covered 3% of its power consumption (EurObserv'er, 2012). As the German powerhouse has decided to curb digestors expansion, sector growth will have to be powered by investments in other European Union countries. Italy is one of the most anaerobic digestion-friendly countries with its highly attractive incentive legislation. According to Terna, Italy's energy transport operator, the number of biogas plants (all sources taken together) rose from 707 (773.4 MWe) in 2011 to 1.471 (1.342 MWe) in 2012 (EurObserv'er, 2013). Increasing energy efficiency of biogas plant will be crucial for the sector's future growth, avoiding monetary incentives. From this point of view, direct injection in gas network could give a new impetus to the sector (EurObserv'er, 2013).

2.2 Mesophilic and thermophilic anaerobic digestion

AD process is largely determined by the substrate composition and reactor design, as well as operating conditions (Appels et al., 2008). One of the important operating conditions is temperature, that has a relevant effect on the growth rate and metabolism of micro-organisms and hence on the population dynamics in the anaerobic reactor. Moreover, temperature of the process affects also the physico-chemical properties of the components found in the digestion substrate. AD of sludge is usually carried out as one-stage process at mesophilic (30-37°C) or thermophilic (55-70°C) conditions. The AD reactor performances are often relatively similar in both temperatures, with some exceptions. An increasing temperature (thermophilic conditions) has several benefits including an increasing solubility of the organic compounds, enhanced biological and chemical reaction rates, an higher grow rate of methanogenic archaea, better degradation of solid substrates and better substrate utilisation and an increasing death rate of pathogens (Ahring et al., 2002;

Appels et al., 2008; Al Saedi et al., 2009; Jenicek et al., 2012) . In fact, many studies reported improved performance of the reactor with increases in temperature (Ge et al., 2011; Bolzonella et al., 2012; Jenicek et al., 2012). Nevertheless, the thermophilic process has also some disadvantages with respect to the mesophilic one as a) the larger energy demand due to high temperature and b) higher risk of ammonia inhibition. In fact, anaerobic fermentation of wastes with high concentration of ammonia was more easily inhibited and less stable at thermophilic conditions temperatures than at mesophilic ones (Chen et al., 2008).

Conventional anaerobic digesters are operated generally at a mesophilic temperature (35 to 37°C) with long hydraulic retention times (HRTs) of 15 to 20 days on WAS, and more than 20 days on primary sludge (Ho et al., 2013). The long treatment times are a key disadvantage of anaerobic digestion, requiring large tank volumes as well as increased mixing and maintenance costs. Thus, thermophilic anaerobic digestion is an attractive alternative to conventional mesophilic anaerobic digestion. The majority of thermophilic systems are operated at 55°C (Ho et al., 2013; Sundberg et al., 2013), as higher temperatures can result in instability due to ammonia inhibition and reduced operability. The main reason for applying thermophilic temperatures is the better sanitizing effect of the higher process temperature compared to mesophilic temperatures and the need for a lower retention times (Ahring et al., 2002). Thermophilic temperatures are commonly applied throughout Europe for treatment of wastes in large scale biogas plants (Ahring, 1995).

An alternative strategy to obtain better performances is phased anaerobic digestion, with two or more digesters used in series. It is considered as a promising technology for the treatment of wastewater sludges (Zamanzadeh et al., 2013). In the last years a temperature phased anaerobic process (TPAD) with a short thermophilic stage acting as thermal pretreatment, followed by a longer mesophilic one was investigated with the aim to separate microbial groups into two phases (Coelho et al., 2011; Ge et al., 2010). In the first thermophilic unit with short hydraulic retention time, hydrolytic and acidogenic/fermentative bacteria degrade polymers to monomers and produce organic acids. In the second stage (with a longer retention time), these products are then converted into methane by strict cooperation between bacteria and archaea during acetogenesis and methanogenesis (Merlino et al., 2013).

Temperature affects also the microbial diversity of the AD process drastically: in mesophilic conditions, the species richness and the number of different microbial phyla appear to be higher and the species composition very different compared to thermophilic conditions (Ritari et al., 2012).

Differences between mesophilic and thermophilic microbial communities in anaerobic digesters are largely reported (Ike et al., 2010; Shi et al., 2013; Pervin et al., 2013 a; Zamanzadeh et al., 2013). A systematical analysis of 21 mesophilic and thermophilic full scale anaerobic digesters highlighted

that bacterial and archaeal community composition was mainly related to the temperature of the process (Sundberg et al., 2013).

3. Microbiology of AD

The principles of design and operation of anaerobic reactors can be often derived only from engineering standpoint, not generally taking into account processes occurring at microorganism level. Thus, there is a lack of systematic information about the nature and role of anaerobic consortia which impacts on the design and operational stability of the systems (O’Flaherty et al., 2006). Our knowledge about the microbial consortia involved in this process is limited because of a lack of phylogenetic and metabolic data on these predominantly uncultivated microorganisms (Rivière et al., 2009). Despite several studies analyzed microbial communities during mesophilic and thermophilic AD (Rivière et al., 2009; Ike et al., 2010; Regueiro et al., 2012; Ritari et al., 2012; Ho et al., 2013; Shi et al., 2013; Pervin et al., 2013 a; Sundberg et al., 2013; Zamanzadeh et al., 2013), details of the bacterial populations within the identified major phyla and the ecological role of those are yet to be determined (Pervin et al., 2013 b).

AD process is described in Fig.1. A diverse number of Bacteria take part in the hydrolysis and fermentation steps, whereas the oxidation of intermediate fermentation products to acetate is performed by either hydrogen- or formate-producing acetogens (Stams and Plugge, 2009). Finally, methane production derives from acetate and hydrogen by methanogenic Archaea. A balanced interaction between the microorganisms in this degradation chain is crucial for the continuous transformation of the intermediates formed and subsequently an efficient biogas production (Sundberg et al., 2013). The prevailing microorganisms in sludge belonged to *Archaea* and *Bacteria* domains. Studies on microbial population in several anaerobic reactors treating different substrates showed that reactors treating similar substrates grouped together (Regueiro et al., 2012; Sundberg et al., 2013). Diverse uncultivated taxonomic groups were detected in the last years, especially within the domain Bacteria, in anaerobic sludges. Finding key (or dominant) populations that belong to such uncultured lineages at various taxonomic levels (from species to phylum levels) is one of the major advances in the microbiology of anaerobic digestion processes in the past few years (Narihiro and Sekiguchi, 2007).

3.1 Bacteria

Biodiversity of Bacteria in anaerobic digesters is strict correlated to the operative parameters and the typology of substrate. Constituents of more than 20 bacterial phyla have been detected in

anaerobic sludges (Narihiro and Sekiguchi, 2007). 16S rRNA gene clones that were frequently and commonly collected from these sludges were distributed in various prokaryotic taxa such as the phyla Proteobacteria (mainly in the Deltaproteobacteria class), Chloroflexi, Firmicutes, Spirochaetes, and Bacteroidetes (Narihiro and Sekiguchi, 2007). In Nelson et al. (2011), a meta-analysis of microbial diversity observed in sequences collected in different study concerning AD showed that the four major phyla identified in anaerobic reactors were *Chloroflexi*, *Proteobacteria*, *Firmicutes* and *Bacteroidetes*; minor phyla were *Synergistetes*, *Planctomycetes* and *Actinobacteria*. In a recent study on six full-scale AD, Bacterioides, Firmicutes and Proteobacteria were retrieved as dominant, and Chloroflexi were present only in the reactor treating sugar (Regueiro et al., 2012). Sundberg et al. (2013), by analyzing microbial populations from 21 full scale anaerobic digesters by 454 pyrosequencing, showed that Firmicutes and Bacterioides were the major phyla, followed by Actinobacteria, Proteobacteria and Spirochetes, and to a lesser extent by Chloroflexi, Verrucomicrobium and Acidobacterium. Recently, studies on thermophilic systems highlighted the presence of members of phylum Thermotogae (Yabu et al., 2011; Pervin et al., 2013 b).

At present, despite the knowhow of the metabolic functions of already identified Bacteria, there is limited understanding of the link between bacterial community composition and their metabolic contribution to AD process. Also the high abundance of uncultivated microorganisms revealed indifferent studies highlight the need of further insights, especially regarding the thermophilic processes.

The enzyme-mediated conversion of organic polymers, such as polysaccharides, lipids, proteins, and fats, into soluble organic monomers in the first step of hydrolysis is carried out by anaerobic bacteria such as Bacterioides and Clostridium (Liu and Whitman, 2008).

Phylum Chloroflexi is generally associated to nutrient removal in activated sludge processes (Cirne et al., 2006). In the study of Ariesyady et al. (2007) performed on a full-scale anaerobic digester treating sewage sludge, member of the phylum Chloroflexi were linked to the metabolization of glucose. In the same study, Betaproteobacteria were linked to glucose, propionate, butyrate and acetate degradation. Deltaproteobacteria contain some syntrophic species, as propionate-oxidizers or sulfate-reducing bacteria (McInerney et al., 2008). Deltaproteobacteria are also involved in LCFA-degradation (Hatamoto et al., 2007).

Firmicutes and Bacteroidetes are phyla of remarkable importance in the degradation of complex organic matter in anaerobic bioreactors (Merlino et al., 2012). Presence of Bacteroidetes could be relevant to accelerating hydrolysis step (Regueiro et al., 2012); this phylum includes carbohydrate and protein-degrading bacteria (Merlino et al., 2012) and members of this group have been reported to possess the ability to degrade macromolecules such as cellulose (Cirne et al., 2006).

Fermentation is carried out by a broad range of species using a variety of enzymes (Pereyra et al., 2010). Acidogenic bacteria mainly belongs to the phylum Firmicutes, represented mostly by members of the classes Clostridia and Bacilli (Ali Shah et al., 2014). Members of Clostridia were also associated to hydrolytic functions include saccharolytic, proteolytic and lipolytic species (Cirne et al., 2006; Merlino et al., 2012; Pervin et al., 2013a) and they are important hydrogen producers in many anaerobic systems (Hung et al., 2011). *Bacillus* has the ability to degrade a wide range of organic compounds, including proteins and carbohydrates (Merlino et al., 2012). Firmicutes phylum includes also several genera of anaerobic microorganisms well known for their cellulolytic activity (Cirne et al., 2006). Acetogenic bacteria are a relatively understudied distinguished group, that under standard redox potential conditions compete for substrates (H_2 and acetate) with methanogens (Ryan et al., 2010). Cultivated acetogens belongs to the family Syntrophomonadaceae within the phylum Firmicutes (McInerney et al., 2008). These syntrophic acetogenic bacteria have an important role in fatty-acid degradation and work in a synergetic way together with hydrogen-scavenging microbes such as hydrogenotrophic methanogens (Merlino et al., 2012). Members of the Thermotogae excrete hydrolytic enzymes to catalyse a wide range of polysaccharides to acetate, carbon dioxide and hydrogen as the main fermentation products (Pervin et al., 2013 b). Thermotogae produced H_2 via fermenting a variety of organic compounds (Yabu et al., 2011). Members of Synergistetes are linked to the degradation of proteinaceous substrates as well as to acetate utilization (Narihiro et al., 2009; Ariesyady et al., 2007). Members of *Planctomycetes* were identified in wastewater and sludge anaerobic treatments mainly as anaerobic ammonia oxidizers (Anammox) (Chouari et al., 2003).

3.2 Archaea

All methanogens are strictly anaerobic archaea belonging to the phylum *Euryarchaeota*. They are a large and diverse group, all of which are obligate methane- producers that obtain all or most of their energy from methanogenesis (Liu and Whitman, 2008). Details about methanogenic orders and their main physiology are reported in Table 3.

Methanogens are important especially for the obligatory syntrophic interactions driving the acetogenic proton reduction needed for growth of Bacteria on the hydrolyzed substrates (Stams and Plugge, 2009). Differently from Bacteria, most members of Archaeal population retrieved in anaerobic reactors were extensively studied in pure and enrichment cultures.

Archaea can be subdivided into two groups: (i) acetotrophic methanogens, which generate methane by acetate decarboxylation and (ii) hydrogenotrophic species, which form methane by the reduction of H_2/CO_2 (O'Flaherty et al., 2006) (Figure 2). Acetotrophic methanogens are the most important

methanogenic species, as 70% of the total methane generated during AD of domestic sewage is via this pathway (O’Flaherty et al., 2006). Typical morphology of acetotrophic and hydrogenotrophic methanogens is shown in Figure 3.

Table 3 – Summary of methanogenic archaea orders and main physiology

Order	Physiology
<i>Methanopyrales</i>	Hydrogenotrophic; hyperthermophiles
<i>Methanobacteriales</i>	Hydrogenotrophic; mesophilic or thermophilic
<i>Methanococcales</i>	Hydrogenotrophic; mesophilic or thermophilic
<i>Methanomicrobiales</i>	Hydrogenotrophic; mesophilic
<i>Methanosarcinales</i>	Only acetotrophic (<i>Methanosaetaceae</i>), or acetotrophic and hydrogenotrophic (<i>Methanosarcinaceae</i>); mesophilic or thermophilic

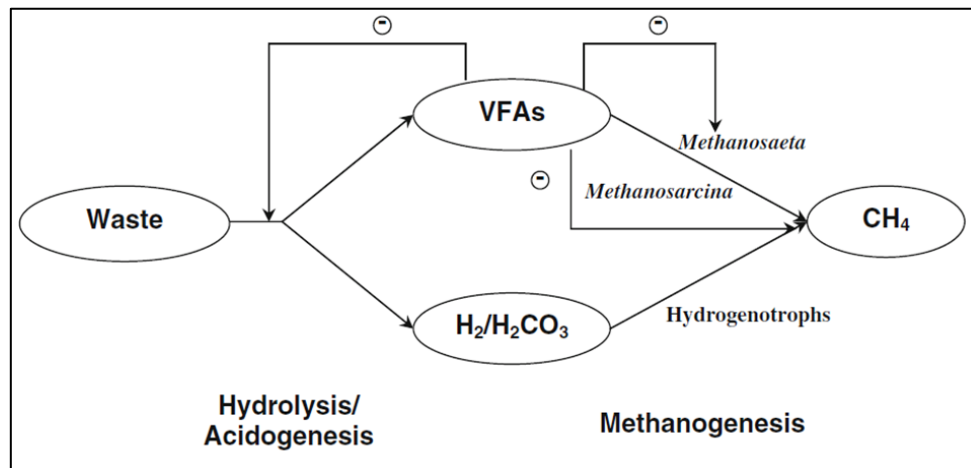


Figure 2 - Simplified kinetic scheme of anaerobic conversion of solid waste. Three groups of methanogens were considered including unified hydrogenotrophic methanogens and two populations of acetoclastic methanogens (*Methanosaeta* sp. and *Methanosarcina* sp.). *Methanosaeta* sp. and *Methanosarcina* sp. are inhibited by high volatile fatty acids concentration (Vavilin et al., 2008 b).

The behavior and activity of both acetotrophic and hydrogenotrophic methanogens during AD have been extensively investigated in different studies (Demirel and Scherer, 2008). One of the major drawbacks of anaerobic digestion is however the sensitivity of the methanogenic consortium to different environmental factors. A change in pH, an increase in salt or organic matter concentration, an alteration of the loading rate or the introduction of a toxic compound often causes system failure (De Vrieze et al., 2012). Their low growth rates compared to the acidogenic and acetogenic bacteria caused often the uncoupling of acetogenic bacteria and methanogens (Gujer and Zehnder, 1983).

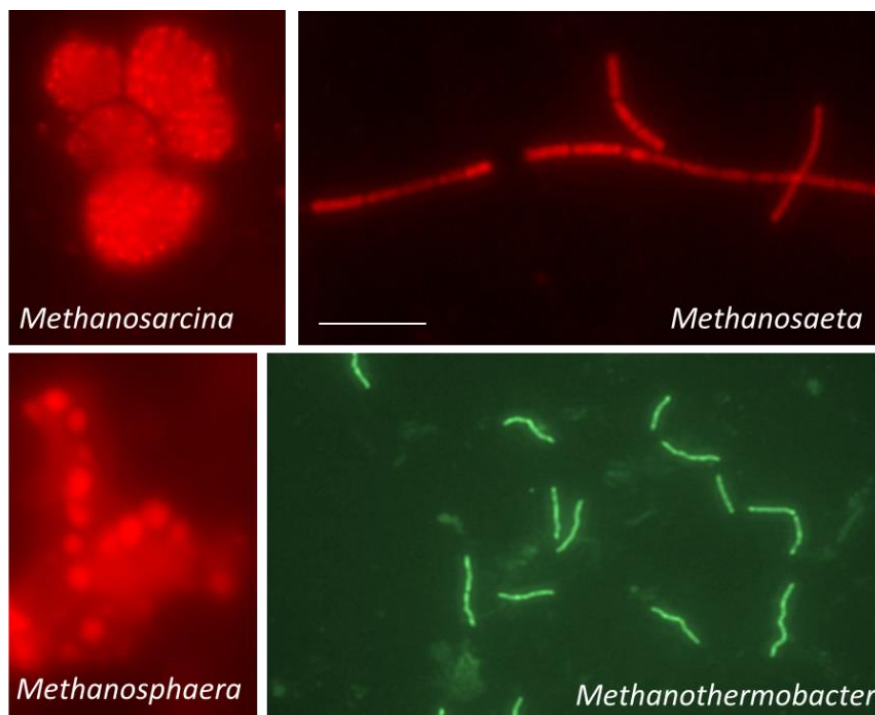


Figure 3 - Characteristical morphology of acetotrophic (*Methanosarcina* and *Methanosaeta*) and hydrogenotrophic (*Methanosphaera* and *Methanothermobacter*) methanogens by Fluorescence in situ Hybridization technique. Bar is 10 μm .

3.2.1 Acetotrophic methanogens

Surprisingly, only two genera of Archaea are known to use acetate as a substrate for methanogenesis (Conklin et al., 2006) and belong to the order Methanosarcinales: *Methanosarcina*, which prefers methylated compounds such as methanol and methylamines to acetate, and *Methanosaeta*, which is a specialist using only acetate (Smith and Ingram-Smith, 2007).

The latter is reflected in a very high affinity for the substrate. For growth, a minimal concentration of only 7–70 μM is needed (Berger et al., 2012). Their growth kinetics are mainly related to acetate and proportional to its concentration. *Methanosarcina* has a greater maximum rate of acetate utilization and maximum growth rate, a greater K_s and a greater yield coefficient compared with *Methanosaeta* (Conklin et al., 2006).

In low acetate environments, *Methanosaeta* prevails on *Methanosarcina* because of its higher affinity for the substrate (Berger et al., 2012), and vice versa. Nevertheless, they are often given as a classic example of how two microorganisms using the same substrate can coexist (Conklin et al., 2006); their simultaneous presence can benefit the AD process due to the higher metabolic versatility of *Methanosarcina*, since it can use almost all substrates for methanogenesis, with the exception of formate (Braguglia et al., 2012). *Methanosaeta* sp. and *Methanosarcina* sp. are inhibited by high volatile fatty acids concentration (Vavilin et al., 2008 b).

Methanosarcina sp. differs however from other methanogens as they are often tolerant against different stressors such as ammonium toxicity, overcharging the loading rate, high salt concentrations and temperature variation (De Vrieze et al., 2012). Vavilin et al. (2008 a) have shown that *Methanosarcina* sp., that it is a coccoid cell, shaped multicellular aggregates containing about 500 cells. Aggregates may resist to inhibition of various compounds (especially VFA and H^+), because of the decreasing of potential inhibiting compounds inside the aggregate.

Also *Methanosaeta*, that it is rod-shaped, can form aggregates as long filaments when grown in acetate with high cell density (Ma et al., 2006; Zhang et al., 2012), and can better compete with other microorganisms for resources in the environment; despite this, thin filaments offer a great surface and seems to be more sensitive to ammonia concentrations than *Methanosarcina* clusters (Demirel and Scherer, 2008).

3.2.2 Hydrogenotrophic methanogens

As described in Table 3, all the methanogens belonging to archaeal orders other than Methanosarcinales are hydrogenotrophic. Hydrogenotrophs can reduce CO_2 to methane with H_2 as the primary electron donor. Many hydrogenotrophic methanogens can also use formate as the major electron donor (Liu and Whitman, 2008).

Despite there is no general rule that accurately define the archaeal population dynamics during mesophilic and thermophilic processes, several studies indicated that acetotrophic methanogenesis was mainly retrieved in mesophilic conditions, while hydrogenotrophic methanogenesis is the main way of methane production in thermophilic conditions (Sipma et al., 2003; Demirel and Scherer, 2008; Krakat et al., 2010). In Ahring et al., (2002) hydrogenotrophic methanogens were the only microbial group, which exhibited higher specific methanogenic activity (SMA) and unchanged MPN (most probable number) at 65°C, compared to 55°C, while the activity and the amounts of other methanogens were significantly reduced. Presumably, it depends only on the hydrogen partial pressure in the system, an important parameter which defines process stability or upsets in an anaerobic digestion process (Demirel and Scherer, 2008). Moreover, hydrogenotrophic methanogenesis is inhibited at low temperature due to an insufficient supply of substrates (Liu and Whitman, 2008).

Hydrogenotrophic methanogens are less sensitive to inhibition than aceticlastic methanogens (Vavilin et al., 2008 b). A wide variety of hydrogenotrophic methanogens belonging to *Methanomicrobiales* and *Methanobacteriales* were detected in anaerobic digestors (Ariesyady et al., 2007; Rivière et al., 2009; Regueiro et al., 2012). Several of the isolated thermophilic hydrogen-utilizing methanogens belonging to the family Methanobacteriaceae were able of using both

hydrogen/carbon dioxide and formate as substrates (Ahring et al., 2002). The most common hydrogenotrophic methanogens in thermophilic digestors include *Methanothermobacter* spp., *Methanobrevibacter* spp. and *Methanoculleus* spp. (Liu and Whitman, 2008).

3.2.3 *Crenarchaeota*

Another archaeal phylum detected in anaerobic sludge is *Crenarchaeota* (Collins et al., 2005); the *Crenarchaeota* species were long thought to consist solely of extreme thermophiles, many of which can metabolize sulfur (Gupta et al., 2011), but they were detected also in mesophilic (Armanet et al., 2008) and psychrophilic habitats (Mchugh et al., 2004). In Collins et al., (2005) the intimate relationship between crenarchaeal clusters and *Methanosaeta* cells in granular sludge suggested that these organisms may interact metabolically with acetate-utilizing methanogens. Recent findings have suggested the broad distribution of mesophilic *Crenarchaeota* and their roles in ammonia oxidation in the environment (Nicol and Schleper, 2006). In Zhang et al., (2012), *Crenarchaeota*, instead of the methanogens, dominated the archaeal communities in anaerobic digestion, but were not related to ammonia oxidation, so more efforts are needed to understand their functions in anaerobic environments.

3.3 *Syntrophic metabolism*

Syntrophic metabolism plays an essential role in the recycling of organic matter to methane and carbon dioxide anaerobic environments like digesters (McInerney et al., 2008). The degradation of natural polymers such as polysaccharides, proteins, nucleic acids, and lipids to CO₂ and CH₄ involves a complex microbial community that interacts syntrophically (Sieber et al., 2012)

During AD, main syntrophic interactions are acetogenesis, in which propionate, longer-chain fatty acids, alcohols, some amino acids, and aromatic compounds are syntrophically metabolized to the methanogenic substrates hydrogen, formate, and acetate, and hydrogenotrophic methanogenesis, where the main syntrophic interactions are established between hydrogen-producing bacteria and hydrogenotrophic methanogens. With increasing population of hydrogenotrophs the hydrogen partial pressure decreases (Vavilin et al., 2008 b), making more favorable the reactions of hydrogen production by fermentative bacteria. The interaction between H₂-producing organisms and H₂-consuming organisms is named interspecies hydrogen transfer (Liu and Whitman, 2008). Interspecies hydrogen transfer between the acetogenic bacteria and the hydrogenotrophic methanogens is considered to be essential for the oxidation of fatty acids in anaerobic conditions (Hattori, 2007). Several syntrophic fatty acid-oxidizing bacteria including a butyrate oxidizer and a propionate oxidizer have been isolated and characterized. Imachi et al. (2000) isolated and co-

cultured the propionate-oxidizing *Desulfotomaculum* with *Methanothermobacter thermoautotrophicus*. Hatamoto et al. (2008) detected butyrate-degrading bacteria belonging to Syntrophaceae species, *Tepidanaerobacter* sp., and *Clostridium* spp. in methanogenic sludge. In addition to these syntrophs, it has been discovered that several bacteria (syntrophic acetate-oxidizing bacteria, SAO) can also oxidize acetate syntrophically when hydrogenotrophic and/or formate-utilizing methanogens are present (Hao et al., 2010; Mayumi et al., 2011).

Besides VFA degradation, also cellulose or protein degradation are linked to H₂ and CO₂ production. Acceleration of cellulose degradation in a methanogenic co-culture between *Clostridium clariflavum* and *Methanothermobacter thermoautotrophicus* was recently described (Sasaki et al., 2012). Moreover, Sasaki et al. (2011) reported the syntrophic interaction between proteolytic *Coprothermobacter proteolyticus* and hydrogenotrophic *Methanothermobacter thermoautotrophicus*. The latter interaction will be further discussed in chapters 2 and 5.

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Chapter 2

Identification and role of proteolytic *Coprothermobacter* spp. in different thermophilic anaerobic systems: a review

Gagliano M.C.

1. Introduction

Thermophilic bacteria have recently attracted great attention because of their potential application in improving different biochemical processes like anaerobic digestion of various substrates, wastewater treatment or hydrogen production. In particular, they possess enzymes with enhanced thermostability and hence may have a potential industrial application (Ollivier et al., 2000). Although proteolytic activity seems to be a common characteristic among mesophilic bacteria, only few proteolytic thermophiles have been characterized so far (Kerstens et al., 1994; Cai et al., 2011). *Coprothermobacter* spp. are gram negative anaerobic thermophilic bacteria detected at high temperatures (from 50 to 70°C). Member of this genus were found to have strong protease activity to degrade proteins and peptides, and they are important hydrogen producers. Their presence in an anaerobic system provides useful indications about the microbial pathways occurring during the process, and the establishment of syntrophic associations between *Coprothermobacter* and hydrogenotrophic archaea can significantly improve process performances. This chapter reviewed the literature related to this genus, describing their phylogeny, metabolism and role in anaerobic processes.

2. Isolation and phylogenetic classification of *Coprothermobacter*

The first member of the genus *Coprothermobacter* was originally isolated from a thermophilic (55°C) digester fermenting tannery wastes and cattle manure (Ollivier et al., 1985) (Fig.1 a). The isolate was classified as a member of the genus *Bacteroides* and named *Thermobacteroides proteolyticus*, strain BT. In 1993 Rainey and Stackebrandt proposed *Thermobacteroides proteolyticus* as the type species of the new genus *Coprothermobacter*, belonging to a deep-rooting phylum of the domain Bacteria and not to the Bacteroides-Cytophaga phylum as previously considered. Because the isolation source, to the species described in Ollivier et al. (1985) was given the name of *Coprothermobacter*.

Kerstens et al. (1994) carried out a further characterization of the species, with the isolation of *Coprothermobacter proteolyticus*, strain I8 from biokitchen waste digestate resulting from an anaerobic composting process at 55°C.

A new species, *Coprothermobacter platensis*, was isolated from a methanogenic mesophilic reactor treating a protein-rich wastewater (Etchebehere et al., 1998) (Fig.1 b). Recently, Tandishabo et al. (2007) isolated *Coprothermobacter* strain IT-3 from an enrichment culture containing organosolv

lignin, characterised by an higher hydrogen production with respect to the other type strains (Tandishabo et al., 2012).

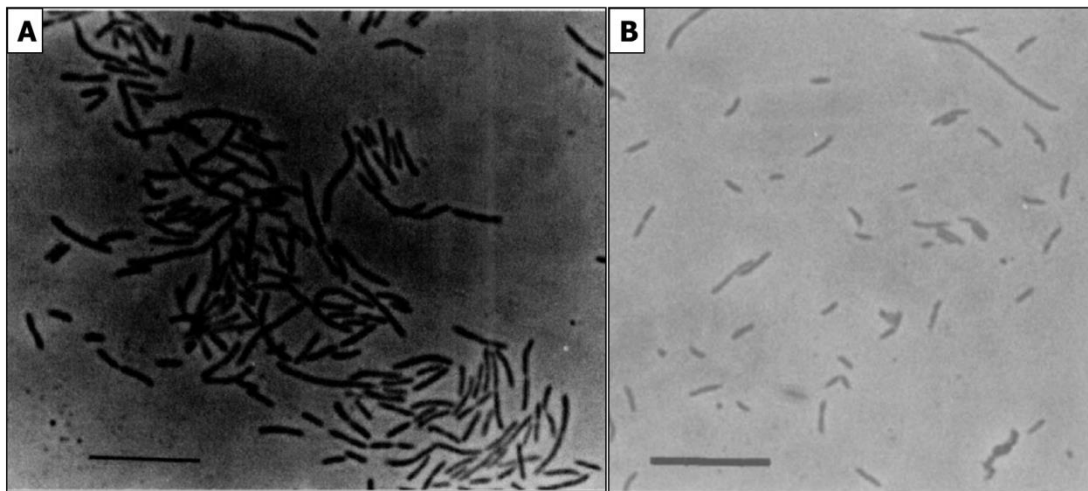


Figure 1 – Phase contrast micrographs of *Coprothermobacter proteolyticus* strain BT (in A, from Ollivier et al, 1985.) and *Coprothermobacter platensis* 3T (in B, from Etchebehere et al., 1998). Bar is 10 µm

In the present bacterial taxonomic system, the genus *Coprothermobacter* is classified into the phylum *Firmicutes*, class *Clostridia*, and affiliated with family *Thermodesulfobiaceae*, which is differently branched from families including most of aminoacid degrading bacteria in the phylum *Firmicutes* (Sasaki et al., 2011). Despite this, Etchebehere et al. (1998) highlighted for the first time a possible phylogenetic relationship between the genus *Coprothermobacter* and the *Thermotogales*. Tang et al. (2004) showed by 16S rRNA gene clonal analysis of a mixed microbial community growing in a thermophilic anaerobic municipal solid-waste digester the occurrence of two OTUs phylogenetically assigned to the phylum *Thermotogae* and closely related to *Coprothermobacter proteolyticus*.

In the work of Kunisawa (2010) regarding the phylogenetic classification of *Thermodesulfovibrio yellowstonii*, *Coprothermobacter proteolyticus* was treated as representing a separate phylum, ‘*Coprothermobacter*’. In this work, orthologous relationships of genes among different genomes were examined, and the comparison between gene arrangements of different bacterial phyla showed a consistent grouping of the major phyla, except for ‘*Coprothermobacter*’.

Placing of *Coprothermobacter proteolyticus* within *Clostridia* is not supported by either ribosomal protein-based phylogeny (Yutin et al., 2012) or whole-genome analysis (Beiko, 2011; Nishida et al., 2011).

An automated computational pipeline for the identification of r-protein genes, applied to 995 completely sequenced bacterial genome, was developed by Yutin et al. (2012). The phylogenetic

tree produced from this alignment (Fig.2) showed that the topology of the r-protein tree is generally compatible with the commonly accepted bacterial taxonomy, but several notable deviations existed.

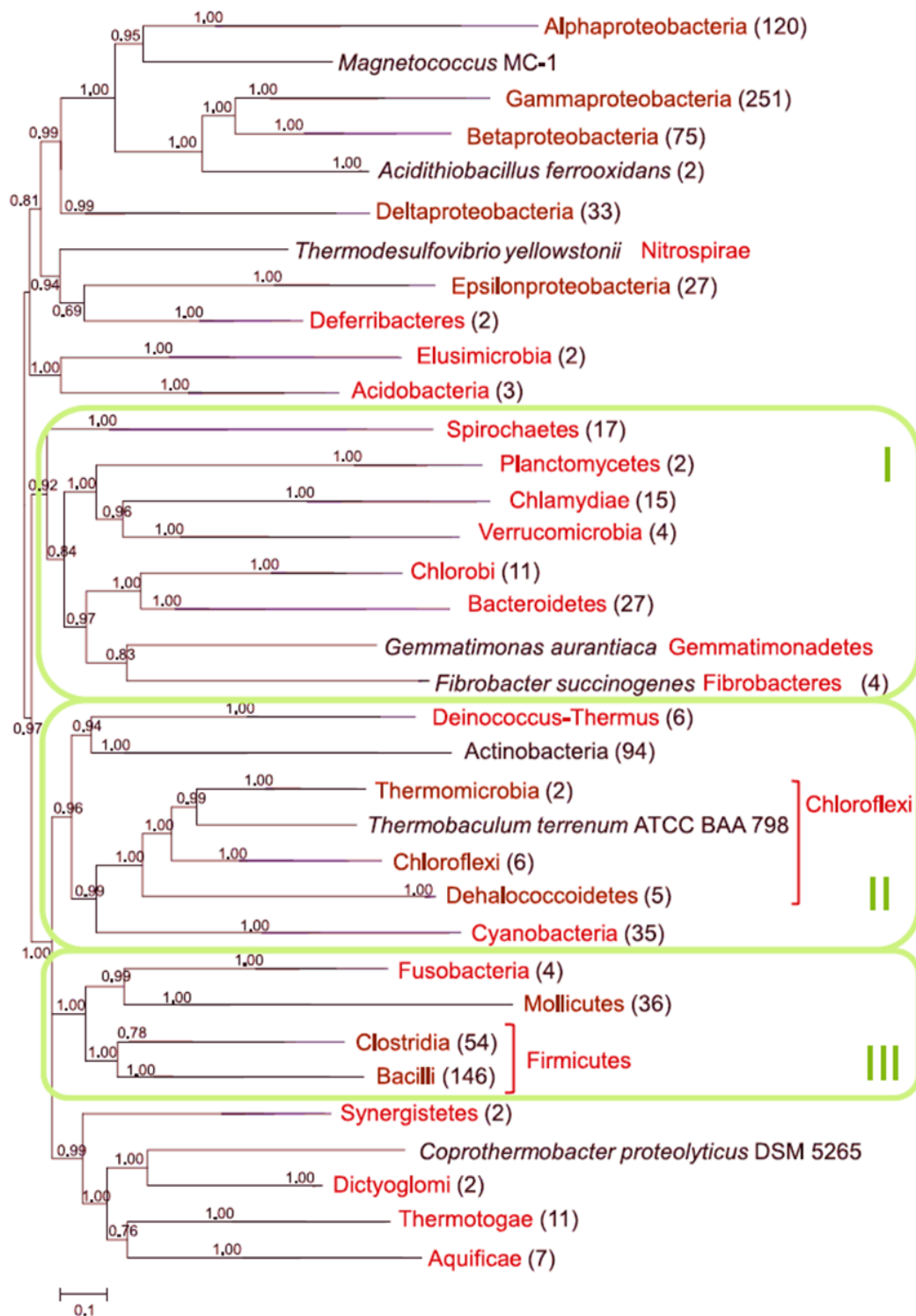


Figure 2 - Bacterial phylogenetic tree reconstructed from a concatenated alignment of 50 nearly ubiquitous r-proteins. Green boxes denoted as I, II, and III mark three putative “megaphyla” resulted from the study (Yutin et al., 2012).

In particular, *Coprothermobacter proteolyticus* DSM 5265 was placed in the *Dictyoglomia-Thermotogae-Aquificae* group, sister to *Dictyoglomia*.

Beiko et al. (2011) by analyzing a set of over 1000 genomes using new tools and techniques for taxonomic classification, highlighted that *Coprothermobacter* group showed a multitude of affiliations that are more consistent with their ecology than with small subunit ribosomal DNA-based taxonomy. With a distance-based approaches (as the neighbor-joining algorithm) they calculated an unusually positioned genome in the set for *C. proteolyticus*, which branches with other major thermophile-containing phyla *Dictyoglomi*, *Synergistetes*, and *Thermotogae* near the base of the bacterial tree. It is unclear whether this association is driven by legitimate genetic affinities, or is simply a consequence of a lack of affinity of these groups for other phyla in the tree. Additionally, using a model-based phylogenetic analysis of orthologous genes to calculate the intergenomic affinities of species, Beiko and colleagues found that the position of *Coprothermobacter* is connected to the three *Firmicute* genera *Bacillus*, *Clostridium* and *Thermoanaerobacter*, but is also linked to *Dictyoglomi*, which are in turn linked to all genera within phylum *Thermotogae*. In the work of Nishida et al. (2011), concerning the phylogenetic position of the anaerobic thermophilic bacterial genus *Dictyoglomus*, a whole-genome comparison analyses that clarified the phylogenetic relationships between *Dictyoglomus* and other bacteria showed that *C. proteolyticus* was excluded from the large cluster of Firmicutes. They demonstrated that *Coprothermobacter* clusters to *Dictyoglomi* and *Thermotogae* and not to *Firmicutes*, indicating that *C. proteolyticus* is not a member of *Firmicutes* but represents another taxonomic group most closely related to *Dictyoglomi*. These results support that *Coprothermobacter*, *Dictyoglomi* and *Thermotogae* diverged from a common ancestor at an early stage of bacterial evolution.

3. Proteolytic activity

Generally, *Coprothermobacter* is associated with the microbial population of anaerobic digesters because of its proteolytic properties.

Proteolytic activity of *Coprothermobacter* spp. is largely reported. Ollivier et al. (1985) showed that *C. proteolyticus* strain BT grew well on peptides, as demonstrated by H₂ production and glucose degradation rate when rumen fluid or trypticase peptone were added to culture media; fermentation of sugars was poor and acetate was the major volatile acid produced. The fermentation products were acetic acid, H₂, and CO₂, along with smaller quantities of isobutyric, isovaleric, and propionic acids.

In Kesters et al. (1994), *C. proteolyticus* strain I8 fermented a wide variety of proteins (gelatin, bacto-peptone, casein, tryptone and bovine serum albumin) and acetate was a major end-product. Strain I8 ferments sugars as also ascertained for strain BT. By analyzing gelatin degradation by *C. proteolyticus*, Kesters et al. (1993) showed that the gelatin concentration in the medium affected strongly the ammonification: the highest percentage of organic nitrogen conversion to ammonium was observed at a gelatin concentration ranging between 2.7 and 10 g/l. At higher concentrations (10-50 g/l) ammonification was reduced but higher production of acetate was observed. Moreover, *C. proteolyticus* expressed its proteolytic activity also when grew poorly.

Etchebehere et al. (1998) described that cells of *C. platensis* were proteolytic, because growth was observed with different proteinaceous substrates. They also highlighted an extracellular protease activity, which increased during growth.

In the metaproteomic study on cellulose methanization process (Lü et al., 2014 b), *C. proteolyticus* was described as scavenger and/or predator performing proteolysis and fermentation. Among the numerous identified proteins, 22 putatively involved in ammonia assimilation and amino acid biosynthesis and in peptidase activities were affiliated to *C. proteolyticus*, highlighting its intensive proteolytic activity during the process. The latter suggested a role in degrading several distinct proteins sources during cellulose degradation, like EPS, the abundant extracellular enzymes or other secreted proteins and dead cell material generated during the incubation by the counter-selection of poorly competitive strains. Moreover, Lü and colleagues further supported this proteolytic activity by other retrieved protein functions. In particular, seven ABC transporter clusters were attributed to *C. proteolyticus* including three related to peptide transport, suggesting an important peptide import/export activity.

3.1 Biotechnological exploitation of proteolytic properties

Enzymes from extremophiles offer the opportunity to expand the reaction conditions compatible with biocatalytic conversions (Toplak et al., 2013).

C. proteolyticus showed high levels of intracellular and extracellular protease activity (Majeed et al., 2013; Lü et al., 2014). For these proteolytic properties, *Coprothermobacter* may be a good candidate for facilitating treatment and processing of protein-rich wastewater under high temperature.

A comprehensive survey of the protease complement (or degradome) in the genome of *C. proteolyticus* was recently reported (Cai et al., 2011); it possesses a core degradome structure that may be common in the thermophilic bacteria, as shown by the comparison with *Moorella thermoacetica* and *Thermoanaerobacter tengcongensis*, the most closely related sequenced species

in the family *Thermoanaerobacteriaceae*. Functional characterization of these enzymes in this bacterium may provide a better understanding of the mechanisms of physiological adaptation to hot temperature and a better assessment of its potential application to wastewater processing.

On the basis of the results obtained in the study of Klingenberg et al. (1991), whom analyzed properties of several extremely thermostable proteases from various thermophilic microorganism, Antranikian and Klingenberg (1994) were the first to isolate and patent a proteolytic enzyme extracted from *C. proteolyticus*. They discovered a serine-protease, showing an extraordinary thermostability as well as thermoactivity, with an optimum temperature in the range of 75 to 95° and a pH optimum in the range of 6.5-10. Protease was also resistant to inhibition from different chemicals (Ethylenediaminetetraacetate, iodoacetate and phenylmethylsulphonylfluoride) (Klingenberg et al., 1991). Due to the unique properties of the protease, it was added to a detergent composition comprising one or more surfactants or additionally enzymes.

Another possible application of protease from *C. proteolyticus* was described in Majeed et al. (2013), which is the first report of the biochemical characterization of a recombinant protease from *C. proteolyticus*. The enzyme is a serine protease with an alkaline pH optimum and functions at elevated temperature. The protease also has the desirable property of retaining high activity in the presence of a wide variety of surfactants, thus indicating potential utility of this enzyme in detergent applications.

The production, purification and biochemical characterization of a *C. proteolyticus* protease in *E. coli*, namely proteolysin, was recently described by Toplak et al. (2013). Proteolysin can hydrolyze proteins at temperatures as high as 90 °C, and showed an high tolerance towards organic solvents. Because of its thermostability, relaxed specificity, the broad pH range, and resistance to routinely used protein denaturants and DTT, proteolysin is a candidate for proteomics studies when protein digestion under extreme conditions is required.

Palatsi et al. (2011) suggested to enrich the anaerobic digestion process with species of peptide-fermentative microorganisms like *Coprothermobacter* in order to treat successfully substrates as slaughterhouse and residual waters. Regarding this observation, bioaugmentation may be a cost-effective strategy to exploit the proteolytic activity of *Coprothermobacter* and improve degradation processes at high temperatures. Bioaugmentation of thermophilic, anaerobic sludge with *Coprothermobacter* was successfully applied by Lü et al. (2014 a). In general, the proteins degradation rate and efficiency were found to slightly increase with the increase of *C. proteolyticus* inoculation density. However, *C. proteolyticus* itself did not improved the final methane yield. On the contrary, when *C. proteolyticus* was added together with a granular anaerobic sludge, the methane yield was significantly enhanced. This could be explained by the fact that *C. proteolyticus*

can produce H_2 from proteinaceous materials and, consequently, promote syntrophic cooperation with hydrogenotrophic methanogens, as described in the next section.

4. Syntrophic association with methanogens

Growth of *Coprothermobacter* spp. on protein substrates is closely associated with hydrogen production and hydrogen is one of the most important carriers in interspecies electron transfer between *Coprothermobacter* spp. and methanogens (Tandishabo et al., 2012). Therefore, *Coprothermobacter* spp. should benefit from the activities of the hydrogenotrophic methanogens in anaerobic digesters. In particular, *Coprothermobacter* can accomplish protein degradation in syntrophic association with hydrogenotrophic methanogenic archaea (Sasaki et al., 2011).

Coprothermobacter activity is improved by the establishment of a syntrophy with hydrogenotrophic methanogens like *Methanothermobacter thermoautotrophicus*, commonly found as component of methanogenic population in many thermophilic anaerobic systems. Hydrogen is the primary energy source of this methanogen, even when in situ hydrogen concentrations are very low (Kato et al., 2008). Sasaki et al. (2011), comparing a monoculture of *Coprothermobacter proteolyticus* to a co-culture with *Methanothermobacter thermoautotrophicus*, reported that in co-culture *Coprothermobacter* growth rate increased 4-fold in presence of *Methanothermobacter* (Fig.3), with respect to a pure culture.

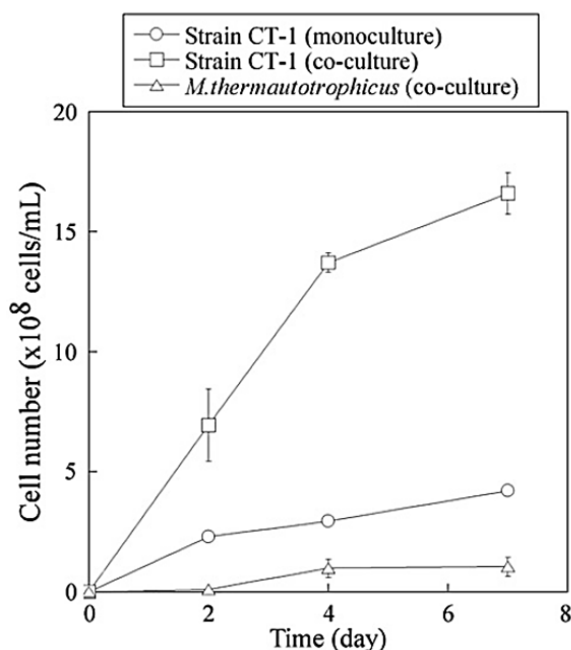


Figure 3 - Time courses of cell numbers in the monoculture (*Coprothermobacter proteolyticus* strain CT-1) and co-culture (*Coprothermobacter proteolyticus* strain CT-1 + *M. thermoautotrophicus*) using casein as the protein substrate. (Sasaki et al., 2011).

Simultaneously, the number of cells of *Methanothermobacter* decreased without affecting methane production rate. In addition, during co-culture the soluble protein content decreased more than in monoculture. This means that the presence of an hydrogenotrophic partner was essential to improve the proteolytic activity of *Coprothermobacter*.

In the work of Lü et al. (2014 b) on cellulose methanisation process, *C. proteolyticus* was proposed as important H₂ producer in the system and might have established efficient syntrophy with *Methanothermobacter*.

Moreover, in a thermophilic digester at 70°C the shift in bacterial population to *Coprothermobacter* dominance corresponded to the predominance of *Methanothermobacter* among Archaea (Ge et al., 2011).

Proteolysis in a thermophilic model bioelectrochemical co-culture (*C.proteolyticus* and hydrogenotrophic *Methanothermobacter thermoautotrophicum* strain ΔH) and single-culture (*C. proteolyticus*) was examined by using a bioelectrochemical system to control electron flow in culture medium (Sasaki D. et al.,2012). In non-bioelectrochemical environments, it has been reported that hydrogen-consuming methanogens help in scavenging hydrogen and increasing proteolytic activity. This study showed that the growth of *C.proteolyticus* and proteolysis were accelerated as the result of increased hydrogen consumption, which occurred because of the better growth of strain ΔH in the thermophilic bioelectrochemical culture system. These observations highlighted the role of *Methanothermobacter* to optimize H₂ consumption avoiding its accumulation and the consequent inhibition of fermentative bacteria.

Coprothermobacter and *Methanothermobacter* were found in the same thermophilic anaerobic systems in several studies (Tang et al., 2008; Tatara et al., 2008; Tang et al., 2011; Ritari et al., 2012; Luo et al., 2013; Lü et al., 2014; Guo et al., 2014).

5.Other metabolic abilities of *Coprothermobacter* spp.

The ability of *C. proteolyticus* and *C. platensis* to use thiosulfate as an electron acceptor was reported by Etchebehere et al. (1998). Thiosulfate reduction, alanine production and hydrogen inhibition of glucose utilization were studied in pure cultures of members of the genus *Coprothermobacter* (Etchebehere and Muxì, 2000). They found that thiosulfate enhanced the growth of the *C. platensis* strain on carbohydrates and proteinaceous substrates, but did not results in an increase in growth with respect to a control culture without a substrate, confirming the inability of the bacterium to use it as an energy source. Hydrogen addition to growing cultures of *C.proteolyticus* and *C.platensis* strains immediately caused growth to cease. It was also shown that

hydrogen accumulation caused inhibition of glucose utilisation thereby explaining the preferential utilization of proteins by member of this genus. Furthermore, both members of the genus *Coprothermobacter* were shown to produce alanine during glucose and pyruvate fermentation. In the work of Tandishabo et al. (2007) the strain IT3 was showed to be involved in cyanide detoxification by means of an extracellular rhodanese activity, and this activity was found also in *C. proteolyticus* and to a lesser extent in *C. platensis*.

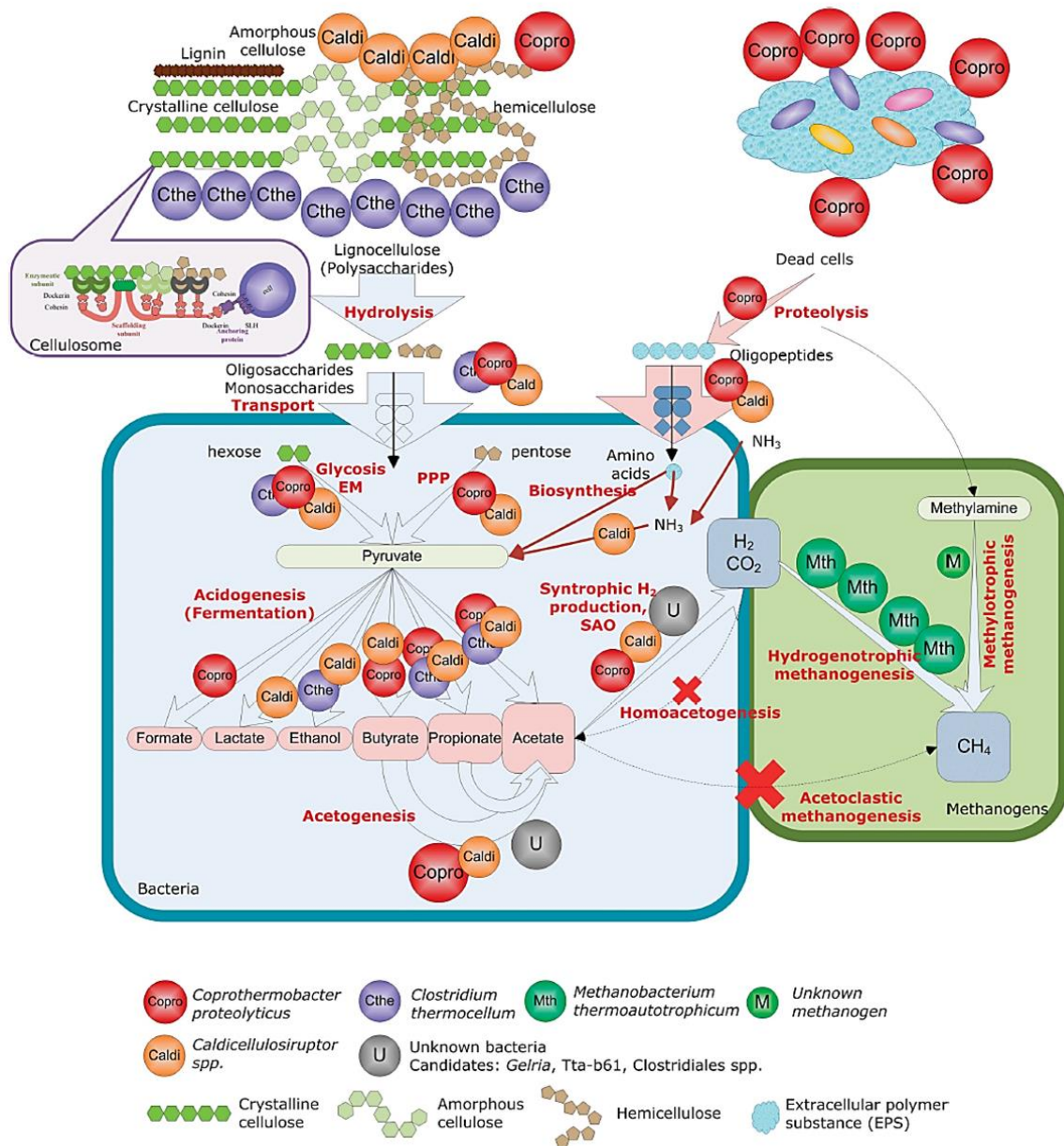


Figure 4 - Functional model for the anaerobic digestion of lignocellulose by complex thermophilic communities, from Lü et al. (2014 b), in which Coprothermobacter (red circle) is involved in several steps.

This ability strongly correlated with thiosulfate reduction in many anaerobic bacteria, and could play an important role in the treatment of wastes from cassava processing, a plant containing cyanogenic glucosides, commonly used as source of calories in developing tropical countries.

A metaproteomic approach revealed that *Coprothermobacter* may be also involved in different pathways during anaerobic degradation of organic matter (Lü et al., 2014 b) (Fig.4). They included fermentative production of pyruvate, formate, butanol and acetate, syntrophic acetate oxidation (SAO) with consequent production of hydrogen, ammonia assimilation, aminoacids biosynthesis and general protein turnover. In addition, a possible role in microcin (a bacterial toxin) synthesis was discovered; several proteins from *C. proteolyticus* related to oxidative stress response or to virulence factor were also identified, but their role remains unclear.

Coprothermobacter was also found to be the dominant bacteria during carbon monoxide biomethanation integrated to anaerobic digestion of sewage sludge, suggesting a possible role in the conversion chain of CO into methane (Luo et al., 2013).

6. Distribution and identification of *Coprothermobacter* in biological anaerobic systems

In recent years, *Coprothermobacter* spp. were identified in several studies focused on the microbial community structure of anaerobic processes, especially under thermophilic conditions.

The molecular enumeration of *Coprothermobacter* spp. was performed on samples from seven anaerobic digesters fed with garbage, dairy cattle manure and food processing waste, using specific primers targeting the 16S rDNA of the genus (Tandishabo et al., 2012). The largest number of *Coprothermobacter* spp. cells was found in a thermophilic anaerobic digester treating dairy cow manure; since dairy cow manure can contain up to 16% of crude protein, *Coprothermobacter* should be associated with the microbial population of digesters because of its proteolytic properties. Lee et al. (2009) detected *Coprothermobacter* spp as the key microbe in the hyperthermophilic acidogenesis step (70 °C) and in the methanogenic step (55°C) of an hyperthermophilic two-phased digestion system treating kitchen garbage and waste activated sludge; 23% of the 16S rDNA clones during acidogenesis step were affiliated to *Coprothermobacter*, and this was correlated to an high protein solubilization efficiency.

Kobayashi et al. (2008) identified *Coprothermobacter* during thermophilic anaerobic digestion of waste activated sludge; 73% of clones were affiliated to this genus, and real-time PCR highlighted that *Coprothermobacter* spp. were the main bacteria in the digester. The same result was evidenced in a following study (Kobayashi et al., 2009) during the thermophilic (70% of total clones) and mesophilic (15.3% of total clones) stages of a TPAD; when an intermediate ozonation between thermophilic and mesophilic stages was introduced, *Coprothermobacter* was not detected in the mesophilic reactor, suggesting that thermophilic bacteria might be killed by intermediate ozonation.

In the study of Cheon et al. (2008) *Coprothermobacter* was detected in a full scale thermophilic digester (55°C) with the random cloning method.

The presence of *Coprothermobacter* was evidenced in coexistence with *Lutispora thermophila* in a thermophilic anaerobic reactor of a two-phased AD treating activated sludge (Pervin et al, 2013), related to amino-acid fermentation. The increased protein hydrolysis was connected to better digestion performance.

In Palatsi et al., (2011) during the anaerobic digestion of slaughterhouse waste mixture, with a metabolism related to long-chain fatty acid degradation, proteolytic activity of *Coprothermobacter* was retrieved together with *Anaerobaculum mobile*.

Coprothermobacter was also associated to carbohydrate fermentation at 55°C during phased anaerobic digestion fed with a mixture of primary and secondary sludge (Zamanzadeh et al., 2013).

Zhang et al. (2009) analyzed the effect of a Focused-Pulsed (FP) sludge pretreatment on the bacterial diversity of a mesophilic full-scale anaerobic digester treating a mixture of primary and waste activated sludge, and showed that *Coprothermobacter* population decreased from 10% to 1% after the pretreatment, in line with the observed dramatic shift from H₂-utilizing to acetoclastic methanogens after FP pretreatment.

Coprothermobacter was the predominant phylotype detected in a thermophilic digester sludge treating municipal solid waste by RNA-Based Stable Isotope Probing (Hatamoto et al., 2008).

Ritari et al. (2012) identified *Coprothermobacter* in a CSTR fed with a mixture of biowaste and sewage sludge.

One OTU affiliated to *C. Proteolyticus* was identified in a bioelectrochemical reactor for methanogenic fermentation of thickened sewage sludge (Sasaki et al., 2013).

Coprothermobacter spp. were also identified in non-conventional anaerobic systems. For instance, 16S rRNA gene sequences closely related to *C. proteolyticus* were found in an anaerobic packed-bed reactor using carbon fiber textiles (CFT) as the supporting material treating an artificial garbage slurry (Sasaki et al., 2007).

In Tatara et al. (2008) 17% of total clones in the liquid and biofilm fractions of thermophilic down-flow anaerobic packed-bed reactor in continuous methanogenesis from propionate were represented by *Coprothermobacter*. In a packed bed reactor treating volatile fatty acids (Ueno et al., 2008) *Coprothermobacter* was the predominant clone in the class of *Thermoanaerobacteriaceae*.

Yabu et al. (2011), detected one OTU related to *Coprothermobacter* during thermophilic dry anaerobic digestion of model garbage with ammonia stripping.

In the study of Tang et al. (2011) a long term (5 years) acclimated microbial community fed with an artificial medium (a mixture of printed copy paper, waste newspaper, dog food, and water) was used

for a thermophilic dry methanogenic systems fed with garbage stillage. *Coprothermobacter* was detected during the process.

Kawagoshi et al. (2005) identified *Coprothermobacter* by DGGE in an hydrogen producing reactor inoculated with six different sources and treating a synthetic medium, while Nissilä et al. (2010) detected *Coprothermobacter* in a batch enrichment of hydrogen producing, cellulolytic cultures enriched from compost material. *Coprothermobacter* was found at the biocathode during hydrogen production in a two-chambered microbial electrolysis cells, representing 19.8% of total clones (Fu et al., 2013 c).

Tang et al. (2008), using DGGE detected 16S rRNA gene sequences belonging to *Coprothermobacter* sp.P1 in a glucose-degrading methanogenic consortium at 65°C.

Some researchers detected *Coprothermobacter* in high temperature petroleum reservoirs. In the work of Nazina et al. (2006) *Coprothermobacter* clones represented 11% of the fermentative bacteria in enrichment cultures (60°C) from formation waters of the high-temperature horizons of Dagang oilfield (China). Lan et al. (2011) identified by DGGE analysis two bands closely similar to *C. proteolyticus* in enrichment cultures (75°C) resulted from production water from a high temperature, water-flooded petroleum reservoir of an offshore oilfield in China.

Mbadinga et al. (2012) detected *Coprothermobacter* in an alkane-dependent methanogenic community derived from production water of a high-temperature petroleum reservoir; its presence was related to thiosulfate reduction. Sulfate reduction ability of this bacteria was also highlighted in Sarti et al. (2009), in which *Coprothermobacter* was detected at high sulfate concentration (3 g SO₄⁻/l) in an anaerobic sequencing batch reactor treating sulfate rich wastewater. *C. proteolitycus* was also found by ARDRA in an anaerobic thermophilic oleate-degrading enrichment culture in the study of Menes and Muxì (2001).

Moreover, *Coprothermobacter* species were identified by clonal analysis in the anode community in thermophilic microbial fuel cells (MFCs): in Jong et al. (2006) and Wrighton et al. (2008), *Coprothermobacter* sequences represented ≈ 15-16% of clones; in Ha et al., (2013), represented 20% of bacterial population in acetate fed thermophilic MFCs, and 3.4% in distillery wastewater fed thermophilic MFCs; in Fu et al., (2013 a and b), was detected in a two-chamber MFCs reactor inoculated with thermophilic anaerobic sludge; in Hussein et al. (2011) *Coprothermobacter* was the dominant bacteria in MFCs treating carbon monoxide and syngas; in Weld and Singh (2011) was surprisingly detected at mesophilic temperatures, probably because the system was connected to a thermophilic anaerobic digester. In all of these studies, it was supposed that its presence was independent by anodic electron transfer, but related to its presence in the startup sludge inoculum (Lovley, 2008), and its ability to do extracellular electron transfer.

Additionally, there are some studies in which 16S rRNA gene sequences belonging to *Coprothermobacter* spp. were retrieved during thermophilic anaerobic digestion, but were not affiliated to this genus (Hori et al., 2006; Sasaki et al., 2006; Hatamoto et al., 2007; Rivere et al., 2009; Krakat et al., 2010).

7. Proteolytic activity and substrate availability

Coprothermobacter growth seems to be related to proteinaceous substrate availability and its level of hydrolyzation.

Tandishabo et al. (2012) showed that *Coprothermobacter* population size in several anaerobic digesters is not only controlled by the thermodynamics of hydrogen production but also by the type of substrates in the respective wastes. They additionally observed that *Coprothermobacter* cells seemed to show a severely decreased ability to proliferate in digesters fed with dairy cow manure compared to the digesters fed with food processing wastes.

Kobayashi et al. (2008) correlated the dominance of *Coprothermobacter* during thermophilic anaerobic digestion of waste activated sludge to the high protein content of sludge (15 g/L).

In a thermophilic anaerobic reactor of a two-phased AD treating activated sludge, a temperature shift from 50°C to 65°C caused a change in bacterial population from *Thermotogae* sp. to *Coprothermobacter* and *Lutispora thermophila*, and their presence was related to an increased hydrolysis, since both microorganism carry out amino-acid fermentation (Pervin et al., 2013). A similar finding was reported by Ge et al. (2011), due to a temperature shift from 55°C to 70°C in a thermophilic anaerobic digester fed with WAS: at 55°C the dominant organism were affiliated to *Clostridia*, *Thermotogae* and *Sphingobacteria*, while at 70°C *Coprothermobacter* outcompetes and dominated in the thermophilic reactor.

Cheon et al. (2007) compared microbial population dynamics in thermophilic methane digesters fed with garbage, sewage sludge and feedstock waste, and *Coprothermobacter* was detected only in the first two digesters, with higher and increasing relative abundance in the reactor fed with sewage sludge, in which the organic loading rate (OLR) was significantly lower with respect to the other reactors.

Lee et al. (2009) connected the presence of *Coprothermobacter* in an hyperthermophilic acidogenic reactor treating kitchen garbage and WAS at 70 °C with the degradation of protein in waste activated sludge at this temperature. Indeed, comparing their results on protein degradation with a reactor fed with only kitchen garbage, they highlighted an increase in protein solubilization efficiency from sludge with respect to kitchen garbage.

Coprothermobacter was detected in a full scale thermophilic digester (55°C), when the substrate changed from garbage to sewage sludge (Cheon et al., 2008). In Sasaki et al. (2007) *Coprothermobacter* was found to be responsible for degradation of low-molecule organic matters in packed-bed reactor degrading organic solid waste.

Guo et al. (2014) compared microbial characteristics of the thermophilic and mesophilic anaerobic digesters exposed to elevated food waste loadings. *Coprothermobacter* was detected in thermophilic digester and its abundance increased with OLR (the OLR of the reactor was gradually elevated with a gradient of 0.5 g VS L⁻¹ d⁻¹ from the initial level of 1.0 g VS L⁻¹ d⁻¹).

In the study of Wagner et al. (2013) nine complex organic substrates from three classes (protein-, lipid-, and cellulose-rich) were investigated in batch experiments in order to evaluate their potential for utilization as substrates for biogas production. *Coprothermobacter* clones were detected during batch digestions fed with dosing feed (obtained from an anaerobic digester), lawn-clippings, carboxymethylcellulose and tree-cut (wood and branches); thus, sludge seems to be a good substrate for *Coprothermobacter* growth, but it was involved in cellulose degradation too.

This feature underlines that the source of proteinaceous substrate is important for *Coprothermobacter* growth.

The relationship between substrate availability and *Coprothermobacter* relative abundance estimated by FISH analysis during thermophilic anaerobic digestion of waste activated sludge will be extensively described in chapter 5.

8. Methods used for *Coprothermobacter* identification

In most of the previously cited studies, *Coprothermobacter* spp. were identified only by applying PCR based approaches, mainly clonal analysis and DGGE, using universal primers for the domain Bacteria. Very few studies developed strategies for the specific identification of members of this genus. Specific primer set for PCR and Real-time PCR were recently designed (Tandishabo et al., 2012; Kobayashi et al., 2009). Regarding its identification by fluorescence in situ identification (FISH), none of probes targeting *Firmicutes* phylum (LGC354 a,b,c probes) targeted *Coprothermobacter proteolyticus* 16S rRNA, and this is in accordance to the phylogenetic classification, as previously discussed (Beiko, 2011; Nishida et al., 2011; Yutin et al., 2012).

Lü et al. (2014 b) designed an oligonucleotide probe for FISH application, Copro925, but the specificity was closely related to 16S rDNA sequences retrieved by clonal analysis in that specific study. For this reason, the design of a specific fluorescent oligonucleotide probe targeting

Coprothermobacter and its identification on anaerobic sludge sample will be extensively described in next chapter.

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Chapter 3

In situ identification of the syntrophic protein fermentative *Coprothermobacter* spp. involved in thermophilic anaerobic digestion process

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1. Introduction

Anaerobic digestion (AD) is one of the few sustainable technologies that both produce energy and treat waste streams. Driven by a complex and diverse community of microbes, AD is affected by different factors, many of which also affect the composition and activity of the microbial community (Weiland, 2010). In recent years, anaerobic processes operating under thermophilic conditions have attracted great attention due to their advantages mainly related to the higher organic matter removal and methane production and total pathogens removal (Montero *et al.*, 2008). Our knowledge about the microbial consortia involved in this process is limited because of a lack of phylogenetic and metabolic data on these predominantly uncultivated microorganisms (Rivière *et al.*, 2009). A deeper knowledge of the identity and function of the microbial components would therefore allow to better control the biological processes.

Coprothermobacter proteolyticus was originally isolated from a methanogenic enrichment culture inoculated from a thermophilic digester (Ollivier *et al.*, 1985). Generally, *Coprothermobacter* is associated with the microbial population of anaerobic digesters because of its proteolytic properties, and for this reason may be a good candidate for facilitating treatment and processing of protein-rich wastewater under high temperature (Cai *et al.*, 2011). Recently, *Coprothermobacter* spp. were identified in several studies focused on the microbial community structure of anaerobic thermophilic reactors. For example, members of this genus were found in a laboratory scale thermophilic anaerobic digester of waste activated sludge (Kobayashi *et al.*, 2008), in a full scale thermophilic sewage sludge digester (Cheon *et al.*, 2008, Rivière *et al.*, 2009), in the hyperthermophilic acidogenesis step during two-phased hyperthermophilic anaerobic co-digestion of waste activated sludge (Lee *et al.*, 2009), and in a two-stage TPAD system under thermophilic conditions (Pervin *et al.*, 2013). Microorganisms related to this genus and affiliated to the family of *Thermodesulfobiaceae* are often observed in methanogenic reactors degrading organic solid wastes (Sasaki D. *et al.*, 2011). *Coprothermobacter* spp., were also detected in the anode biofilm of microbial fuel cells (Wrighton *et al.*, 2008), or during anaerobic fermentative biohydrogen production (Kawagoshi *et al.*, 2005). Growth of *Coprothermobacter* spp. on protein substrates is also closely associated with hydrogen production (Tandishabo *et al.*, 2012). In particular, *Coprothermobacter* can accomplish protein degradation in syntrophic association with hydrogenotrophic methanogenic archaea (Sasaki K. *et al.*, 2011).

In order to study and monitor natural anaerobic ecosystems, as well as anaerobic engineering processes, in situ detection methods as fluorescence in situ hybridization (FISH) are widely used for the identification, quantification and, in combination with other techniques, characterization of

phylogenetically defined microbial populations. Only microscopy-based tools can indeed link isolated extracted DNA markers with intact, individual cells in their natural environments and also exclude the biases often observed with nucleic acids extraction and PCR. In spite of this applicative potential of FISH analysis, no specific probe for in situ biomonitoring of *Coprothermobacter* spp. is available so far. In this study a new oligonucleotide probe was designed, targeting most of the members of the genus *Coprothermobacter* and optimized its application on thermophilic sludge samples through the design of specific helper probes.

2. Materials and Methods

Microorganisms and cell fixation. The following reference strains were used in this study: *Coprothermobacter proteolyticus* strain BT (DSM 5265) and *Sulfurihydrogenibium rodmanii* strain UZ3-5 (DSM 19533) were used in FISH probe optimization as positive and negative controls respectively. Cell material was collected from liquid active cultures and immediately fixed in formaldehyde (FA, 2% v/v final concentration) for 3 h at 4°C. Fixed samples were filtered through polycarbonate membrane filters (pore size 0.2 µm, diameter 47 mm, Millipore) by gentle vacuum (< 0.2 bar) and the filters were stored at -20°C until further processing.

Sludge samples. Thermophilic sludge samples were collected from laboratory-scale anaerobic digesters fed with thermal pretreated waste activated sludge (WAS), operated in semi-continuous and maintained at the constant temperature of 55 °C, as described in the work of Gianico *et al.*, (2013). The sampling of sludge was performed during steady-state operating conditions. Aliquots of 1.5 mL of mixed liquor (ranging from 15 to 30 TS g L⁻¹) were either immediately frozen at -20°C for further DNA extraction or fixed with paraformaldehyde and ethanol for FISH analysis as described in Amann *et al.*, 1990.

Design of oligonucleotide probes and helpers. Probe and helpers sequences and stringency conditions are reported in Table 1. Oligonucleotide helpers and probe were purchased from Bio-Fab Research (Italy). FISH probes were labeled with 5'- Cy3. Probe and helpers coverage, efficiency and design were evaluated using the software Sfold (Ding *et al.*, 2005, <http://sfold.wadsworth.org>), TestProbe (Quast *et al.*, 2013, <http://www.arb-silva.de/search/testprobe/>) and mathFISH (Yilmaz *et al.*, 2011, <http://mathfish.cee.wisc.edu/index.html>). The candidate target regions were chosen through the analysis of rRNA secondary structure generated with the software sFold. Probes specificities were checked in silico against the SILVA 16S rRNA database (version 115). Finally, to test the

possible effectiveness of probes in hybridization, FISH in silico was simulated through the software mathFISH, which calculates the hybridization efficiency of a probe with respect to a target sequence on the basis of thermodynamic mathematical models of FISH. The sequence alignment with TestProbe was done using the database SSU Parc, that contains all aligned sequences with an alignment identity value equal and above 50.

FISH. FISH assays were performed on filter-harvested cells according to previously published procedure (Pernthaler *et al.*, 2001). Domain specific probes, ARC915 probe for *Archaea* and EUB338mix probes for *Bacteria* (Table1), were also applied in order to further check for the specificity of the novel probe. DAPI (4',6-diamidino-2-phenylindole) fluorescent staining was also simultaneously performed for determining total cell numbers. FISH on fixed sludge samples was performed as previously described (Braguglia *et al.* 2102). To identify thermophilic *Methanobacteriales*, the protocol was modified by applying an enzymatic pretreatment with pseudomurein endopeptidase as described in Nakamura *et al.* (2006), in order to improve probe penetration through cell wall.

Microscopy and Signal quantification. Samples were examined by epifluorescence microscopy (Olympus BX51). Fluorescence signal was quantified on at least 10 microscopic images taken from the samples with a digital camera (Olympus XM-10) and the software Cell F. The fluorescence signal intensity was considered as mean gray value (pixel intensities) using ImageJ software package (version1.37v, Wayne Rasband, National Institute of Health, Bethesda, USA, <http://rsb.info.nih.gov/ij/index.html>).

Genomic DNA extraction and PCR amplification of 16S rRNA genes. DNA was extracted from ≈ 700 mg of thermophilic sludge taken at the end of the digestion process, using the protocol reported in Rossetti *et al.* (2008). The concentration and purity of the genomic DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). 16S rDNA genes were amplified using primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) for *Bacteria*, as described in Rossetti *et al.* (2003).

Cloning and sequencing of 16S rDNA. Cloning of PCR products was carried out using pGEM-T Easy Vector System (Promega, USA) into *Escherichia coli* JM109 competent cells (Promega, USA) according to the manufacturer's instructions. Positive inserts were amplified from recombinant plasmids from white colonies by colony PCR, using the sequencing primers T7

(TAATACGACTCACTATAGGG) and M13 (22 mer, Promega, USA) (TCACACAGGAAACAGCTATGAC), as described in Chen *et al.*(2003). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Netherlands). 16S rDNA inserts were sequenced and sequence identities determined. Results of clonal analysis are reported in Figure 1. All the 16S rDNA sequences belonging to the *Coprothermobacter* genus differed by <2%. One 16S rDNA full sequence of *Coprothermobacter* was submitted to GenBank under the accession number KF971872.

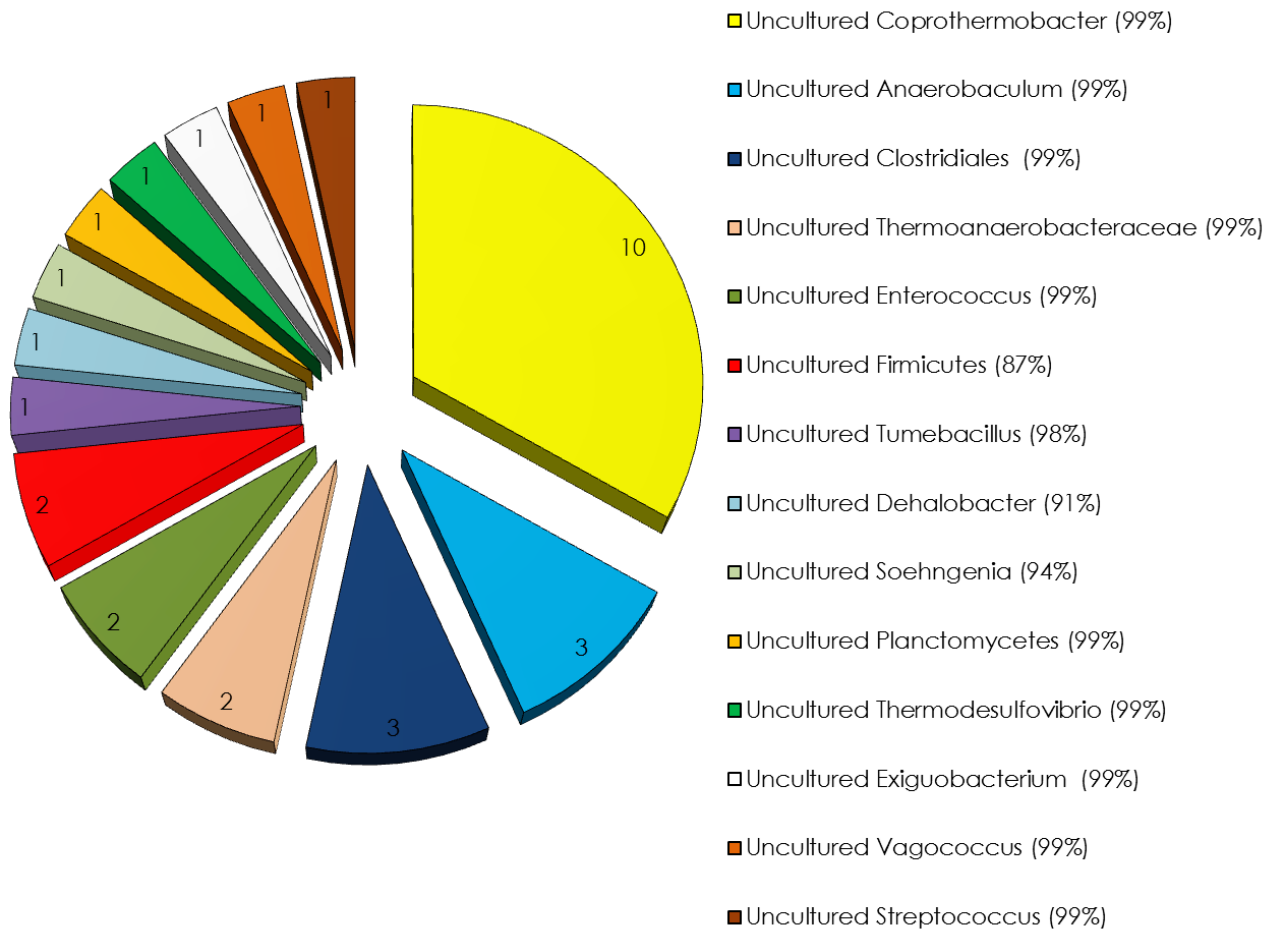


Figure 1 - Bacterial clones from thermophilic digested sludge. A total number of 30 clones were examined. *Coprothermobacter* related sequences were the most abundant. Identity of sequences are reported in brackets.

3.Results

3.1 Design of specific FISH probe for *Coprothermobacter* species

In the present bacterial taxonomy, the genus *Coprothermobacter* is classified into the phylum Firmicutes. Nevertheless, as described in chapter 2, *Coprothermobacter* represented a taxonomic group mostly closely related to *Dictyoglomi* and *Thermotoga*. Indeed, none of probes targeting

Firmicutes phylum (LGC354 a,b,c probes) targeted *Coprothermobacter proteolyticus* 16S rRNA (as ascertained by testing the probes sequences in TestProbe), and no hybridization was obtained in our sludge samples. In order to detect *in situ* members of this genus, a specific FISH probe was produced. Probe design for the detection of *Coprothermobacter* species was performed on the basis of a target region identified by the software CaSSis in the work of Bader *et al.* (2011, supplementary material). The signature sequence with the best group coverage in the phylogenetic tree of *Coprothermobacter* found by CaSSis was used in our study to design a FISH probe, by comparing this sequence with 16S rRNA gene sequences retrieved from our clonal analysis (Figure 1), and with sequences present in the last release of SILVA database. This sequence, covering nucleotide 485-503 (E.coli numbering), was complementary to 70.9% of the sequences of *Coprothermobacter* present in the SILVA SSU 115 database (CTH485 probe in Table 1). The sequence alignment with TestProbe showed that CTH485 probe fully matched also with 20 outgroup sequences belonging to unculturable microorganisms, but all the sequences from the database are about ≈500 bp and some of these have a low Pintail value.

3.2 CTH485 FISH probe optimization

Pure culture. *Coprothermobacter proteolyticus* strain BT (DSM 5265) was utilized as positive control in FISH probe optimization. *Sulfurihydrogenibium rodmanii* strain UZ3-5

(a)	Probe sequence	3' - ATGGGGTCATCTTTCCT - 5'
	Target sequence	5' - TACCCAGTAGAAAGGA - 3'
	<i>Coprothermobacter proteolyticus</i> DSM 5265	5' - ===== - 3'
	<i>Sulfurihydrogenibium rodmanii</i> strain GV2-1C1	5' - =====gA===== - 3'
	<i>Sulfurihydrogenibium rodmanii</i> strain UZ-5 DSM19533	5' - =====gAU===== - 3'
(b)	Probe sequence	3' - GTGCTCCCCCGCCAATTCCT - 5'
	Target sequence	5' - AGGAATTGGCGGGGAGCAC - 3'
	<i>Coprothermobacter proteolyticus</i> DSM 5265	5' - =====UC=g= - 3'

Figure 2 - Difference alignments for CTH485 (a) and ARC915(b) probes. 16S rRNA sequences at the target sites of the probes are displayed for representative reference organisms. Upper-case letters indicate nucleotide changes leading to strong mismatches; lower-case letters indicate changes leading to weak mismatches.

was used as negative control for CTH485 probe even though this probe had three mismatches with 16S rRNA gene sequences of cultured closest relatives (Figure 2 a). The optimal formamide concentration for probe stringency was determined by performing a series of FISH experiments at different formamide increments starting at 0% formamide (Figure 3). The optimal formamide concentration was the highest concentration before the signal intensity decreased. The optimal stringency for probe CTH485 resulted 30% (Figure 4 a). No fluorescence signal was detected when this probe was applied to *Sulfurihydrogenibium rodmanii* strain UZ3-5.

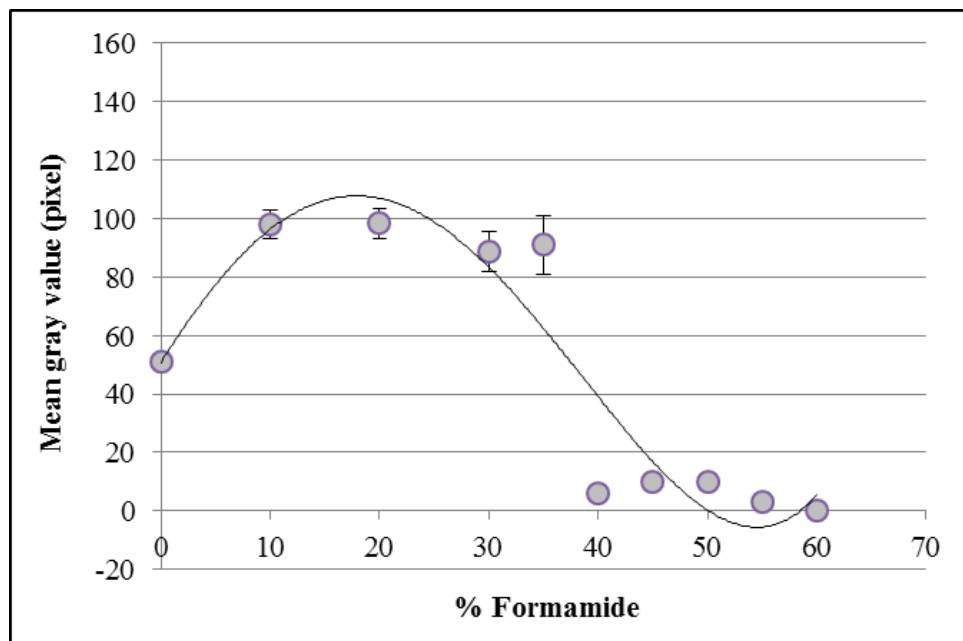


Figure 3 - Probe binding profile of CTH485 probe on pure culture of *Coprothermobacter proteolyticus*. The relative strength of hybridization was determined at increasing concentrations of formamide by quantification of fluorescence signal intensity.

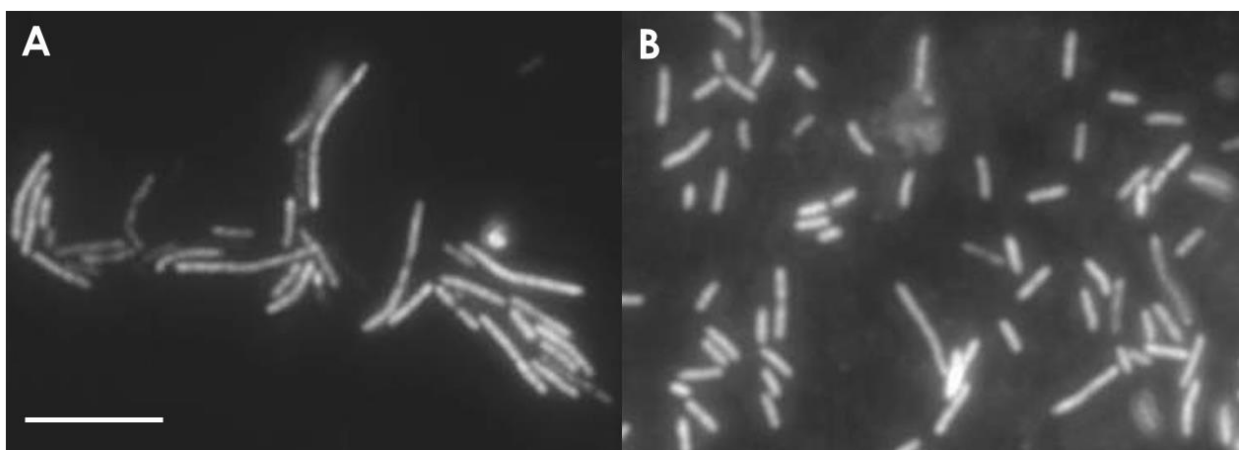


Figure 4 – FISH analysis of *Coprothermobacter proteolyticus* (DSMZ 5265) using CTH485 probe (a), and of thermophilic anaerobic sludge (b) using the probe together with helper CTH439. Bar is 5 μ m.

Thermophilic anaerobic sludge. CTH485 probe, even though optimized and validated on a pure culture of *Coprothermobacter proteolyticus*, did not properly work when thermophilic sludge was analysed: the fluorescence signal was very weak and was not clearly distinguishable from the background fluorescence. Therefore, on the basis of the ensemble centroid secondary structure of 16S rRNA of *Coprothermobacter proteolyticus* (Figure 5), the accessibility of the target region of the probe was evaluated. Two unlabeled helper oligonucleotides were therefore designed to open the helix and make the target more accessible for the probe. Among a range of options, helpers hCTH429 and hCTH439 were chosen (Table 1), both on the basis of the melting temperature values (58.6 and 62.3 °C respectively, higher than the melting temperature of 48°C of CTH485) and on the results obtained from the in silico test of sequences in mathFISH (as described in Materials and Methods).

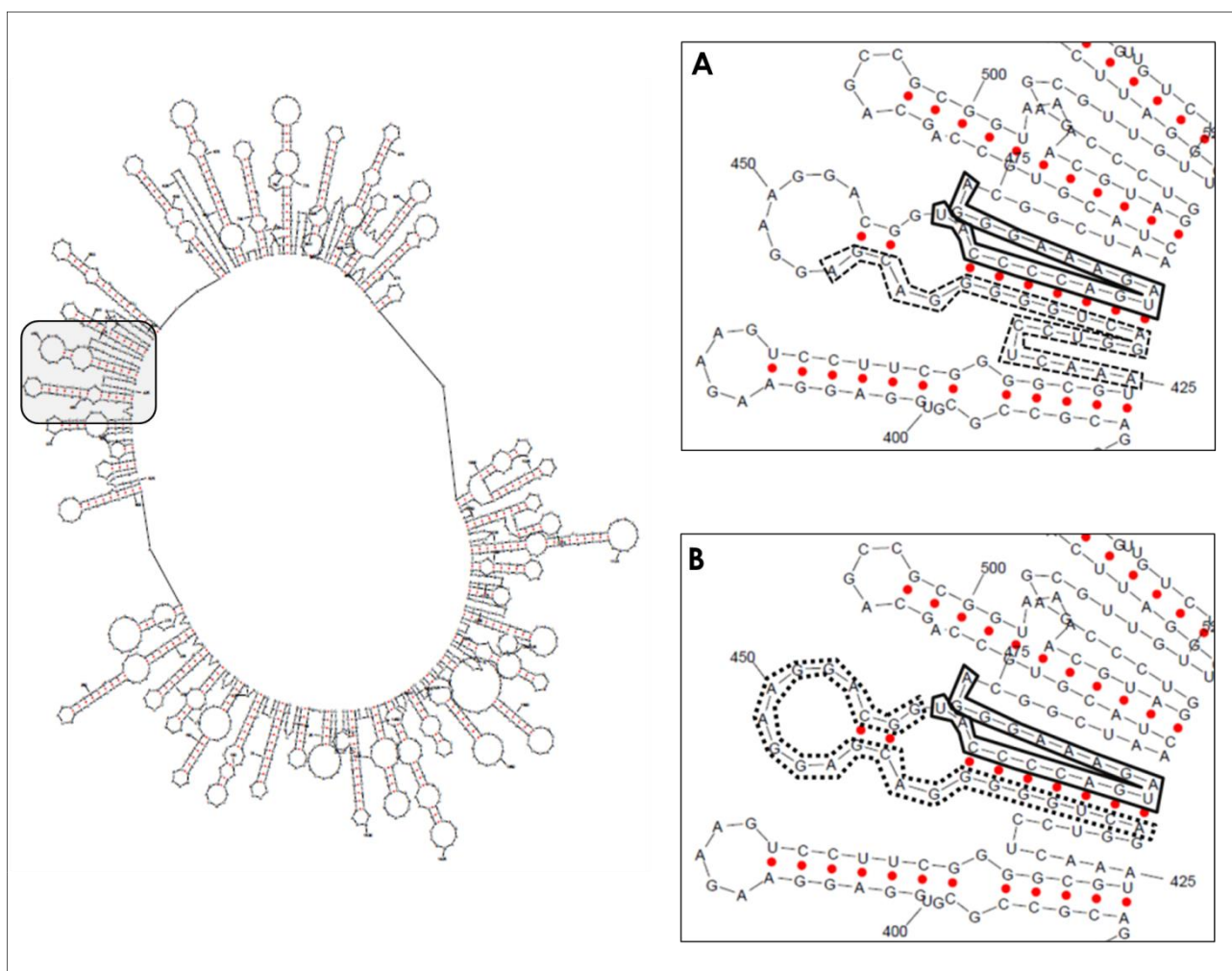


Figure 5 - Design of unlabeled oligonucleotide helpers with the software Sfold. Ensemble centroid 16S rRNA secondary structure of *Coprothermobacter proteolyticus* (on the left) was obtained and the target region of CTH485 probe, that falls inside the helix 16, was analyzed for the design of oligonucleotide helper CTH429 (A) and CTH439 (B) (dashed lines).

Table 1 – List of probes and helpers used in this study.

Name	Target group	E.coli numbering	Probe sequence (5'-3')	Formamide (%)	Coverage (% of target sequences)	Reference
CTH485	<i>Coprothermobacter</i>	485-503	TCCCTTTCTACTGGGGTA	30	71.3	This study
hCTH429	CTH485 helper	429-451	TCGTCCCCCAGTCCAGGAGTTT	-	69.9	This study
hCTH439	CTH485 helper	439-461	CCGTCCCTTCCTCGTCCCCCAGT	-	60.2	This study
ARCH915	<i>Archaea</i>	915-934	GTGCTCCCCCGCCAATTCCT	20, 35	79.1	Crocetti <i>et al.</i> , 2006
MB311	<i>Methanobacteriales</i>	311–333	ACCTTGCTCTCAGGTTCCATCTCC	30	94.8	Crocetti <i>et al.</i> , 2006
EUB388 I, II and III	<i>Bacteria</i>	338 - 355	GCTGCCTCCCGTAGGAGT, GCAGCCACCCGTAGGTGT and GCTGCCACCCGTAGGTGT	20	91.9, 0.6 and 1.4	Amann <i>et al.</i> , 1990

Table 2 – Relative abundance of *Coprothermobacter* identified with probe CTH485 and hCTH439 out of total bacteria or out of total DAPI stained cells. The operative parameters of the three anaerobic thermophilic reactors fed with thermal pretreated activated sludge are detailed in Gianico *et al.*, (2013).

Digester n°	%Coprothermobacter/total cells	%Coprothermobacter/total bacteria	OLR (g VS L ⁻¹ d ⁻¹)	HRT (d)
1	43.3	93	1	15
2	49.7	65	1.8	8
3	42.9	69	3.7	8

When CTH485 probe was applied together with each of both helpers on pure culture of *Coprothermobacter*, similar results were obtained as in the case of the sole probe application with the exception of a shift of the fluorescence signal decay at around 40% of formamide. A remarkable difference was instead observed with the combined application of probe with helpers to fixed samples originating from thermophilic anaerobic sludge digester. In presence of hCTH429 and hCTH439 helper probes, cells displayed good fluorescence signal. The fluorescence signal was higher in presence of hCTH439 with respect to hCTH429 (+15%). At stringency rising to 40% formamide, fluorescence signal was detectable only in presence of hCTH439. This signal was 16% lower compared with the fluorescence signal intensity registered at 30% formamide in the same conditions. The use of probe CTH485 together with hCTH439 at 30% formamide (Figure 4 b) was therefore the optimal solution for the analysis of thermophilic anaerobic sludge. The observed difference of fluorescence emission of CTH485 probe when applied on pure culture and on thermophilic sludge is likely due to physiological changes of 16S rRNA 3D structure of *Coprothermobacter proteolyticus* in a complex habitat like anaerobic digester operating under thermophilic conditions. The application of probe CTH485 and hCTH439 to thermophilic sludge samples taken from three anaerobic digesters fed with thermal pretreated WAS led to the identification of the majority of bacterial population (Table 2 and Figure 6). The relative abundance of *Coprothermobacter* retrieved in the different sludge samples ranged between 60 and 90% out of total bacteria (Table 2).

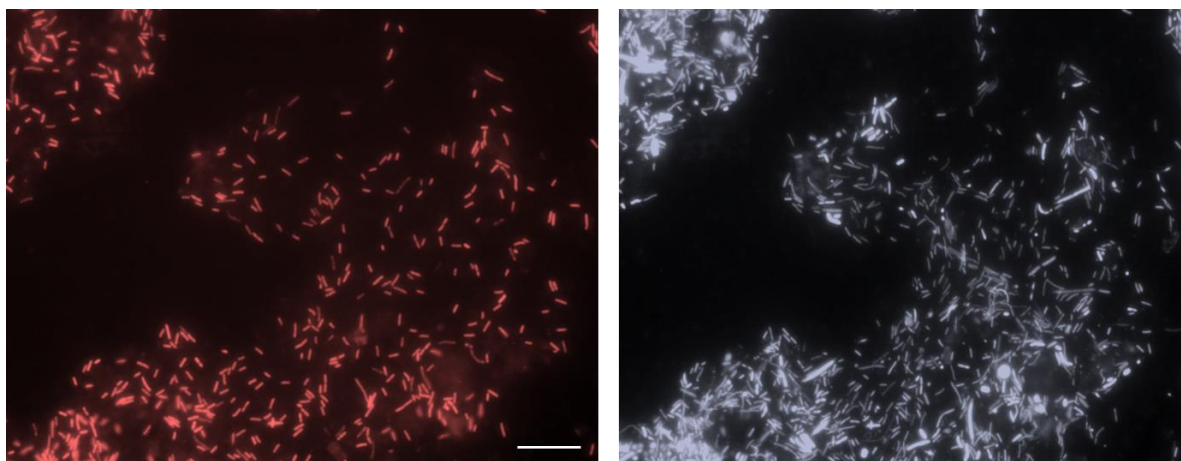


Figure 6 - *Coprothermobacter* cells in thermophilic sludge visualized with probe CTH485 + hCTH439, and DAPI staining of the same field. Bar is 10 µm

3.3 Aspecific binding of *Coprothermobacter* spp. with ARC915 probe

It is worth to noting that an aspecific binding of the probe ARC915 with *Coprothermobacter* cells was observed in this study. The aspecific binding was observed either when the probe was applied

on thermophilic sludge samples or tested directly on the pure culture of *Coprothermobacter proteolyticus* strain BT, working at the stringency conditions widely applied for ARC915 probe (20% and 35% of formamide, Figure 7).

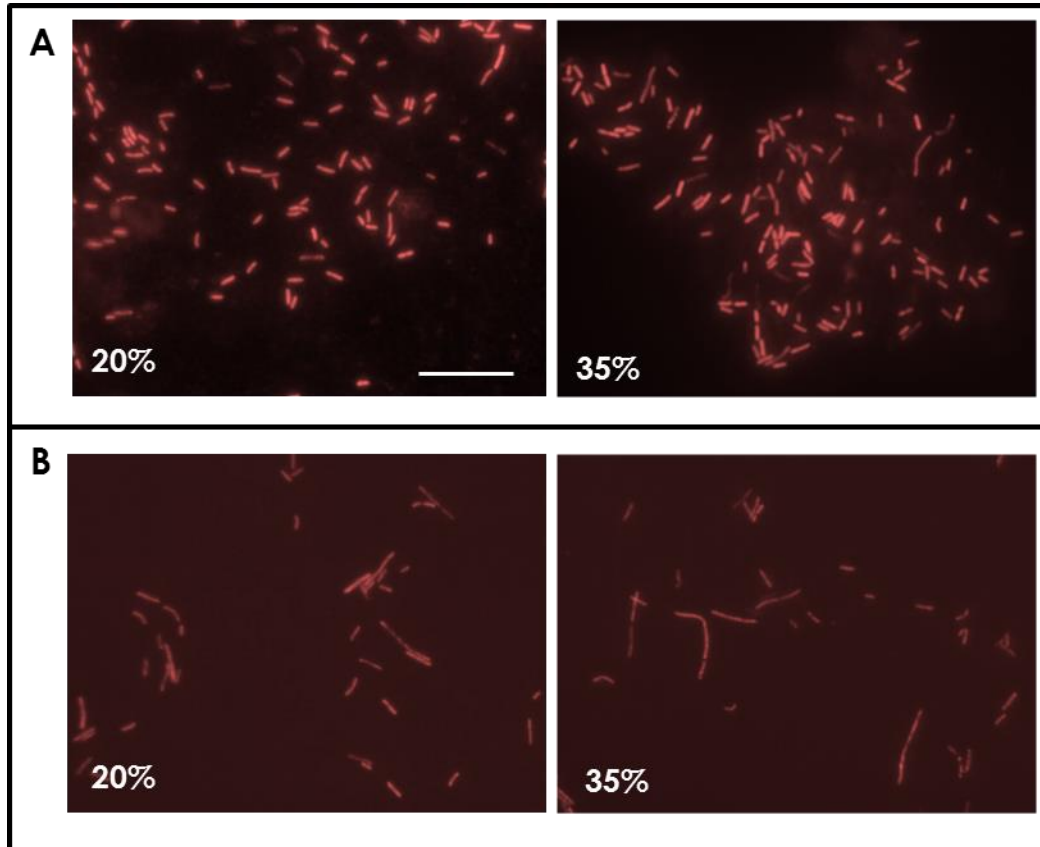


Figure 7 - Aspecific binding of *Coprothermobacter* with ARC915 probe observed in thermophilic sludge samples (A) and in a pure culture of *Coprothermobacter proteolyticus* DSM5265, at two different stringencies conditions (20% and 35%). Bar is 10 μ m.

Hybridization of *Coprothermobacter* cells in thermophilic sludge samples with CTH485 and ARC915 probes is shown in Figure 8 a. The different morphology of archaeal cells, with respect to *Coprothermobacter*, in thermophilic sludge, was highlighted using a pseudomurein endopeptidase pretreatment (Nakamura et al., 2006) and the specific probe MB311, as shown in Figure 8 b. This issue had never been previously reported because in all previous studies the occurrence of this microorganism was ascertained only by constructing 16S rDNA clonal analysis (Kawagoshi et al., 2005; Cheon et al., 2008; Kobayashi et al., 2008; Wrighton et al., 2008; Lee et al., 2009; Rivi re et al., 2009; Sasaki D. et al., 2011; Luo et al., 2013) or by qPCR (Tandishabo et al., 2012) without FISH validation. The position and typology of the mismatches of the 16 rRNA of *Coprothermobacter* spp. with the target sequence of ARC915 probe are reported in Figure 2 b, in which the sequence of *Coprothermobacter proteolyticus* DSM5265 was used as model. Two strong

mismatches (U/T:U/T and C:C) and one weak mismatch (g:u/t) are located at the extremity of the target string and this may explain the incomplete binding of the ARC915 probe.

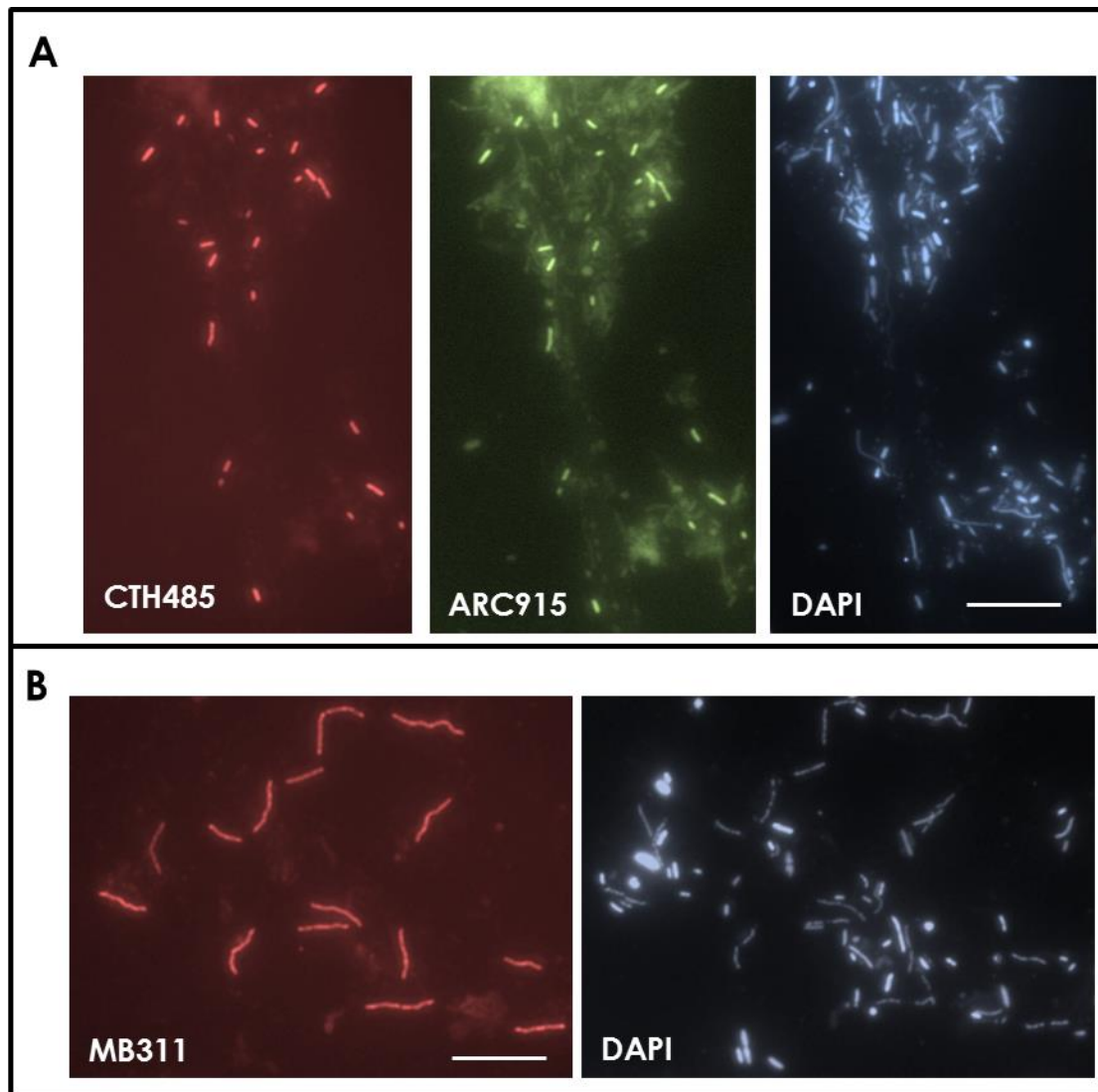


Figure 8 – *Coprothermobacter* cells hybridised with probe CTH485+hCTH439 and ARC915 at 30% formamide (A); archaeal cells belonging to *Methanobacteriales* visualized with MB311 probe at 35% formamide, after enzymatic pretreatment with pseudomurein endopetidase, as described in materials and methods (B). Bar is 10 μ m.

Mismatches at the ends of a probe are significantly more stable than mismatches in the central section, where positional dependence is minimal (Yilmaz *et al.*, 2008). Cover specificity and mismatch weight determination with TestProbe showed that ARC915 probe cover most of the sequences of *Coprothermobacter* spp. present in database (88.7%), and the weight of the mismatches was 2.3. The positional weight distribution used in TestProbe did not consider the different thermodynamic parameters involved in hybridization against different organisms, like free

energy value of target site, probe folding free energy and the mismatch position, as reported in Yilmaz *et al.* (2008). According to these observations, an evaluation of mismatch stability with the mismatch analysis tool of the software mathFISH was done, using the sequence of ARC915 against *Methanothermobacter thermoautotrophicus* strain Δ H 16S (accession number 074260.1) as target sequence and *Coprothermobacter proteolyticus* 16S (accession number 074653.1) as non target sequence (Figure S6 a,b).

(A)

	<i>M. thermoautotrophicus</i>	<i>C. proteolyticus</i>
$\Delta G^{\circ}_{\text{overall}}$	-25.1 kcal/mol	-16.7 kcal/mol
$[\text{FA}]_{\text{m}}$	74.8 %	37.9 %
Hybridization Efficiency	1.0000	1.0000

(B)

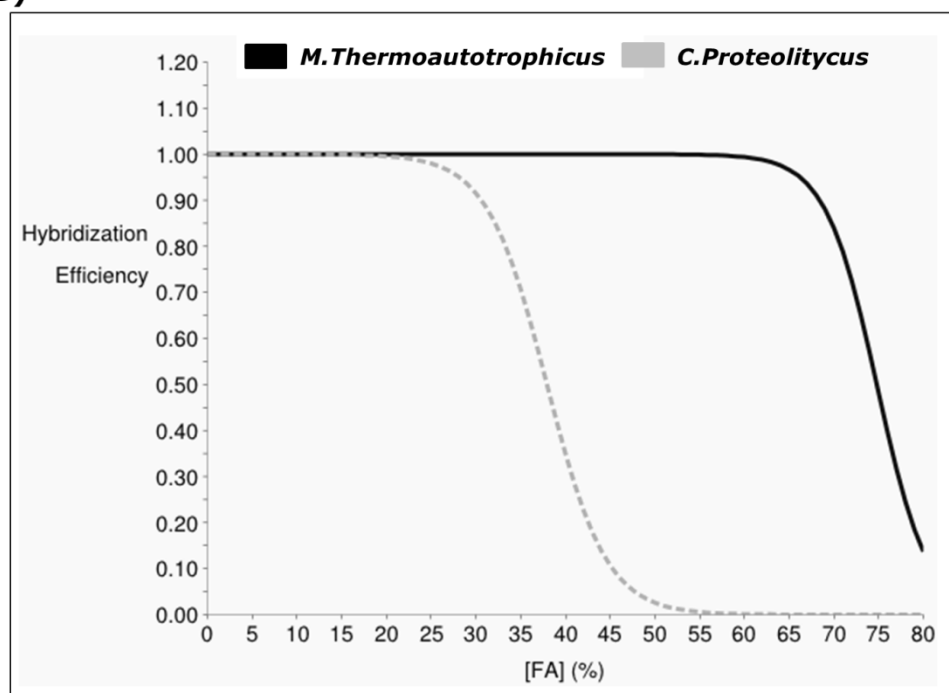


Figure 9 –Mismatch analysis with mathFISH software package for ARC915 probe sequence against 16S rRNA sequence of *Methanothermobacter thermoautotrophicus* strain Δ H and of *Coprothermobacter proteolyticus* strain DSM5265. In (a) and (b), for this derivation, probe and target site sequence were hybridized *in silico* at 46°C with 1 M [Na⁺]. In table (A) the following values are reported: (1) $\Delta G^{\circ}_{\text{overall}}$ is the result of three parameters: ΔG°_1 the free energy change of hybridization assuming a linear probe structure and a fully accessible target site; ΔG°_2 is the free energy change that defines the stability of intramolecular DNA-DNA interactions within the probe. The formation of this structure competes with probe-target duplex formation; ΔG°_3 quantifies the accessibility of the target with respect to the secondary rRNA structure; (2) $[\text{FA}]_{\text{m}}$ is the melting formamide concentration; (3) the hybridization efficiency is calculated at 0% formamide. The graph (B) is the plot of hybridization efficiency as a function of formamide concentration.

Overall ΔG_0 values and the hybridization efficiency trend showed that the probe hybridized with non target organism as well as the target until $\approx 30\%$ FA. According to the *in silico* results, an hybridization on thermophilic sludge samples with ARC915 probe at 30%, 40% and 50% of formamide was done. As seen in figure 4 a, the probe bound both target (archaeal thin filaments) and non-target cells (*Coprothermobacter* little rods, detected also with EUB338mix and CTH485 probes) with the same fluorescence emission obtained at 30% formamide. At 40% of formamide a marked difference in fluorescence emission between target and non-target cells was observed (Figure 4 b), but only at 50% of formamide *Coprothermobacter* cells were no longer detectable (figure 4 c), together with a quite loss of fluorescence from archaeal cells which anyhow did not affect the amount of detected cells (data not shown).

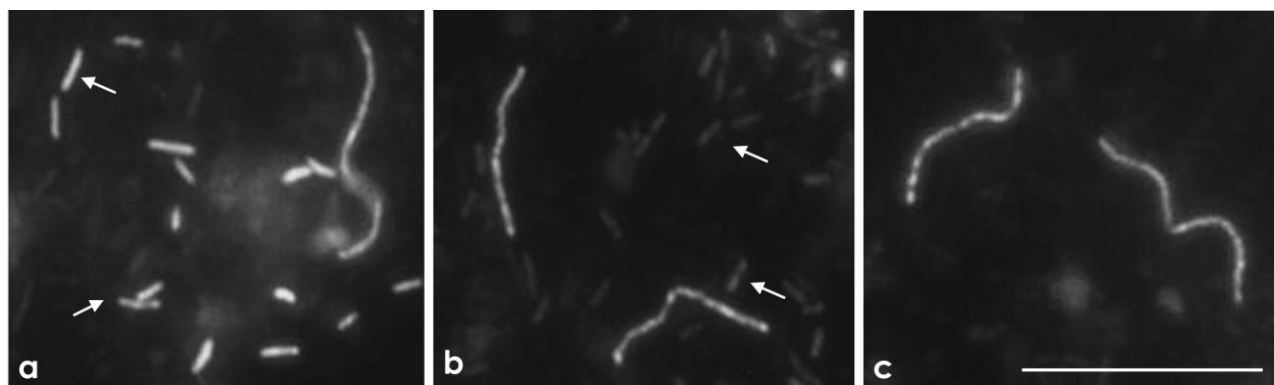


Figure 10– FISH analysis of thermophilic sludge using ARC915 probe at three different stringency conditions, 30% (a), 40% (b) and 50% (c). Cells of *Coprothermobacter* spp. (non-target; some are arrowed) are the short rods whereas thin filaments are *Archaea* cells (target cells). Bar is 10 μm .

4. Discussion

Among the array of molecular methods applied in environmental samples, FISH is appreciated as a versatile, inexpensive, and quantitative tool to analyze microbial community structure. However, the unavailability of specific probes or the lack of binding with already available probes may adversely impact the results. In this study *Coprothermobacter* spp. were highlighted by FISH in thermophilic anaerobic sludge samples (Table 2) only by using unlabeled helper probes. Diversely from PCR-based approaches previously employed for the detection and quantification of *Coprothermobacter* (Tandishabo *et al.*, 2012), FISH analysis allowed the cell counting of the target organism and the estimation of its relative abundance out of total biomass.

The difference between identification in pure culture or in sludge is probably due to the different mechanisms of adaptation that thermophilic microorganisms carry out depending on whether they

live alone or in syntrophy (Nakamura *et al.*, 2006; Sasaki K. *et al.*, 2011). There are strong thermo-adaptive mechanisms playing a role in shaping the 16S rRNA nucleotide composition and assembly inside the 30S subunit of ribosome in prokaryotes living at extreme temperatures.

The GC content of ribosomal 16S RNA has been indeed reported to be proportional to the bacterial growth temperature (Nakashima *et al.*, 2003). For a structured RNA, a region with higher GC content is likely to have more stable secondary structure, because the number of hydrogen bonds between base pairs into the secondary structure is significantly higher (Nakashima *et al.*, 2013). GC content for 16S rRNA of *Coprothermobacter* is about 60%, in line with the GC content reported for various thermophilic organisms (Yamane *et al.*, 2011). Moreover, thermophilic microorganisms can achieve 16S rRNA thermostabilization by chemical modification of nucleotides like methylation of the 2'-OH group of sugar residues, that are located exclusively within the most conserved regions of the mature rRNA, in presence of high salt concentration and with the strong interaction of specific sites of 16S with ribosomal proteins (Torchet and Maurel, 2007).

Moreover, Deutscher (2003) showed that although rRNA is quite stable, under certain physiological conditions it can be extensively degraded. Li and Breaker (1999) described that the susceptibility of RNA to degradation by natural enzymes increases at high temperature, because the 2'-OH of the ribose is activated to attack the phosphate, causing strand cleavage. Thus, in growing cells, the stability of rRNA would most likely be a consequence of protection by ribosomal proteins against the action of RNases.

The ribosome is not a static molecule, and conformational changes of ribosomal proteins could take place during 16S rRNA binding in the 30S subunit assembly and during protein synthesis (Wilson and Noller, 1998; Burton *et al.*, 2012). In particular, ribosomal proteins from thermophilic microorganisms possess special features with respect to the mesophilic ones, for the interactions with 16S rRNA, to ensure the stability of the ribosome at high temperatures (Gruber *et al.*, 2003), like higher numbers of highly mobile binding sites or the presence of more positively charged residues (Burton *et al.*, 2012). The higher affinity of several ribosomal proteins (S8, L1, S4 and S7) for the 16S rRNA in thermophilic microorganisms was extensively studied (Gruber *et al.*, 2003), as adaptation of thermophiles to fold and maintain the appropriate rRNA tertiary structure (Burton *et al.*, 2012). Overall, a closer interaction between proteins and rRNA can likely prevent the degradation at high temperatures in natural environments. This may reasonably affect the accessibility of the 16S rRNA, and the simple denaturation may not be enough. The latter suggests that a target-structure-based interpretation may partially solve these issues.

16S rRNA can exist in several structures; prediction of RNA secondary structure by free energy minimization has been the standard for over two decades. Here we have used the approach from

Ding *et al.* (2005), with the generation of the ensemble centroid secondary structure of 16S rRNA using the software SFold. Because RNA can exist in several structures, that can be grouped into ensemble by similarity, the centroid is the structure in the entire ensemble that has the minimum base-pair distance to the structures. Thus, the ensemble centroid improves the specific predictions of the secondary structure because is representative for a set of structures.

As described in Fuchs *et al.* (2000), the most effective signal enhancement is achieved by directly adjacent helper oligonucleotides and by helpers targeting the region complementary to the probe target site. Moreover, helper should have the exact specificity of the probe. In this work, unlabeled oligonucleotide helpers were designed by combining these three requirements; as shown in figure 3, hCTH439 was both adjacent and complementary to the target region, and this was most likely the reason why it worked better than hCTH429. Despite this, hCTH439 covered 10% less sequences in SILVA database with respect to hCTH429. Therefore, considering that hCTH429 also worked at 30% formamide, they can alternatively be used according to the species of *Coprothermobacter* present in the sample. So, while there are several available software that can design specific probes taking into account all the factors involved in, a specific investigation about rRNA secondary structure is a valuable tool to understand “hybridization dynamics” and improve probes efficiency. Regarding the aspecific binding of ARC915 probe to members of *Coprothermobacter spp.*, given the proved and frequently reported coexistence of this microorganism with hydrogenotrophic *Archaea* in thermophilic environments (Sasaki D. *et al.*, 2011; Sasaki K. *et al.*, 2011), this issue is of particular interest and suggest the need to a cautious characterization approach in analyzing such complex environments. Considered these results, our suggestion is to refine the stringency every time, depending on the kind and the complexity of sample and the abundance of *Coprothermobacter* and *Archaea*, using the probe CTH485 as a reference to highlight the non-target cells.

5. Conclusions

The in situ detection of the protein fermentative *Coprothermobacter* allows to efficiently analyse its syntrophic interaction with hydrogenotrophic methanogens and its role in thermophilic biotechnological processes.

Despite the difficulties in identifying *Coprothermobacter* cells in their natural environment, in situ detection targeting rRNA in thermophilic sludge is still an indispensable molecular tool because of its unique ability to: (1) allow microorganisms to be studied in their natural context, highlighting possible cell-to-cell interactions inside the sludge floc structure; (2) identify the metabolically active cells and its variation in relation to operational parameters during the digestion process; (3)

highlight possible changes in cell morphology compared to pure cultured cells; (4) refine and confirm the data collected from PCR amplification and clonal analysis which may lead to a under/overestimation of members of microbial population.

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Chapter 4

Evaluation of pretreatments effects on sludge floc structure

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1.Introduction

In wastewater treatment plants (WWTP) the activated sludge process is commonly used for biological degradation of organic substances in the wastewater stream. This process is based on carbon degradation by bacteria contained in the activated sludge flocs, under aerobic conditions. These flocs are composed of microorganisms, extracellular polymeric substances (EPS), colloids, mineral particles and ionic components such as divalent cations (Comte et al., 2006). The generated waste activated sludge is then gravity thickened in the secondary tank, mixed with the primary sludge and then treated in the sludge line in order to be stabilized and disposed of. Anaerobic digestion technologies have traditionally been employed to reduce of volume and weight of sludge produced in WWTP and produce energy in the form of biogas (Farooq et al., 2009). Anaerobic degradation of viable, biological solids like waste activated sludge (WAS) requires the hydrolysis of particulate matter to soluble substrates, rate-limiting step of the whole process (Pavlostathis and Giraldo-Gomez, 1991). For this reason, in order to accelerate the low rate of biodegradation and to enhance the digestibility of WAS, a pretreatment could improve the disintegration of biomass and cell walls, facilitating the release of intracellular matter in the aqueous phase. Among these pretreatments, ultrasonic cavitation, ozone, and high-pressure homogenization are largely targeted at the floc disintegration, while thermal hydrolysis process has the potential to produce Class A pathogen-free biosolids as defined by United States CFR 40 Part 503.32 US EPA. Because the thermal hydrolysis process holds the combined benefits of decreasing biosolids quantity and increasing biosolids acceptability, it is of great interest to the wastewater industry (Wilson and Novak, 2009). Thermal hydrolysis is a well-known and widely applied technology used at laboratory and full scale (Laurent et al., 2010) and allows degradation of the gel structure and release of linked water, improving the digestibility of the sludge. Most studies report an optimal temperature in the range of 160-180 °C and treatment times from 30 to 60 min; pressure associated may vary from 600 to 2500 kPa (Carrère et al., 2010). Ultrasounds are currently applied as pretreatment also at full-scale on sewage treatment plants. The action of the ultrasonic treatment is to disrupt mechanically the sludge floc matrix and the cell structure by means of shear forces due to cavitation phenomena. Low frequency ultrasounds (20–40 kHz) have been widely investigated on laboratory, pilot and full-scale level as sludge digestion pretreatment (Grönroos et al., 2005; Braguglia et al., 2006; Nickel and Neis, 2007; Show et al., 2007; Feng et al., 2009). The aim of this work was to evaluate and compare the effect of low frequency ultrasounds and thermal hydrolysis on sludge floc structure in terms of solubilization, floc reduction and microbial population , to better understand dynamics of sludge anaerobic digestion processes described in next sections.

2. Materials and methods

Sludge. Waste activated sludge (WAS) samples were obtained from the municipal “Roma-Nord” wastewater treatment plant, characterized by an organic load of about 700,000 p.e., high sludge age (20 days) and a COD average value of incoming sewage of 200 mg/L. The activated sludge was sampled directly from the oxidation tank.

Ultrasound pretreatment. The disintegration by ultrasound was performed with an ultrasonic processor UP400S (dr. Hielscher, Germany) operating at 300 W and 24 kHz. Sonication energy input was set at 0.4 - 0.5 kWh kg⁻¹ dry solid on 500 mL of waste-activated sludge (2.9 - 4.7 % TS) placed in 1 L beaker with the probe allocated at 3 cm above the beaker bottom. The specific energy input is a function of ultrasonic power, ultrasonic duration, and volume of sonicated sludge and TS concentration, and can be calculated following the equation reported elsewhere (Gallipoli et al., 2014).

Thermal pretreatment. Thermal pretreatment was carried out by a bench scale autoclave Laboklav 25b, allowing a temperature increase by steam mode up to $T = 134\text{ }^{\circ}\text{C}$ and $p = 3.12\text{ bar}$. Once temperature was reached, the pretreatment time lasted 20 min, on 400 mL of sample.

Matter composition. Soluble COD (sCOD), measured in duplicate, was determined by photometric determination of the chromate consumption by organic compounds (COD Cell Test by Spectroquant Merck) subsequent to digestion in a concentrated sulphuric acid solution for 2 h at 148°C (EPA method 410.4). To analyze colloidal phase, sludge aliquots were filtered through glass filters with 1.2 µm pores (GF/C Whatman); the supernatant was used for protein and carbohydrates determination. Protein content was calculated by means of the Bicinchoninic Acid Protein Assay Kit (Sigma–Aldrich BCA-1). Carbohydrates colorimetric determination was carried out as described in Braguglia et al. (2012). Fructose was used as standard. The absorbance of standards and samples was determined spectrophotometrically at 480 nm (Perkin Elmer Lambda Bio 20). The degree of sludge disintegration after pretreatments (DD_{COD}) was calculated as the ratio of the soluble COD increase after pretreatment to the maximum possible soluble COD increase (Braguglia et al., 2006). Sludge filterability was estimated using a capillary suction apparatus supplied by Triton Electronics Ltd., England. A stain- less-steel tube with an inner radius of 0.535 cm and filter paper (Whatman No. 17) was used. Each sludge was analyzed 5 times and the results were averaged

before the results were standardized to the TS concentration. The charge density determinations were performed by a Particle Charge Detector PCD02 (Mütek GmbH, Herrsching). The PCD operates on the principle of the “streaming current detector”. Since the streaming current is proportional to the electric charge of the colloids, it may provide an indication of charge-related particle destabilization in a manner similar to zeta potential (Abu-Orf and Dentel, 1997). The method adopted to measure the surface sludge density on the liquid fraction of sludge samples is fully described in Braguglia et al. (2009).

Flocs dimensional analysis. The floc dimensional analysis of untreated and sonicated WAS was performed by contrast phase microscopy examination at 100X (Zeiss Axioskop). Average floc size was estimated from at least 100 images randomly taken on at least triplicate samples. Images were processed with IMAGEJ software (version1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA, available in the public domain at <http://rsb.info.nih.gov/ij/index.html>), by measuring the major axis of flocs. Floc size distribution was referred to five different intervals from 0 to 1500 μm (0–50, 50–150, 150–500, 500–1000, 1000–1500).

Fluorescence in situ hybridization. For microbial community analysis by FISH, aliquots of 1.5 mL of sludge were fixed with paraformaldehyde and ethanol Amann et al. (1990). FISH analysis was performed as previously described in Braguglia et al. (2012). Oligonucleotide probes specific for *Bacteria* (EUB338mix probes) and *Archaea* (ARC915 probe) domains were used. Samples were examined by epifluorescence microscopy (Olympus BX51). Fluorescence signal was quantified on microscopic images taken from the samples with a digital camera (Olympus XM-10) and the software Cell F. DAPI (4',6-diamidino-2-phenylindole) fluorescent staining was also simultaneously performed for determining total cell numbers. Area measurements of the hybridized cells were reported as a portion of the area covered by total DAPI stained cells in each field. Area measurements were performed on at least 10 different JPEG images (or other image format with 8 bit size of 1388x1040 pixels) using ImageJ software package (version1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA, available in the public domain at <http://rsb.info.nih.gov/ij/index.html>) as described in Braguglia et al. (2012).

3. Results and discussion

3.1 Effect of ultrasounds on sludge flocs

Assessment of the ultrasound effect on sludge flocs was done by dimensional, chemical and microbial analysis. Dimensional analysis was carried out by measuring the flocs dimensions in the untreated and different pretreated sludges within five different size classes from 0 to 1500 μm (Fig. 1). It is worth to noting that most flocs in the untreated secondary sludge had dimensions ranging from 150 to 500 μm . Applying ultrasound at 20 kHz for 2 min (corresponding to E_{spec} of 2700 kJ/kg TS) and 4 min (E_{spec} of 5200 kJ/kg TS) smaller flocs with size lower than 150 μm were the most abundant. On the contrary, by doubling sonication time and consequently the energy (10700 kJ/kg TS) sludge disintegration occurred with a relevant increase of small flocs, especially in the range 0–50 μm , and an almost total absence of large flocs with 150–1000 μm size.

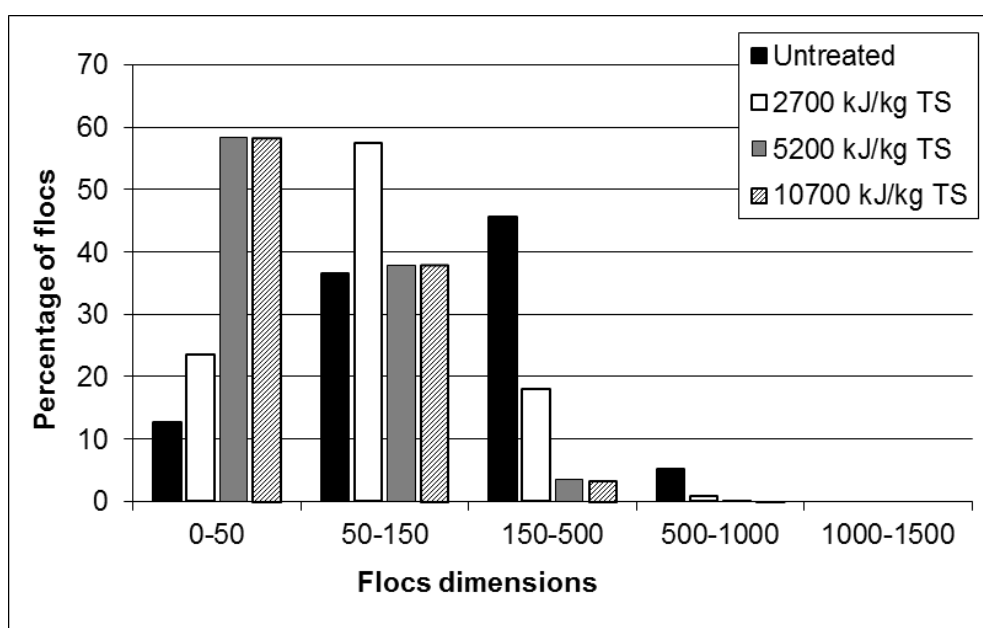


Figure 1. Dimensional distribution of sludge flocs before and after sonication at 20 kHz at increasing energy input.

Microscopy images taken before and after the different sonication pretreatments are shown in Fig.2. Ultrasound floc disintegration and organic material dispersion increased with time and energy. Application of low energy (5200 kJ/kg TS, Fig.2 c) caused moderate sludge disintegration: small aggregates and dispersed material were present. By increasing sonication energy (Fig.2 d) floc disintegration was more intense, and only the backbone of the flocs was visible. Mostly dispersed material was highlighted in background. The most efficient disintegration of sludge was probably

due to marked cavitation phenomena because of the high energy input that generate intense shear forces.

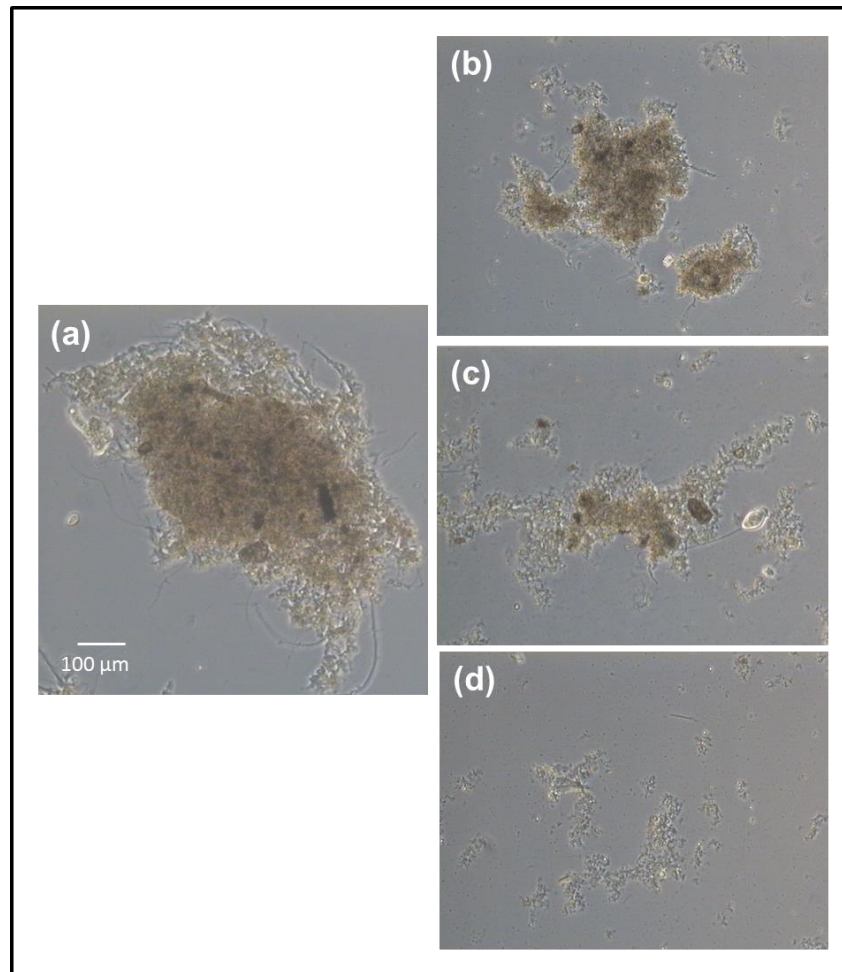


Fig.2. Phase contrast microscopy micrographs of activated sludge flocs before (a) and after ultrasound pretreatments for 2 (b), 4 (c) and 8 (d) minutes.

The increase of soluble COD, proteins and carbohydrates in solution was indeed due to the release of extracellular material during floc disintegration, and the release increase with sonication time and the energy applied (Table 1). As a consequence of the release of colloidal polymers as proteins and carbohydrates in solution, sludge filterability worsened progressively, and after 8 min sonication the normalized CST value increased by nearly 100 times with respect to the untreated sludge, confirming the sludge disintegration. Moreover, As expected, all activated sludge samples carried a net negative surface charge, mainly due to ionisation of functional groups such as carboxylic, sulphate and phosphate of the polymeric substances as proteins and carbohydrates. The results of Table 1 showed that increased amounts of polymeric materials in solution due to sonication were associated with a more negative surface charge. A strict linear correlation between particle charge density and CST was observed, indicating that the increase of colloidal particles in solution due to

pretreatment affected negatively the filterability of sludge, according with previous studies (Neyens et al, 2004; Braguglia et al., 2009).

Table.1 Effects of ultrasound pretreatment

	Untreated	2 ' min (2738 kJ/kg TS)	4 ' min (5275 kJ/kg TS)	8 ' min (10588 kJ/kg TS)
sCOD (mg/L)	15	552.5	1175	1597.5
DD COD (%)	-	2.9	6.2	8.5
Proteins (mg/L)	91.6	434.6	606.6	1014.6
Carbohydrates (mg/L)	4.4	35.5	69.3	111.2
Charge density (mC/gST)	99	1185	1552	2559
CST (s L g TS⁻¹)	0.4	17.5	25	39

FISH analysis of untreated and pretreated samples was carried out to assess effect on microbial population. Since *Archaea* and *Bacteria* are the prevailing microorganisms in activated sludge, their quantification may provide useful informations about cell inactivation caused by the pretreatments. As seen in Fig.3, the effects of ultrasounds on biomass changed with the energy applied.

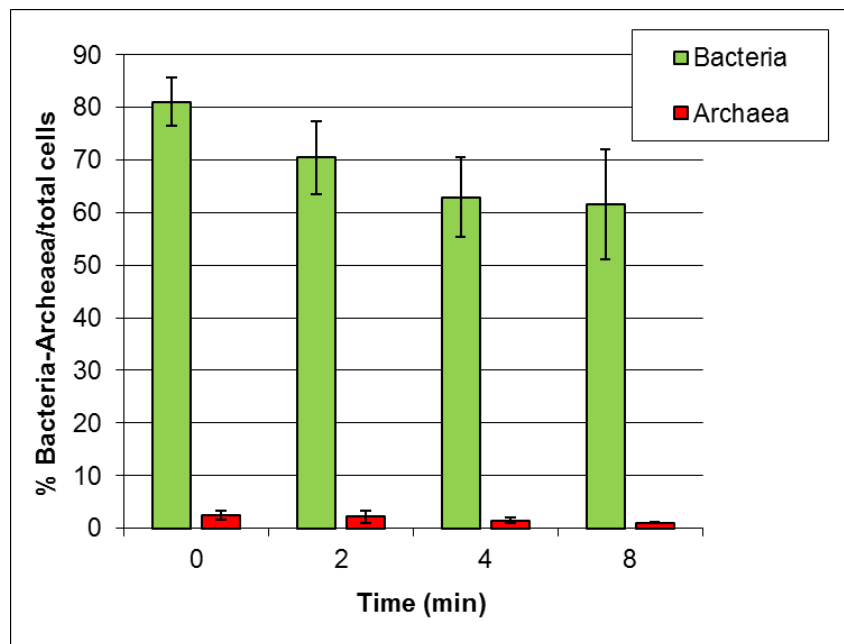


Figure 3 - Amount of total Bacteria and Archaea out of total DAPI stained cells in untreated and sonicated sludge, after different sonication times.

Overall, the relative abundance of *Bacteria* and *Archaea* in sonicated samples decreased with the increase of energy applied, with respect to the untreated sludge. The most significant decrease was retrieved for archeal population, which was estimated to be 12%, 41% and 57% for the three

sonication times. Bacterial population decrease of samples sonicated 4 and 8 minutes was almost the same. Low frequency ultrasounds shear forces by disrupting the floc structure caused slight cell lysis and/or inactivation, too.

3.2 Effect of thermal hydrolysis on sludge flocs

Thermal hydrolysis pretreatment had no significant effect on floc disintegration in terms of size reduction. In fact, dimensional analysis and microscopic investigation on sludge flocs (Fig.4 a and b) showed that flocs dimensions and structure did not change. Flocs are quite compact in size, not so different from untreated sludge. No dispersed material in background was observed. However, high temperatures do not present the mechanical effect of sludge breakage and material dispersion does not occur after thermal pretreatment.

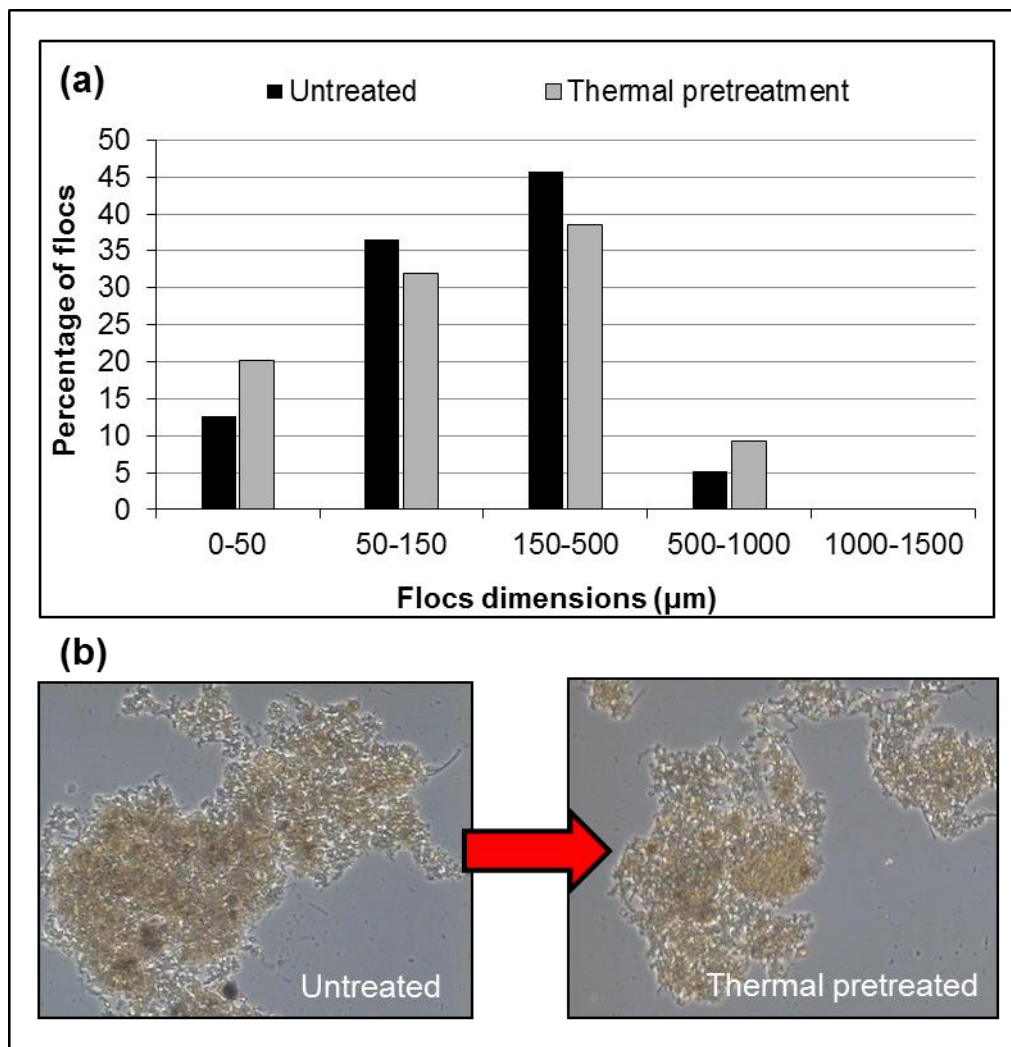


Fig.4. Dimensional distribution (a) and phase contrast microscopy micrographs (b) of activated sludge flocs before and after thermal pretreatment.

Table 2. Effects of thermal pretreatment

	Untreated	Pretreated
COD_{sol} (mg/L)	45	4,340
DD_{COD} (%)	-	21.8
Proteins (mg/L)	30	1,822
Carbohydrates (mg/L)	2.7	418
CST (s.L/gTS)	0.7	66
Particle charge density (mC/gTS)	124	4,241

Nevertheless, solubilization phenomena and changes on surface properties occurred during thermal pretreatment (Table 2), although the floc structure remained unchanged.

The efficiency in disintegrating was highlighted by the increase of soluble COD and colloidal polymers as proteins and carbohydrates with pretreatment. Floc deterioration and solubilization resulted in filterability worsening, and CST increased around 10 times with respect to untreated sludge (Table 2). An increase of the specific charge occurred after pretreatment, due to the increased amount of colloidal and polymeric material released in solution (Table 2).

FISH analysis showed no fluorescence signal in samples after thermal pretreatment. The absence of fluorescence signal after thermal hydrolysis was likely related to cell lysis (and therefore RNA degradation).

3.3 Comparison between pretreatments

Ultrasound treatment at 20 kHz was found to be more effective in sludge disintegration, in terms of floc destruction (Figure 1 and 2) with respect to thermal hydrolysis, that resulted more effective in solubilizing extracellular polymeric substances (table 2) without affecting the integrity of the flocs (figure 4). Significantly higher soluble matter release in terms of sCOD after thermal pretreatment, with respect to ultrasound pretreatment (figure 5) highlighted that this issue is strictly dependent by the energy applied. Indeed, the energy applied during thermal hydrolysis was about 25000 kJ/kg TS, more than twice with respect to the higher energy applied with ultrasounds.

Moreover, the lack of fluorescence signal after FISH analysis of thermal pretreated sludge highlighted the occurrence of cell degradation/inactivation, with a consequent production of additional proteinaceous material over EPS from sludge, that increased the soluble organic matter in soluble and colloidal fraction. High temperature and pressure in thermal pretreatment likely induced a floc “de-structuration” and “emptying”, leaving intact the floc matrix. The high shear forces generated by the implosion of cavitation bubbles after the application of ultrasounds at low frequency caused a more marked impact on sludge disintegration, giving also satisfactory

performances in terms of soluble organic matters release considering the lower energy applied with respect to thermal pretreatment. Considering the applicability of these technologies on a large scale, a good compromise between applied energy and solubilization performances achieved should certainly be found.

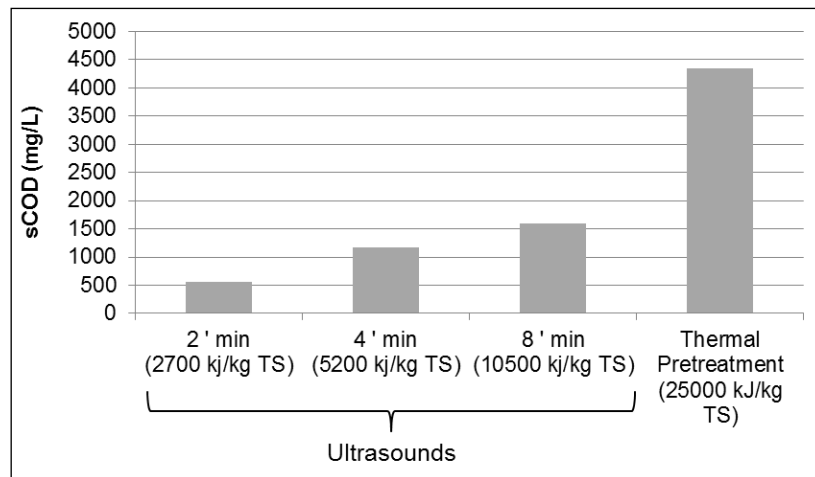


Fig.5 . Soluble COD trends after different pretreatments applications.

4.Conclusions

Mechanical (ultrasounds) and thermal pretreatments are currently applied to improve anaerobic digestion process, but the diversified performances and biogas gains due to pretreatment suggest to investigate more in detail both the effect of the pretreatment on the biomass, and the microbial population involved in particular when pretreated sludge is fed to the anaerobic digesters. The results of this work showed that low frequency ultrasounds application confirmed the effectiveness of this disintegration method against biological and organic components of sludge flocs, maintaining cell integrity if the specific energy is maintained lower than 11000 kJ/kg TS) . Thermal hydrolysis pretreatment permitted to achieve higher performances as regards the release of organic matter in solution, without floc disintegration. Filterability worsened due to these pretreatments because of the release of colloidal charged polymers as proteins and carbohydrates, entrapped in the activated sludge biomass.

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Chapter 5

Thermophilic anaerobic digestion of thermal pretreated sludge: role of microbial community structure and correlation with process performances

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1. Introduction

The anaerobic digestion (AD) of organic wastes still gathers a great interest due to the global needs for waste recycling and renewable energy production, in the form of biogas (Luo et al., 2013). AD has been evaluated as one of the most energy-efficient and environmentally beneficial technology for bioenergy production (Weiland et al., 2010). In a worldwide perspective, anaerobic digestion of sewage sludge is far the most widespread use of anaerobic digestion (Ahring et al., 2002). AD involves several microbial groups forming interdependent microbial consortia. Overall, four major steps can be distinguished. In the first hydrolysis step, both solubilisation of insoluble particulate matter and biological decomposition of organic polymers take place. Acidogenesis and acetogenesis follow in the second and third step and produce a wide variety of fermentation end-products, which are transformed into methane by methanogenic community in the final step. Hydrolysis is often limited for complex organic matter as waste activated sludge (WAS) requiring the hydrolysis of particulate matter to soluble substrates (Pavlostathis and Giraldo-Gomez, 1991).

Thermal, chemical, biological and mechanical processes, as well as combinations of these, have been studied as possible pre-treatments to disintegrate sludge and accelerate hydrolysis (Ferrer et al., 2008). These pretreatments can disintegrate sludge flocs and cells allowing to a significant solubilization of organic matter, as extracellular polymeric substances (EPS). Thermal hydrolysis is a well-known and widely applied technology used at laboratory and full scale (Laurent et al., 2010) as WAS pretreatment. It allows degradation of the gel structure and release of linked water, improving the digestibility of the sludge (Carrère et al., 2010). Most studies report an optimal temperature range of 160-180 °C and treatment times from 30 to 60 min, while the associated pressure may vary from 600 to 2500 kPa (Carrère et al., 2010).

Anaerobic processes operating under thermophilic conditions (50 to 55°C) are commonly applied throughout Europe for treatment of the organic fraction of municipal solid wastes and for manure treatment in large scale biogas plants (Ahring et al., 2002). Thermophilic AD (TAD) processes have attracted a great attention in recent years due to their apparent advantages, like enhanced organic matter removal, high methane production and foaming reduction (Ho et al., 2013). Moreover, TAD enhances the destruction of pathogens, enabling effluent hygienisation, which might be required in a short time for land application (EC, 2000).

To deepen investigate and control the anaerobic digestion process, the identity and the metabolic potential of the microbial consortia driving the process need to be elucidated. There have been limited molecular-based studies of microbial communities in AD systems, and most of these revealed mostly novel phylotypes (Pervin et al., 2013). Our knowledge about the microbial

consortia involved in this process is indeed limited because of the lack of phylogenetic and metabolic data on these predominantly uncultivated microorganisms.

Coprothermobacter proteolyticus is an anerobic thermophilic microbe affiliated with family *Thermodesulfobiaceae*, which is differently branched from families including most of aminoacid degrading bacteria in the phylum Firmicutes (Sasaki et al., 2012). Nevertheless, Nishida *et al.* (2011) showed that *Coprothermobacter* represented a taxonomic group most closely related to *Dictyoglomi* and *Thermotoga*. *Coprothermobacter* spp. ferments proteins, and this proteolytic activity is largely reported (Ollivier et al., 1985; Etchebehere et al., 1998; Cai et al., 2011; Tandishabo et al., 2012; Lü et al., 2014). Recently, *Coprothermobacter* spp. were identified in several studies focused on the analysis of microbial community structure selected in anaerobic thermophilic reactors treating sewage sludge (Kobayashi et al., 2008, Hatamoto et al., 2008; Lee et al., 2009; Luo et al., 2013; Pervin et al., 2013).

This microorganism has attracted the attention of researchers for its potential applications in high temperature environments, because only a few proteolytic anaerobic thermophiles have been characterized so far (Cai et al., 2011).

Coprothermobacter activity is improved by the establishment of a syntrophy with hydrogenotrophic methanogens like *Methanothermobacter thermoautotrophicus* (Sasaki et al., 2012; Lü et al., 2014), commonly found as component of methanogenic population in many thermophilic anaerobic systems (Yabu et al., 2011; Luo et al., 2013). Hydrogen is the primary energy source of this methanogen, even when in situ hydrogen concentrations are very low (Kato et al., 2008).

The objective of this work was to investigate the structure and dynamics of microbial communities involved in TAD of waste activated sludge, either untreated or thermally pretreated, and to correlate the biological data with process performances and operation parameters.

2. Materials and Methods

2.1 Reactors operation and performance

Sludge. Sludge was sampled from the municipal “Roma-Nord” wastewater treatment plant, serving about 780.000 P.E. with an average flow rate of 4.1 m³/s. Average influent water quality to the WWTP was 250 mg COD/L, 20 mg N-NH₄⁺/L and 4 mg P_{tot}/L. The plant includes primary clarification and activated sludge secondary treatment without nutrients removal. Table 1 reports the average characteristics of the waste activated sludge (WAS), collected directly from the

oxidation tank operating at an average sludge age of 20d. The anaerobic inoculum was collected from the full-scale digester of the plant, fed with mixed sludge.

Table 1 - Characteristics of raw activated sludge.

	<i>Average value</i>
TS (mg/L)	20,800
VS (mg/L)	14,400
VS/TS (%)	69%
pH	6.5
Soluble COD (mg/L)	45
Total COD (g/L)	20.1

Thermal pretreatment. Thermal pretreatment was carried out on 400 mL of sludge sample by a bench scale autoclave Laboklav 25b, operating at $T = 134\text{ }^{\circ}\text{C}$ and $p = 3.12\text{ bar}$ for 20 min, as described in Gianico et al. (2013).

Digester system. The anaerobic digestion system operated for 250 days in semi-continuous mode at different organic loading rate (OLR). Two anaerobic jacketed reactors (7 L) were completely mixed and maintained at the constant temperature of 55°C . One reactor, as control unit, was fed with untreated WAS, and the second one, as experimental unit, was fed with the same sludge after thermal pre-treatment (Fig. 1).

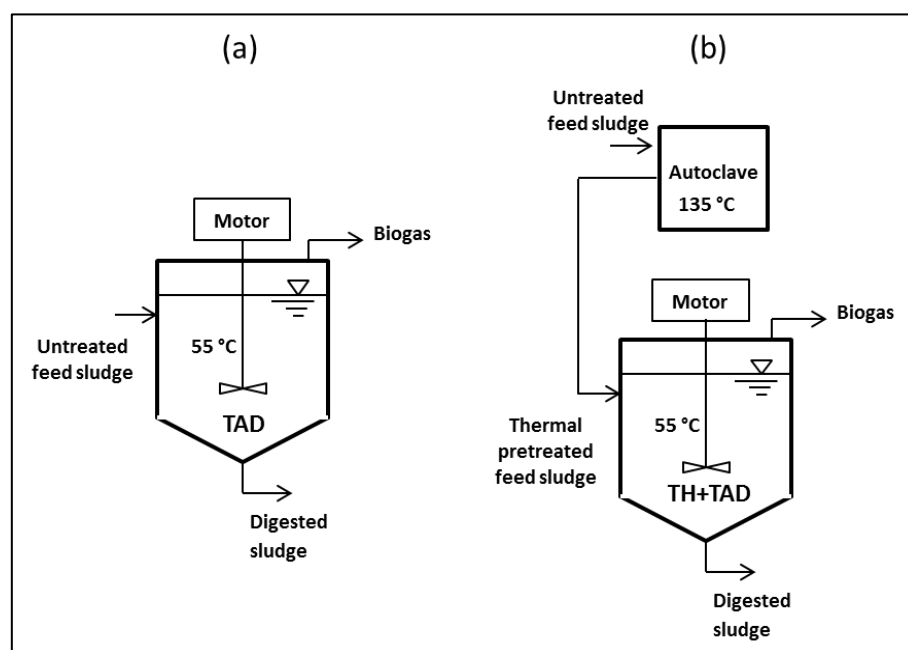


Figure 1 – Schematic diagram of the treatment processes.

Untreated or pretreated sludges were fed manually to the digesters once a day after withdrawing the same volume of digested sludge. Operating conditions of the three anaerobic phases are described in Table 2.

Table 2 - Operating conditions of each anaerobic digestion phase.

Phase #	1	2	3
OLR (g VS L ⁻¹ d ⁻¹)	1.8	1.0	3.7
HRT (d)	8	15	8
Phase lenght (d)	102	103	51

All phases were carried out using the same WAS; the first two phases were carried out with gravity thickened WAS (TS = 20.8 g/L) while the last phase was carried out feeding a dynamic pre-thickened sludge with total solids concentration up to 41 g/L. Pre-thickening of sludge was performed by centrifugation for 2 min at 1100 rpm.

Biogas collection and analysis. The produced biogas was collected by water displacement in a biogas collection unit. The gas meter consisted of a volumetric cell for gas–liquid displacement, a sensor device for liquid level detection, and an electronic control circuit for data processing and display. The methane content in the biogas was measured using a PerkinElmer Auto System Gas Chromatographer equipped with a thermal conductivity detector (TCD) as described in Gianico et al. (2013).

Matter composition. Total and volatile solids (TS and VS) were determined in triplicates according to standard (APHA, 1998). The pH was detected by a portable pH-meter (WTW, pH 330/SET-1). To analyze the soluble phase, the particulate sludge matter was removed by centrifugation (10 min at 5000 rpm), and the resulting centrate was filtrated through 0.45 µm pore size membrane filters. Soluble COD (sCOD) and soluble nitrogen were determined by Cell Test Spectroquant (Merck) as described in Gianico et al. (2013). Ammonia nitrogen was determined according to method 4500-NH₃ C of APHA Standard Methods, 18th edition (1992). To analyze colloidal phase, sludge aliquots were filtered through glass filters with 1.2 µm pores (GF/C Whatman); the supernatant was used for protein and carbohydrates determination. Protein and carbohydrates content were determined by colorimetric BCA and Dubois methods, as described in Braguglia et al. (2012).

2.2 Microbial community analysis

Sample collection. Effluent sludge samples were periodically collected from both reactors during start-up and at steady state operating conditions. Aliquots of 1.5 mL of mixed liquor were either immediately frozen at -20°C for further DNA extraction or fixed with paraformaldehyde and ethanol for FISH analysis as described in Amann *et al.* (1990).

Genomic DNA extraction and PCR amplification of 16S rRNA gene. DNA was extracted from ≈700 mg of thermophilic sludge taken at the end of operation of both systems following the protocol reported in Rossetti *et al.* (2008). The concentration and purity of the genomic DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). 16S rRNA genes were amplified using primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) for Bacteria domain and primers A109F (ACKGCTCAGTAACACGT) and 1386R (GCGGTGTGTGCAAGGAGC) for *Archaea*, using PerfectTaq DNA polymerase kit (5Prime, Deutschland). PCR amplification of 16S rRNA genes of Bacteria was carried out as described in Rossetti *et al.* (2003). The protocol reported in Skillman *et al.* (2004) was applied for 16S rRNA gene amplification with archaeal primers.

Cloning and sequencing of 16S rDNA. Cloning of PCR products was carried out using pGEM-T Easy Vector System (Promega, USA) into *Escherichia coli* JM109 competent cells (Promega, USA) according to the manufacturer's instructions. Positive inserts were amplified from recombinant plasmids obtained from white colonies by PCR using the sequencing primers T7 (TAATACGACTCACTATAGGG) and M13 (TCACACAGGAAACAGCTATGAC). PCR amplification was carried out as described in Chen *et al.* (2003). PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Netherlands). A total of 90 clones, 30 for bacteria and 60 for archaea, were initially selected for a first-pass sequence analysis of clone inserts with 530F as a sequencing primer. Further, a representative of each taxonomic group was fully sequenced on both strands with 926R, 519R and 907R sequencing primers. 16S rDNA full sequences were submitted to GenBank under the accession numbers reported in Table 5.

Fluorescence in situ hybridization (FISH). FISH on fixed sludge samples was performed as previously described (Braguglia *et al.* 2102). The probes used in this study are listed in Table 3. The sequence of the PCR primer SYN961R from the study of Horz *et al.* (2006) was used to generate a

Table 3- Oligonucleotide probes sequence, target microbial groups and stringency conditions used in this study.

Name	Target group	Probe sequence (5'-3')	Formamide (%)	Reference
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	20 or 35	Stahl and Amann, 1991
EUB338, EUB338-II, EUB338-III	Bacteria	GCTGCCTCCCGTAGGAGT, GCAGCCACCCGTAGGTGT, GCTGCCACCCGTAGGTGT	20 or 35	Amann et al., 1990
BET42a	Betaproteobacteria	GCCTTCCCACATTCGTTT	35	Manz et al., 1992
GAM42a	Gammaproteobacteria	GCCTTCCCACATTCGTTT	35	Manz et al., 1992
HGC69a	Actinobacteria	TATAGTTACCACCGCCGT	35	Roller et al., 1994
ALF968	Alphaproteobacteria	GGTAAGGTTCTGCGCGTT	20	Neef, 1997
CF319a	Bacteroidetes	TGGTCCGTGTCTCAGTAC	35	Manz et al., 1996
LGC354a, LGC354b, LGC354c	Firmicutes	TGGAAGATTCCCTACTGC, CGGAAGATTCCCTACTGC, CCGAAGATTCCCTACTGC	35	Meier et al., 1999
DELTA485A, DELTA495B, DELTA495C	Deltaproteobacteria	AGTTAGCCGGTGCTTCCT, AGTTAGCCGGCGCTTCCT, AATTAGCCGGTGCTTCCT	35	Lücker et al., 2007
TM7905	TM7	CCGTCAATTCCTTTATGTTTTA	35	Hugenholz et al., 2000
PLA886	Planctomycetales	GCCTTGCGACCATACTCCC	35	Neef et al., 1998
cPLA886	PLA886 competitor	GCCTTGCGACCGTACTCCC	-	Neef et al., 1998
CTH485	Coprothermobacter	TCCCTTTCTACTGGGGTA	30	Gagliano et al., 2014
hCTH439	CTH485 helper	CCGTCCTTCCTCGTCCCCCAGT	-	Gagliano et al., 2014
SYN961	Synergystetes	GTTCTTCGGTTTGATCG	20	This study
MSMX860	Methanosarcinales	GGCTCGCTTCACGGCTTCCT	45	Raskin et al., 1994
MS821	some Methanosarcina	CGCCATGCCTGACACCTAGCGAGC	40	Raskin et al., 1994
MX825	Methanosaetaceae	TCGCACCGTGCCGACACCTAGC	50	Raskin et al., 1994
MX825b	Methanosaetaceae subgroup	TCGCACCGTTGCCGACACCTAGC	50	Crocetti et al., 2006
MX825c	Methanosaetaceae subgroup	TCGCACCGTGCTGACACCTAGC	50	Crocetti et al., 2006
MG1200b	most Methanomicrobiales	CRGATAATTCCGGGGCATGCTG	20	Crocetti et al., 2006
MB311	Methanobacteriales	ACCTTGTCTCAGGTTCATCTCC	30	Crocetti et al., 2006
MC1109	Methanococcales	GCAACATAGGGCACGGGTCT	45	Raskin et al., 1994
MC504	Methanocaldococcaceae	GGCTGCTGGCACCGACTTGCCCA	55	Crocetti et al., 2006
cMC504	MC504 competitor	GGCTGCTGGCACCGAACTTGCCCA	-	Crocetti et al., 2006

FISH probe targeting the phylum *Synergystetes* and members of the genus *Anaerobaculum*; coverage and efficiency of the probe were evaluated using the software TestProbe (Yilmaz et al.,

2014, <http://www.arb-silva.de/search/testprobe/>, SSU database version 117) and mathFISH (Yilmaz et al., 2011, <http://mathfish.cee.wisc.edu/index.html>). To identify thermophilic *Methanobacteriales* with ARC915 and MB311 probes, the protocol was modified as described in Nakamura et al. (2006) by using an enzymatic pretreatment of fixed samples with pseudomurein endopeptidase (Pei) in order to improve probe penetration. A recombinant form of Pei (rPeiW) originated from cloning and expression of pseudomurein endoisopeptidase gene from *M. wolfeii* DSM2970 (peiW) was used.

Microscopy and Fluorescence Signal quantification. Samples were examined by epifluorescence microscopy (Olympus BX51). Fluorescence signal was quantified on microscopic images taken from the samples with a digital camera (Olympus XM-10) and the software Cell F. DAPI (4',6-diamidino-2-phenylindole) fluorescent staining was also simultaneously performed for determining total cell numbers. Area measurements of the hybridized cells were reported as a portion of the area covered by total DAPI stained cells in each field. Area measurements were performed on at least 10 different JPEG images (or other image format with 8 bit size of 1388x1040 pixels) using ImageJ software package (version 1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA, available in the public domain at <http://rsb.info.nih.gov/ij/index.html>) as described in Braguglia et al. (2012).

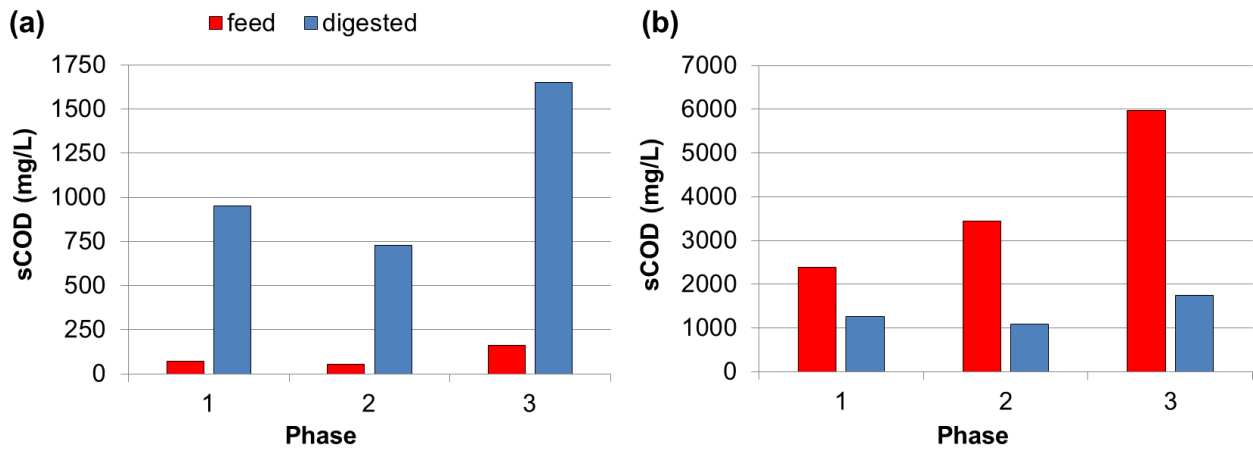
3. Results and Discussion

3.1 TAD performances

During digestion, VS degradation occurred in both anaerobic reactors fed with untreated and thermal pretreated sludge. With regard to the digestion of the untreated sludge (control reactor), organics removal was $42 \pm 2\%$ in the first two digestion phases, and decreased up to $38 \pm 1\%$ by increasing the OLR in the 3rd phase (Tab.4). The integration of a thermal pretreatment allowed to obtain a slight gain of +7% in VS removal, only at low OLR. In the control reactor, the solubilised organic matter increased and accumulated markedly, probably due to the higher rates of organics hydrolysis with respect to the anaerobic conversion rate. On the contrary, in the parallel experimental unit conducted by feeding pretreated sludge, the quite high initial soluble COD, resulting from pretreatment, was progressively removed (Figure 2). Generally, all the experimental reactors showed more than 55% sCOD removal whereas no removal occurred in the control reactors.

Table 4 – Steady state anaerobic digestion performances.

	Phase #1 OLR=1.8 kgVS/m ³ d		Phase #2 OLR=1.0 kgVS/m ³ d		Phase #3 OLR=3.7 kgVS/m ³ d	
	Untreated	Pretreated	Untreated	Pretreated	Untreated	Pretreated
VS_{destruction} (%)	42±1.4	45±1.6	43±1.5	46±1.8	38±1.1	37±1.3
Specific biogas production (Nm³/kgVS_{fed})	0.31±0.03	0.32±0.03	0.26±0.02	0.36±0.04	0.31±0.02	0.32±0.03
Methane content in biogas (%)	70	69	60	66	69	70
Methane production rate (Nm³/m³ digester d)	0.38±0.01	0.41±0.01	0.15±0.01	0.22±0.02	0.74±0.02	0.80±0.02

**Figure 2** – Mean sCOD values of feed and digested sludge, in the three different phases, during the digestion of untreated (a) and thermal pretreated (b) sludge. For the reactor fed with pretreated sludge, there was always a removal from the initial high value of soluble COD resulting from thermal pretreatment.

Due to the high removal of soluble organic material, the cumulative biogas production (Fig.1) and in particular methane production rates (Tab.4) always increased in the experimental reactors in comparison to the control reactors (Figure 3). The gap in biogas production increased with time according to the extent of sCOD removal.

The specific biogas production of the control reactor was in the range 0.26-0.31 Nm³ kg⁻¹ VS_{fed}, according to the typical thermophilic WAS digestion (0.25–0.50 Nm³ kg⁻¹ VS_{fed}). The specific biogas production of experimental reactor was higher and varied from 0.36±0.01 Nm³ kg⁻¹ VS_{fed} for the initial phases at low OLR, decreasing to 0.32±0.01 Nm³kg⁻¹ VS_{fed} by increasing OLR (Table S2). In all the digestion phases methane accounted for more than 60% of biogas composition. The highest methane gain (+47%) was obtained during the second digestion phase with thermal

pretreated sludge at long HRT, that probably facilitate the establishment of different, fruitful, conversion pathways of the released organic matter into methane.

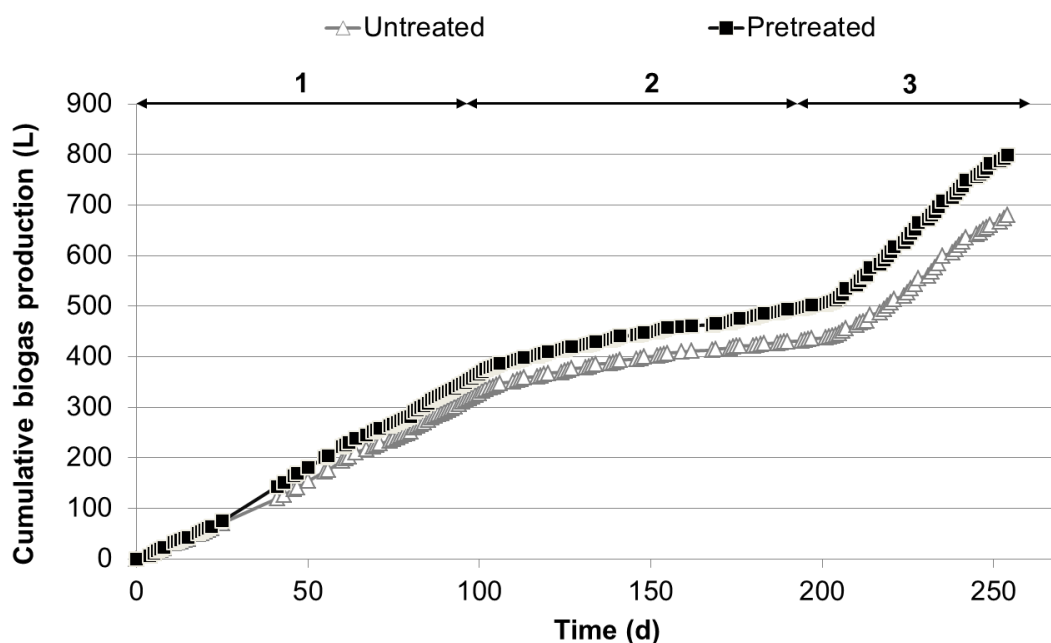


Figure 3 – Cumulative biogas production of the control reactor fed with untreated sludge (grey) and of the experimental reactor fed with pretreated sludge (black), during digestion time.

3.2 Identification of key microbial populations

Clonal analysis. Sequencing the 16S rDNA amplicons obtained with PCR primers for both bacterial and archaeal members of the microbial community revealed the occurrence of known thermophilic microorganisms. The number and the affiliation of the screened clones are reported in Table 4. The bacterial 16S rRNA gene sequences fell into five different taxonomic groups (Fig.4 a). Most of the clones were affiliated to *Coprothermobacter proteolyticus*. This microorganism is a protein fermentative bacterium originally isolated from a thermophilic (55°C) digester fermenting tannery wastes and cattle manure (Ollivier et al., 1985). The majority of archaeal clones were affiliated to *Methanosarcina thermophila* and *Methanothermobacter thermoautotrophicus* species (Tab.4, Fig.4 b), commonly identified as dominant methanogens in several thermophilic processes (Yabu et al., 2011; Ho et al., 2013; Luo et al., 2013). Overall, it is well known that the presence of hydrogenotrophic methanogens (as *M. thermoautotrophicus*) can promote the growth and thermodynamically improve the degradation rate of fermentative bacteria by establishing different syntrophic associations under thermophilic conditions (Sieber et al., 2012).

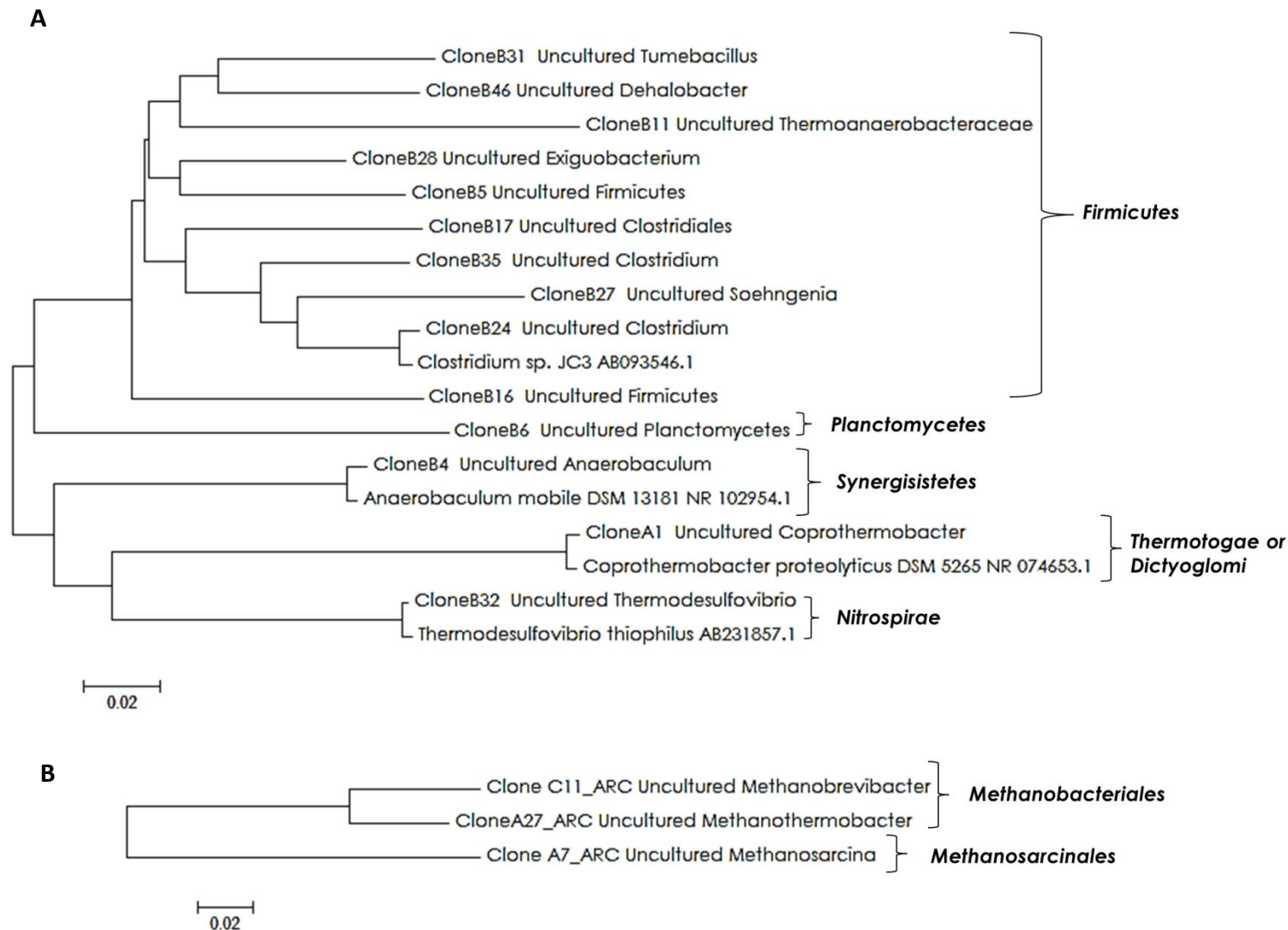


Figure 4 - 16S rRNA gene-based phylogenetic tree of bacterial (a) and archaeal (b) clones using the Neighbor-Joining method [Saitou and Nei, 1987]. The evolutionary distances were computed using the Maximum Composite Likelihood method [Tamura *et al.*, 2004]. Evolutionary analyses were conducted in MEGA6 [Tamura *et al.*, 2013]. In (a), *Clostridium* sp. JC3, *Coprothermobacter proteolyticus* DSM5265, *Anaerobaculum mobile* DSM 13181, and *Thermodesulfobacterium thiophilus* GeneBank sequences were used as references.

Coprothermobacter, as hydrogen-producing bacteria can establish a syntrophic association with *Methanothermobacter*, and they were found to coexist in the same anaerobic system in several studies (Sasaki et al., 2007; Tatara et al., 2008; Yabu et al., 2011; Palatsi et al., 2011; Luo et al., 2013; Lü et al., 2014).

Three bacterial clones were affiliated to *Anaerobaculum mobile* (within Synergistetes phylum in Fig.4 a), a moderately thermophilic peptide-fermenting bacterium previously isolated from an anaerobic lagoon (Menes and Muxì, 2002). This motile bacterium also ferments a range of carbohydrates and organic acids with acetate, H₂ and CO₂ as end products (Mavromatis et al., 2013). It was found together with *Coprothermobacter* and *Methanothermobacter* (Menes et al., 2001; Hatamoto et al., 2008; Yabu et al., 2011; Palatsi et al., 2011).

Table 4 – Clones number and affiliation for bacterial (a) and archaeal (b) members of the microbial population estimated by 16S rRNA gene clonal analysis at the end of the digestion process.

(a)

Accession number	Affiliation	Similarity (%)	no. of clones
KF971872	<i>Coprothermobacter Proteolyticus</i>	99	10
KJ626491	<i>Anaerobaculum mobile</i>	99	3
KJ626485	<i>Clostridium sp. JC3</i>	99	1
KJ626486	<i>Uncultured Clostridium</i>	99	1
KJ626490	<i>Uncultured Clostridium</i>	95	1
KJ626484	<i>Uncultured Thermoanaerobacteraceae</i>	99	2
KJ626496	<i>Enterococcus faecium</i>	99	2
KJ626487, KJ626489	<i>Uncultured Firmicutes</i>	87	2
KJ626481	<i>Uncultured Tumebacillus</i>	98	1
KJ626482	<i>Uncultured Dehalobacter</i>	91	1
KJ626483	<i>Soehngenia saccharolytica</i>	95	1
KJ626488	<i>Uncultured Planctomycetes</i>	99	1
KJ626492	<i>Thermodesulfovibrio thiophilus</i>	99	1
KJ626493	<i>Exiguobacterium aurantiacum</i>	99	1
KJ626494	<i>Vagococcus fluvialis</i>	99	1
KJ626495	<i>Streptococcus equinus</i>	99	1
Total			30

(b)

Accession number	Affiliation	Similarity (%)	no. of clones
KF971874	<i>Methanosarcina thermophila</i>	98	34
KF971873	<i>Methanothermobacter thermoautotrophicus</i>	99	25
KF971875	<i>Methanobrevibacter arboriphilus</i>	98	1
Total			60

Clostridium sp. strain JC3, together with the other clones affiliated to *Clostridiales*, *Soehngenia saccharolytica*, and *Tumebacillus* are carbohydrates degraders. *Clostridium* sp. strain JC3 is important in the anaerobic hydrolysis of cellulose in anaerobic digestion of activated sludge, as described in Syutsubo et al., (2005). Lü et al., (2014) reported the coexistence of cellulolytic bacteria belonging to the class *Clostridia* together with *Coprothermobacter* and *Methanothermobacter* in the anaerobic cellulose degradation under thermophilic conditions.

Members of the genus *Thermoanaerobacteraceae* are acetate oxidizing syntrophs (SAO) (Sieber et al., 2012), that may compete with acetotrophic methanogens (as *Methanosarcina thermophila*) for acetate utilization. *Thermodesulfovibrio thiophilus* was described as an obligately anaerobic, thermophilic bacterium that reduce sulfate and other sulfur compounds, but in the absence of sulfate can syntrophically degrade organic substances, such as lactate, ethanol and propionate, in close association with hydrogenotrophic methanogens (Sekiguchi et al., 2008).

Members of *Planctomycetes* were identified in wastewater and sludge anaerobic treatments mainly as anaerobic ammonia oxidizers (Anammox) (Chouari et al., 2003); the contemporary presence of proteolytic bacteria, that is directly related to high ammonia production, may explain their presence.

Microbial population dynamics by Fluorescence in situ hybridization. FISH analysis was further employed to evaluate the abundance of each microbial component and to follow the population dynamics during the entire digestion process in the control and experimental reactors. As shown in Fig.2, even though a similar microbial composition was finally observed in both reactors, the dynamics during the reactor operation strongly differed. Among Bacteria mainly members of *Coprothermobacter* group were found (Fig.5 a). In the control reactor (Fig.5 a), *Coprothermobacter* relative abundance was lower with respect to other bacteria during the first phase, then reached a relative abundance of about 30% in the middle of phase 2. On the other hand, in the reactor fed with pretreated sludge (Fig.5 b) *Coprothermobacter* became the main component of the bacterial population ($\approx 50\%$ out of total cells) already at the end of the first phase, remaining stable with OLR variation.

In all the previous studies in which *Coprothermobacter* was identified during anaerobic thermophilic sludge digestion (Kobayashi et al., 2008, Hatamoto et al., 2008; Lee et al., 2009; Luo et al., 2013; Pervin et al., 2013) its occurrence was only ascertained by PCR based approaches, and it was not found to dominate in every examined digester, as reported by Tandishabo et al., (2012). Therefore, the dominance of *Coprothermobacter* in both reactors indicates that the main way of methane production for the most part of the process goes through proteolysis as fermentative step. In anaerobic systems *Coprothermobacter* was shown to implement its intensive proteolytic activity with extracellular and intracellular proteases (Lü et al., 2014). In particular, it grows well on

peptides (Ollivier et al.,1985), and its abundance may be correlated to the complexity of the organic substrate (Tandishabo et al., 2012). The high abundance of *Coprothermobacter* retrieved in both reactors suggests that extracellular proteinaceous material was abundant in such systems, and specifically that proteins were present in their soluble form (as discussed in next section).

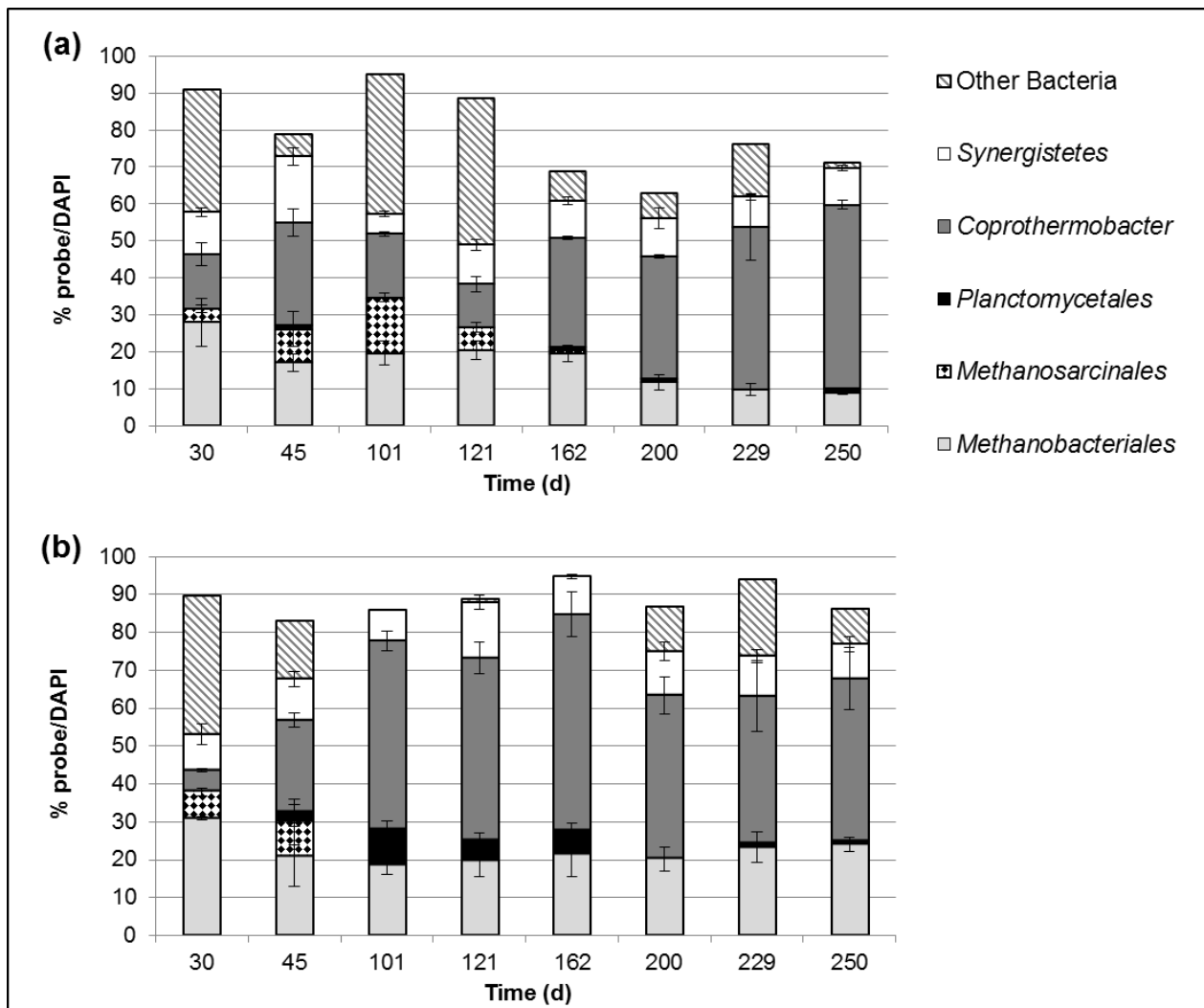


Figure 5 - Microbial population dynamics estimated by FISH analysis during reactor operation in TAD of untreated WAS (a) and thermally pre-treated WAS (b)

Synergistetes, (shown in Fig.6 b), were the other main group of bacteria identified by FISH. Their relative abundance was pretty stable (around 10% out of total biomass, Fig.5 a and b) during all the process, with no significant differences between the two reactors. This result is in line with the clonal analysis which showed the presence of *Anaerobaculum* spp., belonging to *Synergistetes* phylum, in the TAD biomass.

Members of *Planctomycetales*, morphologically resembling Anammox cells (Fig.6 c), were detected in both reactors at very low concentration ($\approx 1\%$ out of total biomass), with the exception of days 101, 121 and 162 (up to 10% of total biomass) in the reactor fed with pretreated sludge. Archaeal population was composed of members of *Methanosarcinales* and *Methanobacteriales* orders. As shown in Figure 5, *Methanosarcinales* spp. were present in both reactors during phase 1; at the beginning of phase 2 *Methanosarcinales* spp. were detected only in the control reactor.

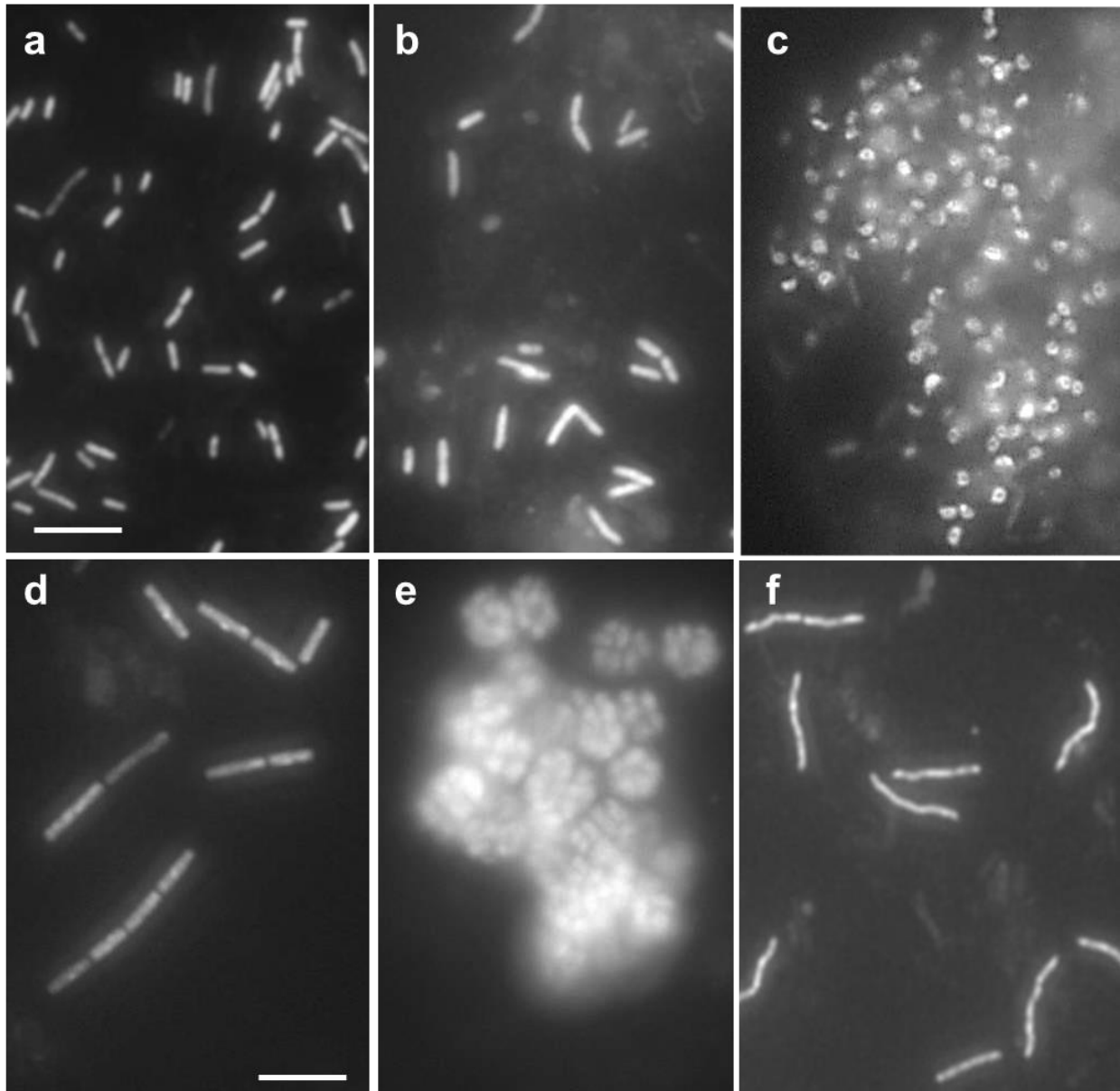


Figure 6 - Cells identified by FISH in TAD processes. (a) *Coprothermobacter* cells identified with probe CTH485 and hCTH439; (b) *Synergistetes* cells identified with SYN961 probe, morphologically resembling *Anaerobaculum*; (c) *Planctomycetales* identified with PLA886 probe, morphologically resembling anammox cells ; *Methanosaeta* filaments (d) and coccoid clusters of *Methanosaeta* (e) identified with MSMX860 probe; (f) *Methanobacteriales* cells identified with MB311 probe using the rPeiW sample enzymatic pretreatment. Bar is 5 µm.

Methanosarcinales identified with MSMX860 probe showed the morphologies of *Methanosaeta* (single rods and filaments) and *Methanosarcina* (coccoid clusters) (Fig.6 d and e). In control reactor, there was a shift from *Methanosarcina*, detected at days 30 and 45, to *Methanosaeta* detected at days 101 and 121. On the contrary, only *Methanosarcina* was detected in the experimental reactor. The remaining part of archaeal population belonged to *Methanobacteriales*, frequently identified as the dominant group during TAD (Krakat et al., 2010, Yabu et al., 2011; Ge et al., 2012). The observed relative abundances of *Methanobacteriales* were comparable in both reactors until the middle of phase 2, then it was significantly higher for the pretreated sample, as similarly observed for *Coprothermobacter* spp. (Fig.5).

Regarding FISH identification of these hydrogenotrophic methanogens, difficulties in the detection of members of the order *Methanobacteriales* were previously reported (Sekiguchi et al., 1999; Kubota et al., 2008; Krakat et al., 2010) because of the impermeability to oligonucleotide probes of the cell walls structural component of this family, the peptidoglycane pseudomurein. Moreover, Nakamura et al. (2006) and Kato et al. (2008) found that *Methanobacteriales* members like *Methanothermobacter thermoautotrophicus* modify cell surface thickness growing in syntrophic co-culture with fermenting bacteria, or in presence of environmental stresses. As detailed in Materials and Methods, this limitation was overcome by applying an enzymatic sample pretreatment with pseudomurein endopeptidase (Pei). The enzymatic pretreatment performed on sludge samples and the application of MB311 probe showed the presence of positive cells which were not previously visualized (Fig.6 f).

Despite there is no general rule that accurately define the archaeal population dynamics during thermophilic processes, several studies indicated that hydrogenotrophic methanogenesis is the main way of methane production (Sipma et al., 2003; Demirel and Scherer, 2008; Krakat et al., 2010), especially when temperature is above 55°C. Nevertheless, the acetotrophic methanogen *Methanosarcina* was previously found in thermophilic reactors (Ho et al., 2013; Lins et al, 2013) and its presence was mainly due to its metabolic versatility. *Methanosarcinaceae* are indeed capable of either hydrogenotrophic or acetoclastic methanogenesis, with the metabolic potential also for acetate oxidation to hydrogen (Ho et al., 2013). However, the hydrogen consumption by *Methanosarcina thermophila* is limited and high concentration of hydrogen inhibits its acetotrophic activity (Ahring et al., 1991). Either *Methanothermobacter thermoautotrophicus* or *Methanobrevibacter arboriphilus* belong to the order *Methanobacteriales*. *M. thermoautotrophicus* was found to coexist in syntrophic association with *Coprothermobacter* spp. in the anaerobic biomass during thermophilic anaerobic processes (Sasaki et al., 2012, Lü et al., 2014).

On the contrary, despite the simultaneous identification of *Methanosarcina thermophila* with other syntrophic bacteria (including *Coprothermobacter*, in Kobayashi *et al.*, 2008) the presence of these two kind of microorganisms seems to be inversely related because of the overlapping of their metabolic functions (Ho *et al.*, 2013).

Therefore, despite 16S DNA sequences of *M. thermophila* were the most abundant retrieved with clonal analysis, FISH analysis showed that this methanogen played a role only in the preliminary stage of the process (Fig.5). Additionally, since the mean acetate concentration in digested sludge during the three digestion phases was negligible (ranging from 10 to 40 mg/L), we can hypothesize that the decrease of the *Methanosarcina* population corresponded to the occurrence of syntrophic acetate oxydizers, as *Thermoanaerobacteraceae* identified during clonal analysis.

On the other hand, simultaneous identification and occurrence of *Coprothermobacter* spp. and *Methanothermobacter* spp. in thermophilic reactors likely suggest the establishment of a strict syntrophic association. Moreover, as shown in Figure 6 f, most of the cells identified by MB311 probe were morphologically similar to *Methanothermobacter*, suggesting a dominance of this microorganism in coexistence with *Coprothermobacter* spp. Since *Anaerobaculum* can carry out the same fermentative reactions of *Coprothermobacter*, its presence is in line with the establishment of a protein fermentative metabolism during TAD process.

The failure in FISH identification of the other members of the phylum *Firmicutes* may be explained by the lack of a full match of the LGC354 mix probes to the target sequences. Most of the *Firmicutes* sequences retrieved in this study showed indeed mismatches with the LGC354 set probes and their application surely produced an underestimation of this phylum in the microbial community. This feature was also described in the work of Pervin *et al.* (2013) on two-phased AD of WAS, in which 43% of the clone sequences affiliated with *Clostridia* did not completely match the LGC354 mix probes.

3.3 Correlation between biomass composition and TAD performances

Protein degradation by *Coprothermobacter*. The predominance of *Coprothermobacter* in bacterial population, and the simultaneous presence of a quite stable population of *Anaerobaculum*, implies the presence of a proteolytic pathway during TAD, likely related to proteinaceous material solubilization and degradation induced by the temperature increase, and to a greater extent by thermal pretreatment integration.

In fact, just the thermal hydrolysis of the feed induced protein solubilization, highlighted by the dramatic increase of soluble ($COD_{0.45\mu m}$) and colloidal ($COD_{1.2\mu m}$) COD as well as the protein

fraction, after thermal pretreatment (Tab.5). In fact, protein fraction of the total colloidal COD varied from 50% for the raw untreated sludge to 62% for the thermal pretreated one. Wilson and Novak (2009) reported that thermal hydrolysis (130 °C) of the protein bovine serum albumin showed similar effects as the anaerobic biological hydrolysis in which proteins are converted into peptides and individual amino-acids. Thermal hydrolysis pretreatment likely allowed to quickly transform the particulate organic substrate into soluble and colloidal compounds as proteins and peptides, without the intervention of the hydrolytic microbial consortia. So, the growth of *Coprothermobacter* cells can be favored under these conditions.

Moreover, the lack of fluorescence signal after FISH analysis of pretreated WAS (data not shown) highlighted the occurrence of cell degradation/inactivation, with a consequent production of additional proteinaceous material over EPS from sludge.

Overall, solubilization of the protein constituents of EPS together with the dead cell material in solution promoted the growth of *Coprothermobacter* in the experimental reactor with respect to the control reactor.

Table 5 – Colloidal fraction analysis of gravity thickened sludge before and after thermal-hydrolysis pretreatment. COD_{prot} was the COD related to the proteins, calculated considering the stoichiometric factor of 1.5; COD_{carb} was the COD related to carbohydrates, calculated according the stoichiometric factor of 1.1

(mg/L)	Raw sludge	Thermal Pretreated sludge
COD_{0.45µm}	45 ± 3	4340 ± 420
COD_{1.2µm}	88 ± 10	4460 ± 435
Proteins	30 ± 2	1822 ± 206
COD_{prot}	45 ± 3	2733 ± 7
Carbohydrates	3 ± 0.3	10 ± 2
COD_{carb}	3.3 ± 0.3	11 ± 2.2

At the same time in the control reactor, digestion temperature was clearly the factor that affected protein release, as highlighted by the accumulation of sCOD during the digestion process due to biological hydrolysis of particulate material (Fig.7 a).

Previous experiences (Lee *et al.* , 2009; Ge *et al.* , 2012; Pervin *et al.*, 2013) evidenced the presence of *Coprothermobacter sp.* in digested sludge due to the high temperature of the process (until 70°C), correlated to the degree of protein solubilization.

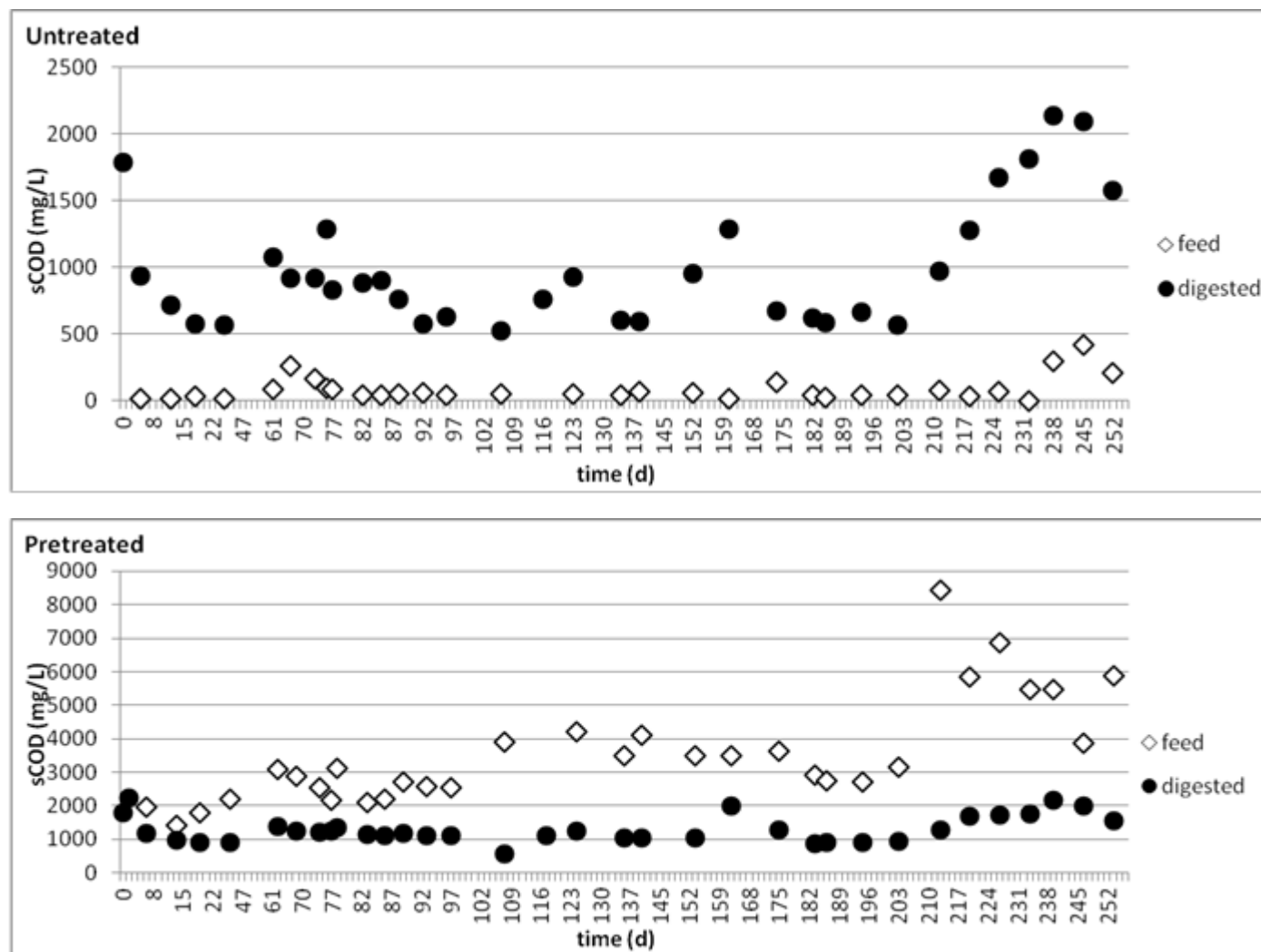


Figure 7 – sCOD values of feed and digested sludge along thermophilic anaerobic digestion of untreated and pretreated sludge.

In Lü *et al.*, (2013), a temperature shift from 35 to 55°C resulted in an abiotic solubilization calculated to account for around 16-20% of the total protein solubilization.

In Menes *et al.* (2001) the persistence of *Anaerobaculum* and *Coprothermobacter* in an enrichment LCFA degrading culture was justified by their ability to use proteinaceous substrates, resulting from dead cell material generated during the process. The same observation about *Coprothermobacter* was made by Lü *et al.* (2014), by analyzing an enrichment culture degrading cellulose.

The presence of a protein degradation pathway during digestion was also highlighted by the concentration of soluble ammonia in the effluent, ranging from 760 to 1340 mg/L during the digestion phases independently on the pretreatment (Tab.6).

Since soluble proteins were immediately de-aminated to ammonia, the soluble ammonia concentration of the pretreated feed was always significantly higher (170-220 mg/L) with respect to the untreated feed (15-20 mg/L).

Although ammonia concentration in the pretreated feed was always higher, the organic load appeared to be the most significant parameter affecting ammonia levels in the anaerobic effluents (Gianico *et al.*, 2013; Wilson and Novak, 2009).

Table 6 – Soluble ammonia concentration in feed and digestate, at steady state conditions of each digestion phase.

phase	Soluble NH ₄ ⁺ -N (mg/L)			
	Untreated		Thermal Pretreated	
	feed	digested	feed	digested
1	15±1	939±87	219±18	1031±98
2	14±1	767±69	165±14	757±64
3	21±2	1344±120	201±20	1225±112

Proteolytic activity of *Coprothermobacter* and the consequent production of ammonia were not influenced by its relative abundance. Ammonification of proteins did not negatively affect the methanogenic activity, because methanogenic cells start to be partially inhibited at 1.7g/L, and total inhibition occurs only at 4.2 g/L (Chen *et al.*, 2008).

As previously described, sCOD removal in the experimental reactor increased with digestion time (Fig.7 b) highlighting the presence of more efficient hydrolytic pathways than in the control reactor. Moreover, the increase in relative abundance of *Coprothermobacter* during TAD of pre-treated sludge was coupled to the enhancement of sCOD removal (Fig.8). On the other hand, comparing the relative abundance of *Coprothermobacter* in both reactors (Fig.5) to sCOD fed in phase 3

(Fig.7), at high OLR, the extremely high values of sCOD corresponded to a decrease of *Coprothermobacter* in the reactor fed with pretreated sludge. In fact, relative abundance of *Coprothermobacter* was around 50 - 57% until sCOD was maintained around 4000 mg/L, then decreased to 38 - 42% out of total cells when sCOD raised up to 6000 mg/L. As seen in Fig.5, the amount of unidentified Bacteria during phase 3 was higher in the experimental reactor than in the control reactor, highlighting that likely different bacterial groups took place in the fermentative step under these conditions. On the other hand, in the control reactor the relative abundance of *Coprothermobacter* raised after increasing soluble organic load (about 200 mg/L) due to the pre-thickening step (Fig.7 a).

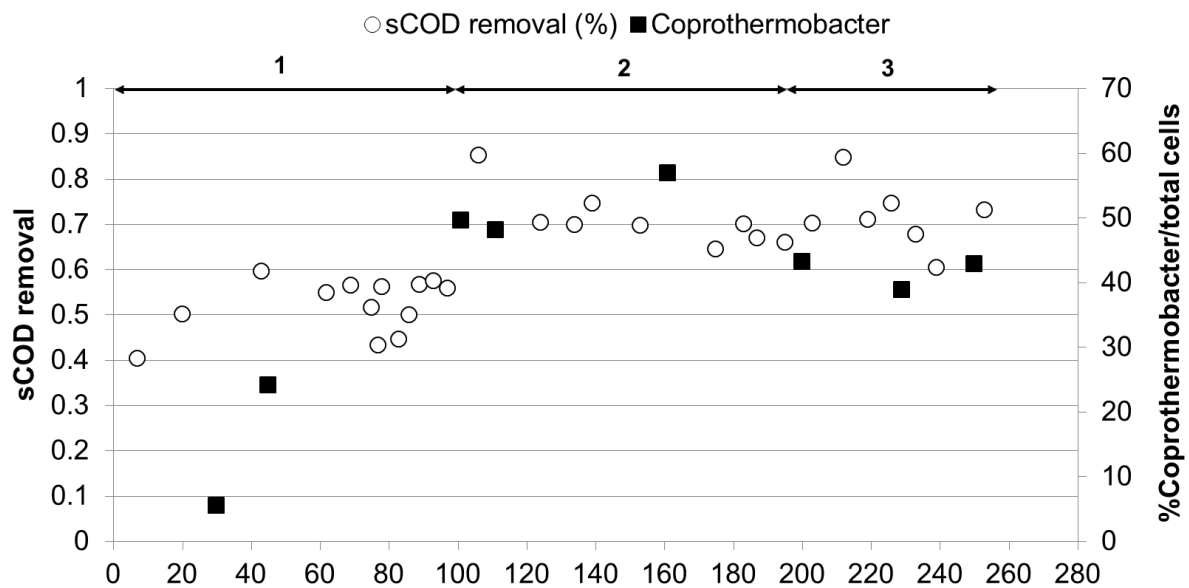


Figure 8 - Correlation between sCOD removal and *Coprothermobacter* relative abundance during TAD of thermal pretreated WAS.

Overall, these observations underline a possible strict correlation between substrate availability and cell activity in *Coprothermobacter* population dynamics during anaerobic digestion, as emerged in previous studies (Cheon et al., 2007; Lee et al., 2009; Tandishabo et al., 2012), particularly relevant by pretreatment integration, that increased significantly the bio-available substrate in the sludge to be digested.

Syntrophic associations and methanogenesis. Sasaki *et al.* (2012), by comparing a monoculture of *Coprothermobacter proteolyticus* with a co-culture with *Methanothermobacter thermoautotrophicus*, reported that in co-culture *Coprothermobacter* growth rate increased 4-fold in presence of *Methanothermobacter*, with respect to a pure culture. Simultaneously, the number of cells of *Methanothermobacter* decreased without affecting methane production rate. In addition,

during co-culture the soluble protein content decreased more than in monoculture. This means that the presence of an hydrogenotrophic partner was essential to improve the proteolytic activity of *Coprothermobacter*. Overall, beyond the relative abundances of these microorganisms recorded in both reactors, the establishment of a stable syntrophic association is the key to obtain greater performances of the entire system. Based on these observations, comparing the relative abundances of these two species during digestion time (Fig.9), emerged that in the experimental reactor the establishment of this syntrophic association occurred already in the early stages of the process (at the end of phase #1), a further evidence that the typology and the composition of substrate had significantly influenced the evolution of this microbial interaction.

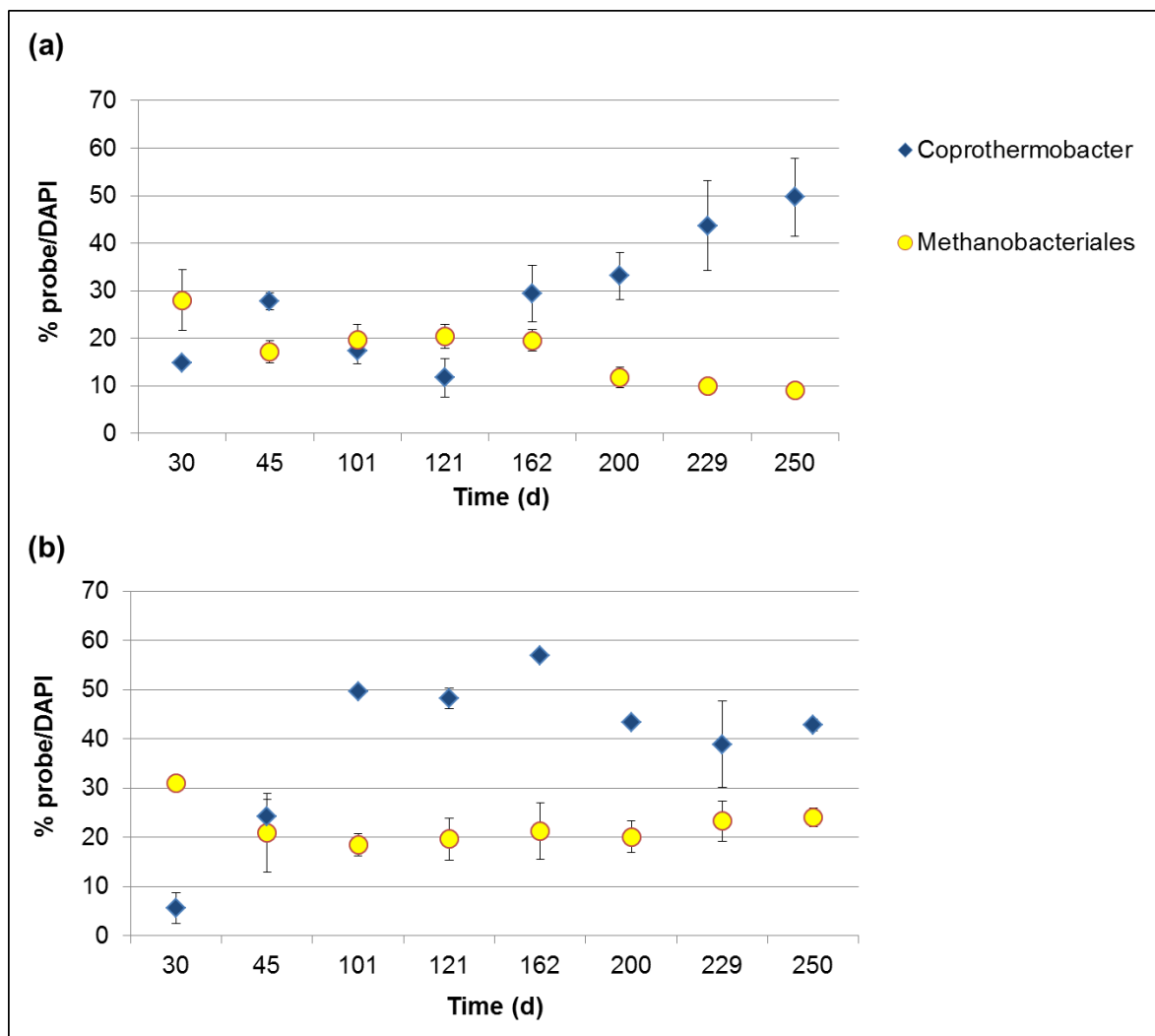


Figure 9 – Focus on *Coprothermobacter*, *Synergistetes* and *Methanobacteriales* population dynamics estimated by FISH analysis during reactor operation in TAD of untreated WAS (a) and thermally pretreated WAS (b).

The earlier establishment of a stable population of *Coprothermobacter* in the reactor fed with pretreated sludge, together with its syntrophic partner *Methanothermobacter*, which relative

abundance was maintained around 20% out of total cells during the process (Fig.9 b), seems to have positively affected the cumulative biogas production (Fig.2), always higher for the experimental reactor in comparison to the control reactor. On the other hand, the progressive decrease of *Methanothermobacter* in the control reactor did not affect the consequent increase of *Coprothermobacter*.

The immediate high activity of *Methanothermobacter* cells (day 30, Fig.9 a and b) was probably crucial for the equilibrium of both systems. These H₂ scavengers methanogens improved the stability of the process decreasing H₂ partial pressure, directly accelerating the kinetics of proteolysis operated by *Coprothermobacter*. The almost steady trends in relative abundance of *Methanothermobacter* during digestion of pretreated sludge indicated that OLR variations did not affect population dynamics under these conditions, while in the control reactor the increase of OLR appeared to be the main factor influencing the decrease of *Methanothermobacter*.

As described in Morgan *et al.* (1997), the success of *Methanothermobacter* in the environment may depend on its ability to maximize either growth rate or growth yield (number of cells per mole of CH₄ produced). *M. thermoautotrophicum* cells can modify their gene expression pattern based on the availability of H₂, choosing between maximum growth rate and maximum growth yield. With a very low H₂ supply, *M. thermoautotrophicum* cultures did not grow but improved methanogenic enzymes expression. With higher H₂ availability, growth occurred, but as long as growth was H₂ limited, the growth yield was improved. When the H₂ supply was not growth rate limiting biomass and CH₄ production increased most rapidly and in parallel.

On the basis of these observations, *Methanothermobacter* population dynamics observed in the experimental reactor are in line with the occurrence of H₂ limitation, likely due to an imbalance between production and consumption.

On the other hand, in control reactor, *Methanothermobacter* decreased during phase 3 (Fig.9 a), without negatively affecting the cumulative biogas production (Fig.2) with respect to the previous phase, allowing to assume that H₂ supply was different under these conditions.

So, methane production is neither related to energy conservation nor to the relative abundance of microorganisms, but to H₂ concentration and its availability during AD.

Members of *Methanosarcinales* were revealed by FISH analysis at the beginning the TAD process. This finding highlighted the occurrence of two different methane production pathways. Afterwards, their disappearance is likely due to the increasing hydrogen concentration caused by the fermentative bacteria activity, that led to gradual inhibition of acetate metabolism (Ahring *et al.*, 1991). It should be mentioned that members of the genus *Methanosaeta* utilize only acetate for methane production. This is reflected in a very high affinity for the substrate: a minimal

concentration of only 7–70 μM is needed for growth (Berger et al., 2012). For this reason, *Methanosaeta* species prevail over members of the genus *Methanosarcina* in low acetate environments.

In the control reactor, the shift from *Methanosarcina* to *Methanosaeta* probably corresponds to the gradual inhibition of acetate metabolism and the transition to a fully hydrogenotrophic methanogenesis. In the experimental reactor, the sole presence of *Methanosarcina* at days 30 and 45 underlines how the different substrate availability due to thermal hydrolysis may accelerate the establishment of a well-defined pathway of methanogenesis through a stable syntrophic association.

4. Conclusions

- Temperature driven hydrolysis and the consequent released bioavailable substrate strongly influenced the composition of the microbial population: the simultaneous presence of syntrophic *Coprothermobacter* and *Methanothermobacter* has determined an efficient conversion of H_2 into methane.
- Thermal pretreatment of feed sludge improved the overall AD performances which were mirrored by different microbial population dynamics.
- Protein availability is an important key-factor to enrich *Coprothermobacter* species and stimulate the syntrophy with *Methanothermobacter* during thermophilic AD processes.
- The potential linked to the selective enrichment of *Coprothermobacter* spp. in mixed anaerobic biomass is valuable for the future development of thermophilic engineered anaerobic systems to overcome hydrolysis limitation and optimize the methane yield by hydrogenotrophic methanogenesis.

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Chapter 6

Microbial diversity in innovative mesophilic/thermophilic temperature phased anaerobic digestion of sludge

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1.Introduction

Anaerobic digestion is a robust and efficient technology for the energetic valorization of various types of biomass (including organic wastes and sewage sludge), and it is expected to play a crucial role in the future of renewable energy production (Lauwers et al., 2013). Anaerobic digestion of organic matter occurs through the sequential cooperation of different microbial groups in order to achieve degradation of a variety of polymeric and monomeric substrates (O’Flaherty et al., 2006). The anaerobic degradation of organic matter proceeds in a series of four metabolic steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. A diverse number of bacteria take part in the first three steps, and methane formation is mainly derived from acetate and hydrogen/CO₂ conversion by methanogenic *Archaea*. A balanced interaction between the microorganisms in this chain is crucial for an efficient biogas production. Thus, a better understanding of the structure and function of microbial communities during AD may help to improve the reactor performances. However, organic particulate matter degradation efficiency of AD of waste activated sludge remains limited, because of the hydrolysis, considered the rate-limiting step of the entire process (Pavlostatis and Giraldo-Gomez, 1991; Bougrier et al., 2006). In order to increase or accelerate the biodegradation rates, various pretreatments (thermal, enzymatic, chemical or mechanical) have been widely investigated (Carrère et al., 2010; Carlsson et al., 2012). Ultrasounds are currently applied as pretreatment also at full-scale on sewage treatment plants. The action of the ultrasonic treatment is to disrupt mechanically the sludge floc matrix and the cell structure by means of shear forces due to cavitation phenomena. Moreover, phased anaerobic digestion in which two or more digesters are used in series is a promising technology for the treatment of wastewater sludges (Zamanzadeh et al., 2013). In the last years a temperature phased anaerobic process (TPAD) with a short thermophilic stage acting as thermal pretreatment, followed by a longer mesophilic one was investigated with the aim to separate microbial groups into two phases (Coelho et al., 2011; Ge et al., 2010). In the first thermophilic with short hydraulic retention time, hydrolytic and acidogenic/fermentative bacteria degrade polymers to monomers and produce organic acids. In the second stage (with a much longer retention time), these products are then converted into methane by strict cooperation between bacteria and archaea during acetogenesis and methanogenesis (Merlino et al., 2013). However, detailed knowledge of the microbial community structure and their function is lacking (Pervin et al., 2013a). TPAD is particularly applicable to AD of activated sludge, as it allows increased performance regarding solids removal and methane production at a moderate energy input, and moderate pathogen removal (Paul et al., 2012). Studies of these microbial communities are limited and knowledge of the ecology and how that may be

related to the system operation is just beginning to develop (Pervin et al., 2013a). Nevertheless, the microbial population is likely to be competent in anaerobic hydrolytic and acidogenic functions, and hence likely to be bacterial rather than archaeal or eukaryotic (Amani et al., 2010). The new approach of this study was based on the idea to sub-divide the anaerobic digestion process into three different stages: 1) an ultrasounds pretreatment to improve hydrolysis, 2) a short mesophilic stage to improve volatile fatty acids formation and 3) a final thermophilic stage to convert these intermediates into methane and contemporarily assure the complete hygienization of the digested sludge. Focus of this study was the characterization of the microbial communities growing in this innovative system, fed by real waste activated sludge, either untreated or ultrasounds pretreated. The study was specifically aimed at understanding the microbial composition of the mesophilic and thermophilic anaerobic biomass under different conditions of temperature, organic loading rate and substrate solubilization and availability. Investigation was performed by Fluorescence In Situ Hybridization (FISH) using a wide range of oligonucleotide probes with broad and narrow specificity.

2. Material and methods

2.1 Reactors operation and performance

Sludge. Waste activated sludge (WAS) samples were obtained from the municipal “Roma-Nord” wastewater treatment plant, characterized by an organic load of about 700,000 p.e., high sludge age (20 days) and a COD average value of incoming sewage of 200 mg/L. The activated sludge was sampled directly from the oxidation tank. The anaerobic inoculum utilized for the start-up of the mesophilic stage was sampled from the full scale digester of the plant treating mixed sludge, while the anaerobic biomass for the thermophilic reactors originated from a previous experimentation with semi-continuous thermophilic system (Gianico et al., 2013).

Sludge pretreatment. The disintegration by ultrasound was performed with an ultrasonic processor UP400S (dr. Hielscher, Germany) operating at 300 W and 24 kHz. Sonication energy input was set at 0.4 - 0.5 kWh kg⁻¹ dry solid on 500 mL of waste-activated sludge (2.9 - 4.7 % TS) placed in 1 L beaker with the probe allocated at 3 cm above the beaker bottom.

Mesophilic/thermophilic dual stage anaerobic digestion. Sludge digestion was carried out using four anaerobic digesters operated in semi-continuous mode. Two reactors, as control line, were used

to carry out the mesophilic/thermophilic digestion of untreated waste activated sludge; the other two reactors, as experimental line, were selected to treat the same sludge, but after sonication (Fig.1). All jacketed reactors ($V=7L$) were completely mixed: the first mesophilic digester was maintained at the constant temperature of $37^{\circ}C$, while the thermophilic reactor was maintained at $55^{\circ}C$. In the first test the organic loading rate (OLR) to the first mesophilic reactor was fixed at $3.9 \text{ kg VS m}^{-3} \text{ d}^{-1}$ and to the successive thermophilic at $1.2 \text{ kgVS m}^{-3} \text{ d}^{-1}$; in the second test, the OLR was increased up to 10 for the mesophilic and $2.5 \text{ kgVS m}^{-3} \text{ d}^{-1}$ for the thermophilic reactor, by decreasing the hydraulic retention time (HRT) in the mesophilic reactor and by pre-thickening the incoming feed sludge. Table 1 lists the operating conditions of the anaerobic digestion tests.

Table 1 – Operating conditions of mesophilic (1°stage) and thermophilic (2°stage) digestion tests.

	Test 1		Test 2	
	1°stage	2°stage	1°stage	2°stage
T ($^{\circ}C$)	$37^{\circ}C$	$55^{\circ}C$	$37^{\circ}C$	$55^{\circ}C$
OLR ($\text{g VS L}^{-1} \text{ d}^{-1}$)	3.9	1.2	10	2.5
HRT (d)	5	10	3	10
Test duration (d)	97	97	81	81

Biogas collection and analysis. The produced biogas was collected by water displacement in a biogas collection unit. The gas meter consisted of a volumetric cell for gas–liquid displacement, a sensor device for liquid level detection, and an electronic control circuit for data processing and display. The methane content in the biogas was measured using a PerkinElmer Auto System Gas Chromatographer equipped with a thermal conductivity detector (TCD) as described in Gianico et al. (2013).

Matter composition. Total and volatile solids (TS and VS) were determined in triplicates according to standard methods (APHA, 1998). The pH was measured by a portable pH-meter (WTW, pH 330/SET-1). To analyze sludge organic content, the particulate sludge matter was removed by centrifugation (10 min at 5000 rpm), and the resulting centrate was filtrated through 0.2, 0.45 and $1.2 \mu\text{m}$ pore size membrane filters. Volatile fatty acid (VFA) were quantified from $0.2 \mu\text{m}$ filtrate (soluble phase), by gas chromatography using PerkinElmer Auto System Gas Chromatographer with flame ionization detector (FID). The GC analyses were performed on a stainless steel column packed with 60/80 mesh Carboxen C, 0.3% Carbowax (Supelco, USA), under the following conditions: injector $200^{\circ}C$, oven $175^{\circ}C$, detector $250^{\circ}C$. Nitrogen was used as a

carrier gas at a flow rate of 30 mL/min. Soluble COD (sCOD), was determined on 0.45 μm filtrate (soluble phase) by Cell Test Spectroquant (Merck) as described in Gianico et al. (2013). Protein content was determined on 1.2 μm filtrate (colloidal phase) by BCA colorimetric method as described in Braguglia et al. (2012).

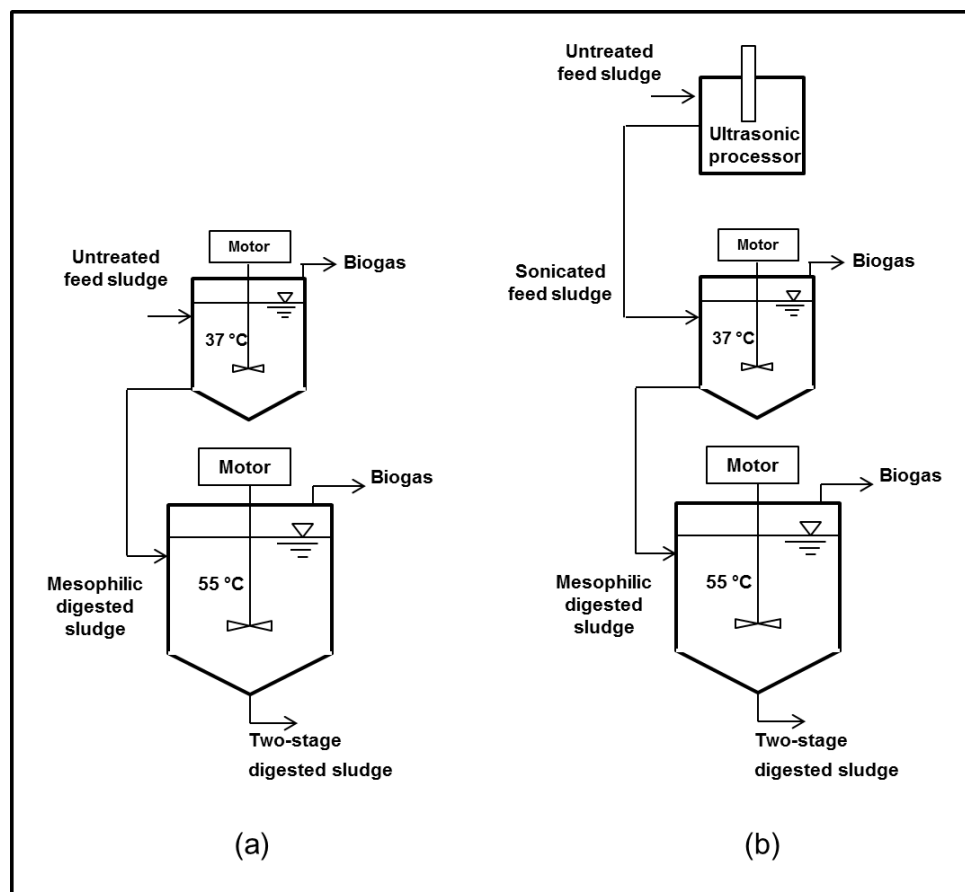


Figure 1 - Schematic diagram of the mesophilic/thermophilic process on untreated (a) and sonicated (b) WAS

2.2 Microbial community analysis

Sample collection. Effluent sludge samples were collected from reactors during the steady state operation of the systems. These samples were fixed with paraformaldehyde and ethanol for FISH analysis as described in Amann et al. (1990).

Fluorescence in situ hybridization (FISH). FISH on fixed sludge samples was performed as previously described (Braguglia et al., 2102). Details of the employed oligonucleotide probes are available at probeBase (Loy et al., 2007). Probes were labelled with Cy3 or FITC fluorophores. To identify thermophilic archaea of the order *Methanobacteriales* the protocol was modified as

described in Nakamura et al. (2006), by applying the enzyme pseudomurein endopeptidase (rPeiW) to improve probe penetration inside cells. The lyophilized rPeiW was provided from Dr. Kohei Nakamura (Laboratory of Environmental Microbiology and Engineering, Faculty of Applied and Life Sciences, Gifu University).

Microscopy and fluorescence signal quantification. Samples were examined by epifluorescence microscopy (Olympus BX51) using filters for FITC (excitation 470-490 nm – emission 520 nm) and for CY3 (excitation 546 nm – emission 590 nm). Autofluorescence was tested by performing a control test on unstained samples observed with filters used for FISH analysis. No autofluorescence was retrieved in all the screened samples. Fluorescence signal was quantified on microscopic images taken from the samples with a digital camera (Olympus XM-10) and the software Cell F. All the hybridizations with specific probes were carried out in combination with DAPI staining to estimate the portion of cells targeted by group specific probes out of the total cells. Area measurements of the hybridised cells were reported as a portion of the area covered by total DAPI stained cells in each field. Area measurements were performed on at least 10 JPEG images (or other image format with 8 bit size of 1388x1040 pixels) using ImageJ software package (version 1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA, available in the public domain at <http://rsb.info.nih.gov/ij/index.html>) as described in Braguglia et al. (2012).

Microbial diversity. The relative abundance of each microbial component calculated by FISH was utilized to describe the biodiversity of mixed microbial communities, estimated with two different parameters commonly employed: Shannon–Weaver index of diversity (H) (Shannon and Weaver, 1963). and Pielou's evenness index (Heip, 1974).

3. Results and discussion

3.1 Reactor performances and microbial population dynamics

The innovative process described here is based on the integration of a mechanical pretreatment before a dual stage mesophilic-thermophilic digestion process (Gianico et al., 2014). The scope of this layout was to improve the hydrolysis and the fermentation steps during the mesophilic digestion of sonicated sludge, while the successive long thermophilic stage could additionally improve organic matter biodegradation obtaining higher methane yield and sludge hygienization. The composition and the structure of the anaerobic biomass selected during both mesophilic and

thermophilic stages were investigated to better understand the effect of the operating conditions on microbial community in order to achieve better process performances.

Mesophilic microbial community. As shown in Figure 2, the bacterial population identified by FISH ranged between 55% and 65% of total DAPI stained cells and was mainly composed of *Proteobacteria*, commonly found as main components of activated sludge (Wilén et al., 2008).

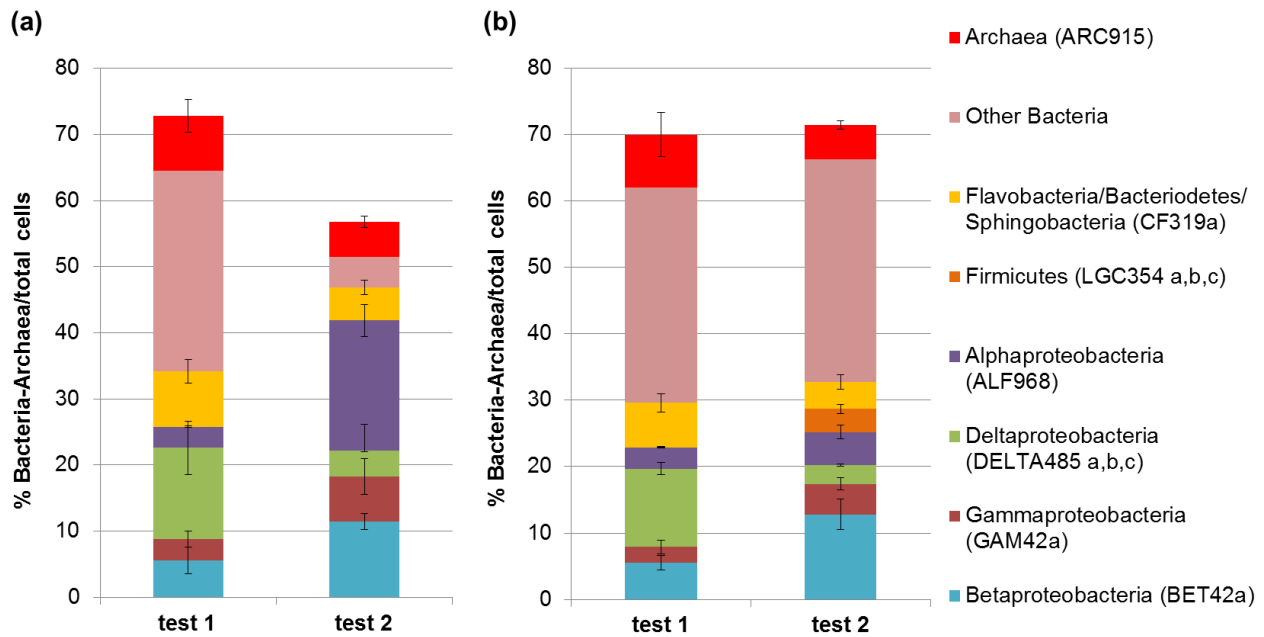


Figure 2 - Relative abundance of *Bacteria* and *Archaea*, out of total cells in mesophilic reactors fed with untreated (A) and sonicated (B) sludge operating at steady state conditions. FISH oligonucleotide probes applied for the analysis are reported in brackets

Changes in HRT did not affect the amount of bacteria retrieved in the anaerobic biomass in the reactor fed with ultrasound pretreated sludge (Fig.2 B), despite the higher organic loading rate (Tab.1). Overall, in the reactor fed with ultrasound pretreated sludge the impact of operative changes was negligible with respect to the control reactor, although slight variations in the relative abundance of individual components were observed. The relative abundance of archaeal population decreased with the increase of OLR in both sonicated and untreated biomass (Fig.2). This is in agreement with the biogas production of the mesophilic reactors, that was higher in test 1 with respect to test 2 (Fig.3). Shortening the HRT of the first mesophilic stage reduced the extent of conversion of substrates in methane, benefiting of the biogas production of following thermophilic stage in test 2 (Fig.3). The latter was confirmed by VFAs measurements in mesophilic samples during the two tests (Fig.4). In test 1 (Fig.4 a, b), only acetate and propionate were detected, but the

concentration was very low (< 30 mg/L). Diversely, during test 2, VFAs were found at higher concentrations, ranging from 0.5 to 1 g/L in the control reactor and from 0.7 to about 2.5 g/L in the reactor fed with sonicated sludge (Figure 4 c-d).

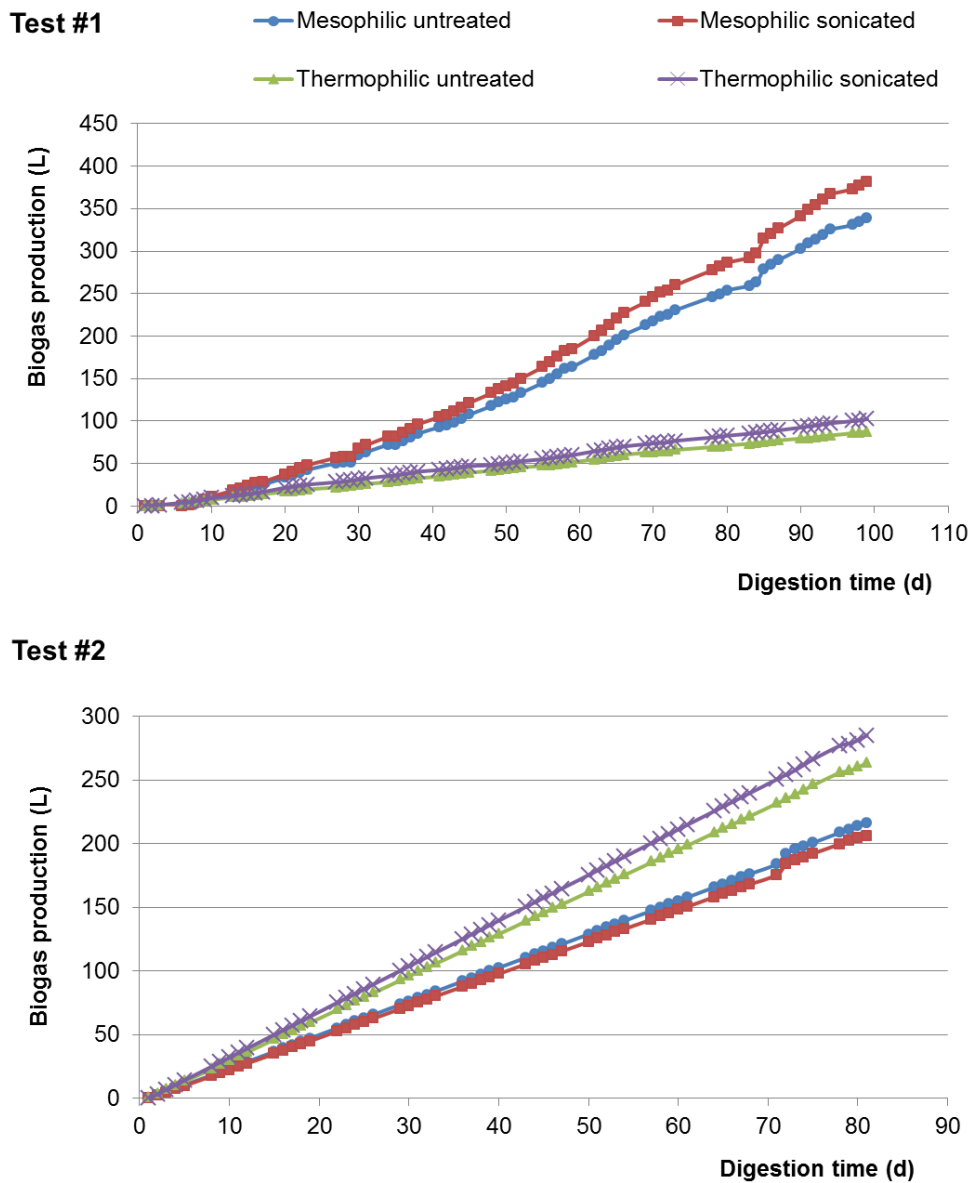


Figure 3 - Mesophilic and thermophilic cumulative biogas production during the anaerobic tests

Additionally, VFAs were present also in the form of butyrate and isobutyrate. These VFAs, conveyed as feed into the thermophilic reactor, enhanced consequently the biogas yield with respect to test 1. This indicated that in the mesophilic stage of test 1 the conversion rate of organic substrates into methane was higher than in test 2, with a consequent loss of the methanogenic potential incoming in the thermophilic reactor. Indeed, in test 1 the mesophilic stage produced the majority of the total biogas during the two stage digestion (Fig.3).

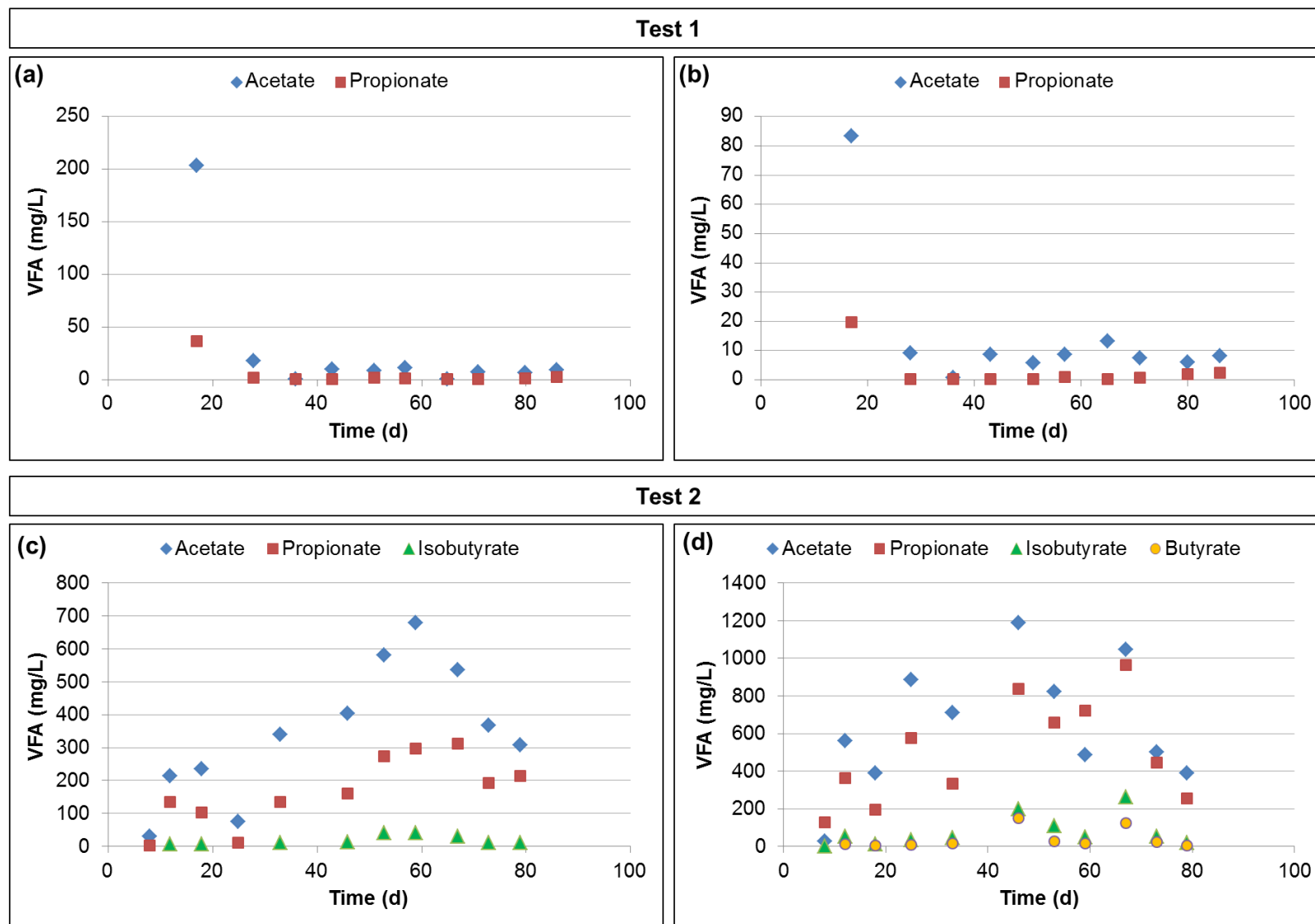


Figure 4 - VFA trends in mesophilic reactors fed with untreated (a and c) and sonicated (b and d) sludge during test 1 and test 2

Shortening the HRT and increasing the OLR of the mesophilic reactor led to the improvement of the performance of the following thermophilic stage in test 2, encountering the original aim of the mesophilic stage.

The application of specific FISH probes for *Archaea* showed the presence of long filamentous and rods of *Methanosaeta* spp. The latter was found in all digestion phases, highlighting the occurrence of acetotrophic methanogenesis as expected in a mesophilic anaerobic system. The occurrence of these microorganisms is strictly related to acetate concentration, because *Methanosaeta* is a specialist in using acetate and grows only at low acetate concentrations. Fluctuations in relative abundance (Fig.5) of this microorganism during the two tests (a decrease during test 1 and an increase during test 2 in both reactors) are mainly due to rapid changes in acetate concentration (which constitutes the largest portion of VFA present in the system) observed during the fermentative step of anaerobic digestion, as shown in Figure 4. The remaining part of archaeal population was not identified by applying the available FISH probes.

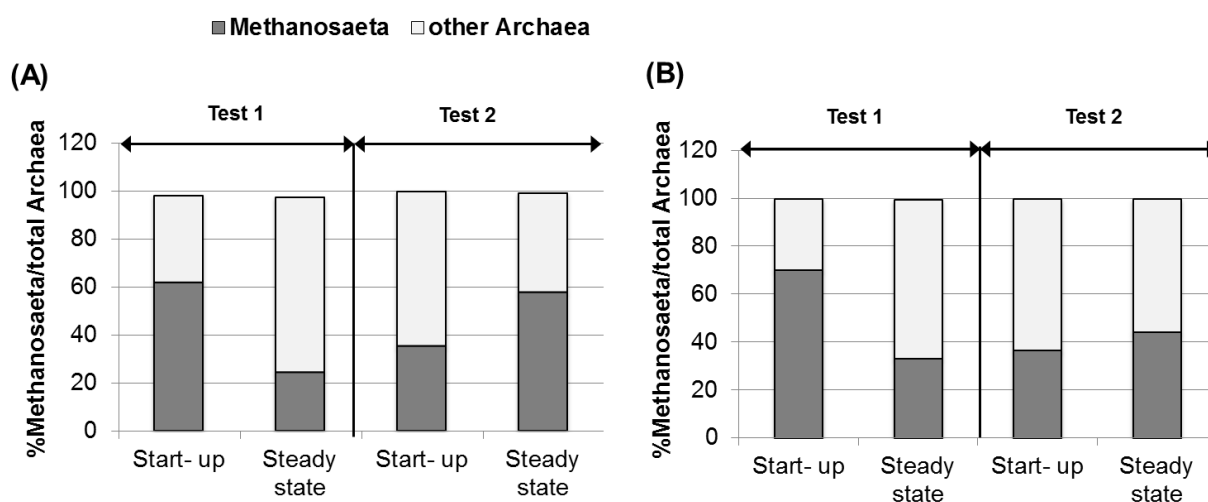


Fig. 5 Relative abundance of *Archaea* (ARC915 probe) and *Methanosaeta* (MX825 a,b,c probes) out of total cells in mesophilic reactors fed with untreated (A) and sonicated (B) sludge for the two digestion tests, at the beginning and at the end of each digestion test

Thermophilic microbial community. As shown in Figure 6, proteolytic fermentative bacteria *Coprothermobacter* and hydrogenotrophic *Methanobacteriales* were found as component in the microbial community. Microbial composition of the inoculum is reported in Figure 6 a. *Methanobacteriales* cells were further identified as *Methanothermobacter* sp. on the basis of the results obtained an archaeal 16S rRNA gene clone library (data not shown). *Coprothermobacter* and *Methanothermobacter* were found to live in strict syntrophic associations in many previous

studies (as described in Chapter 2, section 4). Proteolytic activity of *Coprothermobacter* is well documented (Etchebehere et al. 1998; Tandishabo et al. 2012; Majeed et al. 2013; Lü et al. 2014), and in particular this microorganism is capable to ferment proteins, growing well in presence of peptides (Ollivier et al. 1985).

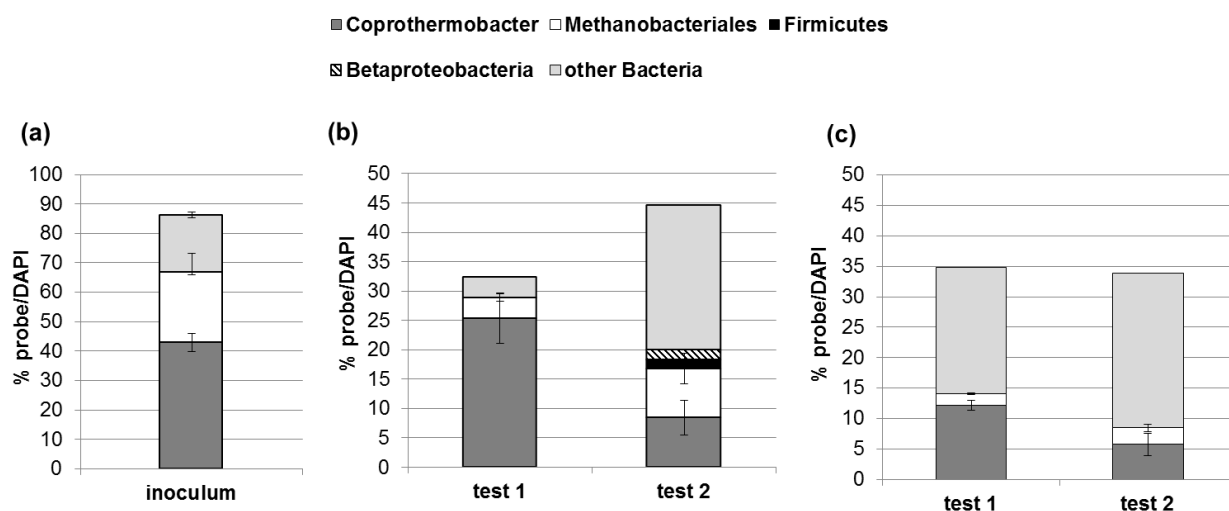


Fig. 6 Relative abundance of *Bacteria* (EUB338mix probes), *Coprothermobacter* (CTH485 probe), *Betaproteobacteria* (BETA42A probe), *Firmicutes* (LGC354a,b,c) and *Methanobacteriales* (MB311 probe) out of total cells in thermophilic reactors fed with untreated (b) and sonicated (c) sludge for the two digestion tests, at the end of each digestion period. In (a) is reported the microbial composition of thermophilic inoculum

Therefore, the solubilization of proteins into small molecules can promote *Coprothermobacter* establishment, especially at high temperatures (Lee et al. 2009). In this mesophilic/thermophilic digestion system, *Coprothermobacter* decreased with increased OLR indicating a failure in acclimation under the new reactor conditions (Fig.6). Sasaki et al. (2011) reported that the growth of *Coprothermobacter* was proportional to protein consumption. Tandishabo et al. (2012) highlighted that *Coprothermobacter* population size was controlled by the type of substrates in the feed. Evidently, fermentative pathways were different with respect to the original thermophilic reactor from which the inoculum was sampled. In particular, sonication pretreatment and/or mesophilic stage generated substrates in which proteic components were not degraded into small molecules. Sonication pretreatment generally seems to affect *Coprothermobacter* relative abundance in test 1 (Fig.6 b-c), with the increase of other bacteria in the system fed with sonicated sludge (Fig.6 c). In test 1, after sonication and mesophilic stage, proteins concentration was about 260 mg/l and the sCOD value increased up to 250 mg/L (Table 2).

Regarding the second test, the OLR increase of the mesophilic stage highly improved sCOD and proteins release and accumulation in the reactor, but this had no positive effect on the maintenance

of *Coprothermobacter* population in the thermophilic reactor. Indeed, as described in Bougrier et al. (2005), after low frequency ultrasounds pretreatment, proteins were made soluble but not completely degraded, as very little organic nitrogen was transformed into ammonium.

Table 2 – Soluble COD and proteins during steady state of dual digestion test 1(a) and test 2 (b)

(a)

	Untreated			Sonicated		
	<i>Feed</i>	<i>1° stage</i>	<i>2° stage</i>	<i>Feed</i>	<i>1° stage</i>	<i>2° stage</i>
CODsol (mg/L)	56	210	930	520	252	871
Proteins (mg/L)	65	258	650	279	264	664

(b)

	Untreated			Sonicated		
	<i>Feed</i>	<i>1° stage</i>	<i>2° stage</i>	<i>Feed</i>	<i>1° stage</i>	<i>2° stage</i>
CODsol (mg/L)	134	1520	2180	2082	2892	2482
Proteins (mg/L)	89	661	1388	1103	818	1471

An additional factor influencing *Coprothermobacter* population was likely the HRT of the mesophilic stage: the shortening of HRT from test 1 to test 2 may have decreased the proportion of protein degradation during mesophilic hydrolysis. The percentage of acetate degradation in thermophilic reactors during test 2, with respect to the incoming sludge from mesophilic reactors is shown in Figure 7. Acetate degradation occurred in both reactors, but was higher in the reactor fed with sonicated sludge.

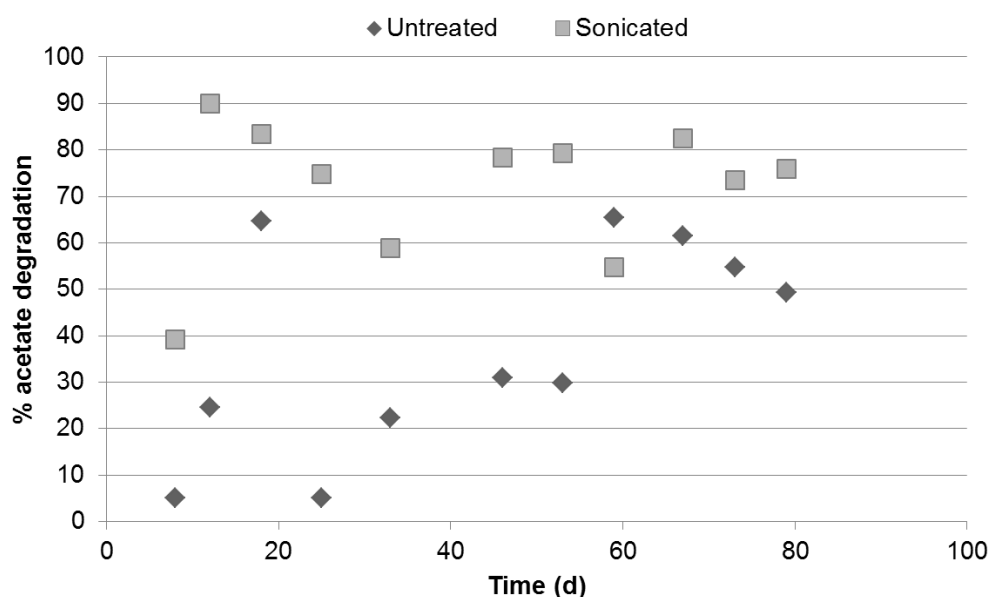


Figure 7 - Acetate degradation in thermophilic stage during test 2, in reactor fed with untreated and sonicated sludge.

Thus, the high concentration of VFA transferred from mesophilic to thermophilic stage in test 2, definitely changed the pathway of methanogenesis. All these observations can easily explain the decrease in relative abundance of *Coprothermobacter* in the end-stage compared to the start-up population. On the other hand, *Methanothermobacter* was the only methanogen retrieved in the anaerobic sludge (Fig.6). This was mainly due to the VFA accumulation from mesophilic stage, because *Methanothermobacter* is less sensitive than acetoclastic methanogens to increases in VFAs concentration (Hori et al. 2006). This means that hydrogenotrophic methanogenesis was the main way of methane production, but the hydrogen production pathway was not driven by *Coprothermobacter* population, and likely other kind of syntrophic associations take place. The hydrogen supply seemed to be the key parameter affecting biogas production, rather than the population size of *Methanothermobacter*, as described in Morgan et al. (1997). The lack of acetoclastic methanogens indicated that acetate was neither cleaved nor oxidized by archaea, and therefore, under these conditions, at high acetate concentration, the most probable way of methane production is the syntrophic oxidation of acetate (SAO) to hydrogen by syntrophic acetate-oxidizing bacteria (not identified in this study), followed by hydrogen removal by *Methanothermobacter* (Karakashev et al. 2006; Ge et al. 2012). SAO is a key pathway at elevated temperatures (Ho et al. 2013). However, some studies have recently found that SAO became predominant in thermophilic or stressed environmental conditions (Hao et al. 2011), but microorganisms involved in this pathway are widely under investigations. As reported by Lü et al. (2014) also *Coprothermobacter* may operate SAO with consequent production of hydrogen, but cooperation with several bacterial species is required. Thus, population composition of start-up thermophilic biomass progressively changed, but strong variations in relative abundances of *Methanothermobacter* and *Coprothermobacter* revealed that microbial community was strongly affected by the overall operative conditions. The decrease of *Coprothermobacter*, together with the higher presence of other bacteria, was in line with the likely occurrence of SAO as methane production pathway. The identity of other bacteria needs to be further investigated to fully describe the methane production process occurring in the thermophilic stage of this dual stage system.

3.2 Biodiversity of mesophilic and thermophilic microbial communities

The relative abundance of each microbial component was utilized to estimate two different parameters commonly employed to describe the biodiversity of mixed microbial communities. Shannon-Weaver diversity index (H') and Pielou's evenness index (E) estimates with FISH data showed a decrease of biodiversity and evenness under thermophilic conditions (Table 3). H index

was lower than 0.7 under thermophilic conditions (compared with 1.3-1.4 estimated in mesophilic samples) and it was flanked by similar trend of the evenness. This finding indicated that thermophilic anaerobic biomass could be therefore more susceptible to sudden changes and less prompt to adapting to operative variations.

Table 3 - Shannon–Weaver (H) and Eveness (E) values calculated for the two digestion tests in dual stage system at the end of each digestion period.

	Mesophilic stage				Thermophilic stage			
	Untreated		Sonicated		Untreated		Sonicated	
	<i>Test 1</i>	<i>Test 2</i>	<i>Test 1</i>	<i>Test 2</i>	<i>Test 1</i>	<i>Test 2</i>	<i>Test 1</i>	<i>Test 2</i>
H	1.4	1.3	1.4	1.4	0.5	0.7	0.6	0.5
E	0.7	0.7	0.70	0.7	0.7	0.5	0.8	0.7

The strong impact of microbial biodiversity on the process evolvement was highlighted in the previous sections: during mesophilic stage, microbial population quickly changed in response to variations in HRT and OLR, switching from methane to VFA production (Fig.3 and Fig.4), with negligible variations of microbial composition and relative abundance.

On the other hand, during thermophilic stage, the start-up microbial population slowly evolved encountering operative variations, changing the way of fermentative hydrogen production.

Differences between mesophilic and thermophilic microbial communities in anaerobic digesters are largely reported (Ike et al., 2010; Shi et al., 2013; Pervin et al., 2013a; Zamanzadeh et al., 2013). A systematical analysis of 21 mesophilic and thermophilic full scale anaerobic digesters highlighted that bacterial and archaeal community composition was mainly related to the temperature of the process (Sundberg et al., 2013). Looking at the distribution of microbial population within *Archaea* and *Bacteria* domains, in the study of Nelson et al. (2011) a meta analysis of microbial diversity in several AD systems revealed that species richness of *Bacteria* is higher than *Archaea*. Generally, in studies based on in situ identification, archaeal relative abundance is lower than bacterial one (Ariesyady et al., 2007; Montero et al., 2009; Krakat et al., 2010). Indeed, hydrolytic and fermentative steps require the cooperation of different bacterial groups that degrade the wide range of soluble organics and convert them into end-products of fermentation. On the other hand, methanogens have a very complex metabolic system that comprise all of enzymes required for methane production, and they modulate this machinery in relation to substrate availability. Moreover, they have different growth kinetics with respect to *Bacteria*. For example, the

hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus*, did not grow when a very low H_2 was supplied, although methanogenesis continued and transcription of genes for methanogenesis was stimulated (Morgan et al., 1997); in this manner it can optimize H_2 utilization obtaining high yield of methane in H_2 limiting environments, without exponential growth. This means that the abundance or the number of components of *Methanothermobacter* population is not directly related to methane yield.

Under mesophilic conditions, acetotrophic methanogenesis is the main way of acetate production. Only the genera *Methanosarcina* and *Methanosaeta* are able to transform acetate into methane (Smith and Ingram Smith, 2007). Their growth kinetics are mainly related to acetate and proportional to its concentration. In low acetate environments, *Methanosaeta* prevails on *Methanosarcina* because of its higher affinity for the substrate (Berger et al., 2012), and vice versa. Nevertheless, their simultaneous presence can benefit the process due to the higher metabolic versatility of *Methanosarcina*, since it can use almost all substrates for methanogenesis, with the exception of formate (Braguglia et al., 2012). *Methanosarcina* and *Methanosaeta* were found also in thermophilic processes, but their activity is often limited because of acetate utilization is mostly exploited by fermentative bacteria during Syntrophic Acetate Oxidation (SAO). Indeed, the syntrophs of acetate oxidizing bacteria and their partner hydrogenotrophic methanogens are able to successfully outcompete the aceticlastic methanogens (Hao et al., 2011). In thermophilic conditions, in the absence of acetotrophic methanogens, microbial communities could still maintain a highly efficient and stable performance without acetate accumulation (Krakat et al., 2010). For this reason, acetate was converted via SAO, and the high hydrogenotrophic activity retrieved in several biogas reactors using acetate as carbon source supported this opinion (Hao et al., 2011). In this study, FISH analysis highlighted the presence of aceticlastic methanogens during the mesophilic stage, while *Methanothermobacter* population was the only species identified during thermophilic stage. As acetate was the main substrate during thermophilic stage in test 2, the original syntrophic association between *Methanothermobacter* and *Coprothermobacter* was replaced by a different hydrogenotrophic pathway, likely related to SAO.

4. Conclusions

Study of microbial populations during the innovative dual stage mesophilic/thermophilic anaerobic digestion indicated HRT of the mesophilic stage as crucial parameter to improve the performance of the following thermophilic stage. Shortening the HRT, a shift from methane to VFA production was observed, in particular by pretreating the sludge with ultrasounds. In thermophilic stage, substrate

composition and availability strongly influenced the composition of the microbial population. In particular, the proteolytic *Coprothermobacter* deriving from thermophilic inoculum drastically decreased, and other bacteria, likely involved in syntrophic acetate oxidation, took place. Species richness was lower under thermophilic conditions compared with the values estimated in mesophilic AD and it was flanked by similar trend of the evenness, indicating that thermophilic microbial communities may require a longer acclimation period before obtaining a stable microbial population being more susceptible to sudden changes and less prompt to adapting to operative variations.

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Chapter 7

Efficacy of of the methanogenic biomass acclimation in batch mesophilic anaerobic digestion of ultrasound pretreated waste activated sludge

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1. Introduction

Sludge digestion is the most common process for waste sludge treatment. The anaerobic mesophilic process (30-37°C) is the most widely used, mainly due to the low energy requirements and high stability of the process (Braguglia et al., 2014). However, the transformation efficiency of the sludge organic particulate matter into biogas is generally low. Renewed interest for anaerobic digestion is derived from the possibility of a significant performance improvement by applying an appropriate sludge pre-treatment, chemical, mechanical or thermal, leading to the breakage of flocs and cell walls and enhancing solids hydrolysis (Khanal et al., 2007). Among the different pretreatments, ultrasounds are widely applied (20-40 kHz) also at full scale for the mechanical disintegration of sludge flocs, as shown in chapter 4. Because of this, they have been examined as a treatment of sludges prior to mesophilic anaerobic digestion and have been shown to achieve improved gas production (Lafitte-Trouquè and Forster, 2002). Moreover, ultrasounds were shown to have an effect on sludge solubilisation and on biogas production during batch anaerobic digestion of sonicated sludge (Bougrier et al., 2005). Start-up is a critical step in the operation of anaerobic digestion, and it requires much attention to keep the system in good condition. Some researches focused on the start-up of anaerobic digestion revealed that the inoculum type is an important factor for a successful start-up (Kobayashi et al., 2009). From the viewpoint of microbial ecology, it is possible that the methanogenic archaeal community in the inoculum affects the evolution of the digester operation, as has been discussed in various reports (Griffin et al., 1998; McMahon et al., 2001, 2004; Kobayashi et al., 2009). In general, methanogenesis is an important and often rate-limiting step in the operation because methanogenic *Archaea* are strictly sensitive and slow growing. Thus, achieving formation of the active methanogenic community during process may depend on the archaeal community present into the inoculum. In this work, we carried out mesophilic batch anaerobic tests using two different inocula, in order to evaluate the effect of abundance and physiological conditions of inoculum archaeal cells on ultimate methane yields of sludge anaerobic process with untreated and sonicated sludge.

2. Materials and methods

2.1 Digesters operation

Sludge. Waste activated sludge (WAS) samples were obtained from the municipal “Roma-Nord” wastewater treatment plant, characterized by an organic load of about 700,000 p.e., high sludge age

(20 days) and a COD average value of incoming sewage of 200 mg/L. The activated sludges was sampled directly from the oxidation tank. Characteristics of raw activated sludges used for the two different digestion tests are reported in Table 1. WAS showed variations in terms of solid concentrations due to seasonal differences in wastewater properties and operations at the WWTP. These variations are the source of the deviation in the properties of feed sludge listed in Table 1, as also described in previous studies (Toreci et al., 2009; Wilén et al., 2008). Indeed, wastewater treatment plants based on the activated sludge process are subject to transient operating conditions (hydraulic retention, flow, etc.) and to seasonal changes in temperature. However, sudden changes affect the microbial activity which might affect flocculation and consequently sludge properties.

Table 1. Characteristics of raw activated sludges and inocula used for the two different digestion tests.

Activated sludges			Inocula		
	Test #1	Test #2		Test #1	Test #2
TS (mg/L)	36.3	14.5	TS (mg/L)	18.9	15.3
VS (mg/L)	18.2	11.7	VS (mg/L)	9.8	10.1
VS/TS (%)	50	80.9	VS/TS (%)	52	66
pH	7.05	6.9	pH	7.98	7.67
Soluble COD (mg/L)	51	15	Soluble COD (mg/L)	373	295

Ultrasound pretreatment. The disintegration by ultrasound was performed with an ultrasonic processor UP400S (dr. Hielscher, Germany) operating at 300 W and 24 kHz. Sonication energy input was set at 0.4 - 0.5 kWh kg⁻¹ dry solid on 500 mL of waste-activated sludge (2.9 - 4.7 % TS) placed in 1 L beaker with the probe allocated at 3 cm above the beaker bottom. The specific energy input is a function of ultrasonic power, ultrasonic duration, and volume of sonicated sludge and TS concentration, and can be calculated following the equation reported elsewhere (Gallipoli et al., 2014).

Anaerobic digestion batch tests. The experimental apparatus is shown in Figure 1. Experiments were carried out on bench scale anaerobic reactors of 0.4 L (working volume) that were operated in batch mode, immersed in a temperature controlled, agitated water bath ($\approx$ 140 rpm) at 37°C. The reactors were fed with a mixture of WAS, either untreated or sonicated, and raw anaerobic inoculum, at different ratio. Two different digestion tests were carried out, with two different type of anaerobic inocula. In test 1, the inoculum was taken from a semi-continuous mesophilic reactor in acidogenic conditions (described in Chapter 6). In test 2, the inoculum was an anaerobic sludge after 35 days of mesophilic batch acclimatation. Characteristics of the two inocula are reported in

Table 1. The digestion period was 20 days, and the pH variation during this period was in the range 7.5- 7.7. The produced biogas was collected by gas impermeable tubes (Tygon R3603, Cole-Parmer, Illinois , USA) connected to flask's septa by drip valves, into 50 mL syringes. The biogas volume was read daily. The methane content in the biogas was measured using a PerkinElmer Auto System Gas Chromatographer equipped with a thermal conductivity detector (TCD) as described in Gianico et al. (2013).



Figure 1 - Experimental configuration for batch anaerobic digestion

At regular time intervals (after 2, 6, 9, 14 and 20 digestion days) one batch reactor containing untreated and another one containing sonicated sludge were stopped, opened and the sludge was analyzed.

The pretreated sludges used as feed for the anaerobic digestion tests were sonicated with a specific energy of 12400 kJ/kg TS for test 1 (corresponding to a DD_{COD} of about 11.5%), and of 4950 kJ/kg TS for the test 2 (corresponding to a DD_{COD} of about 7.2%). The sCOD value of fed sonicated sludge used as feed for both test was about 2000 mg/L.

Matter composition. Total and volatile solids (TS and VS) were determined in triplicates according to standard methods (APHA, 1998). The pH was measured by a pH-meter (Eutech pH700). To analyze the soluble phase, the particulate sludge matter was removed by centrifugation (10 min at 5000 rpm), and the resulting centrate was filtrated through 0.45 μ m pore size membrane filters. Soluble COD (sCOD) and soluble nitrogen were determined by Cell Test Spectroquant (Merck) as described in Gianico et al. (2013). To analyze colloidal phase, sludge aliquots were filtered through glass filters with 1.2 μ m pores (GF/C Whatman); the supernatant was used for

protein and carbohydrates determination. Protein and carbohydrates content were determined by colorimetric BCA and Dubois methods, as described in Braguglia et al. (2012). Acetate levels were measured on sludge samples filtrated through 0.2 µm pore size membrane filters, by ionic chromatography using a Dionex DX-100 chromatograph equipped with AS 14 4-mm (Dionex) column for the detection of anionic compounds.

2.2 Microbial community analysis

Sample collection. Effluent sludge samples were periodically collected from both reactors during start-up and at steady state operating conditions. Aliquots of 1.5 mL of mixed liquor were either immediately frozen at -20°C for further DNA extraction or fixed with paraformaldehyde and ethanol for FISH analysis as described in Amann *et al.* (1990).

Fluorescence in situ hybridization (FISH). FISH was performed on fixed sludge samples as previously described (Braguglia *et al.* 2102). Oligonucleotide probes specific for *Archaea* domain (ARC915 probe), *Methanosarcinales* (MXMX860), *Methanosaetaceae* (MX825 a, b, and c), *Methanosarcinaceae* (MS821), *Methanococcales* (MC504), *Methanomicrobiales* (MG1200b) and *Methanobacteriales* (MB311) were used. Details of probes are available at probeBase (Loy et al., 2007). Samples were examined by epifluorescence microscopy (Olympus BX51). Fluorescence signal was quantified on microscopic images taken from the samples with a digital camera (Olympus XM-10) and the software Cell F. DAPI (4',6-diamidino-2-phenylindole) fluorescent staining was also simultaneously performed for determining total cell numbers. Area measurements of the hybridized cells were reported as a portion of the area covered by total DAPI stained cells in each field. Area measurements were performed on at least 10 different JPEG images (or other image format with 8 bit size of 1388x1040 pixels) using ImageJ software package (version1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA, available in the public domain at <http://rsb.info.nih.gov/ij/index.html>) as described in Braguglia et al. (2012).

3.Results and discussion

3.1 Anaerobic digestion performances

Looking at cumulative methane production during test 1 (Fig.2 a) the overall production was higher with respect to test 2 (Fig.2 b). However, the positive effect of sonication on the process performances was highlighted only for test 2, with a +14% gain of methane (Fig.2 b).

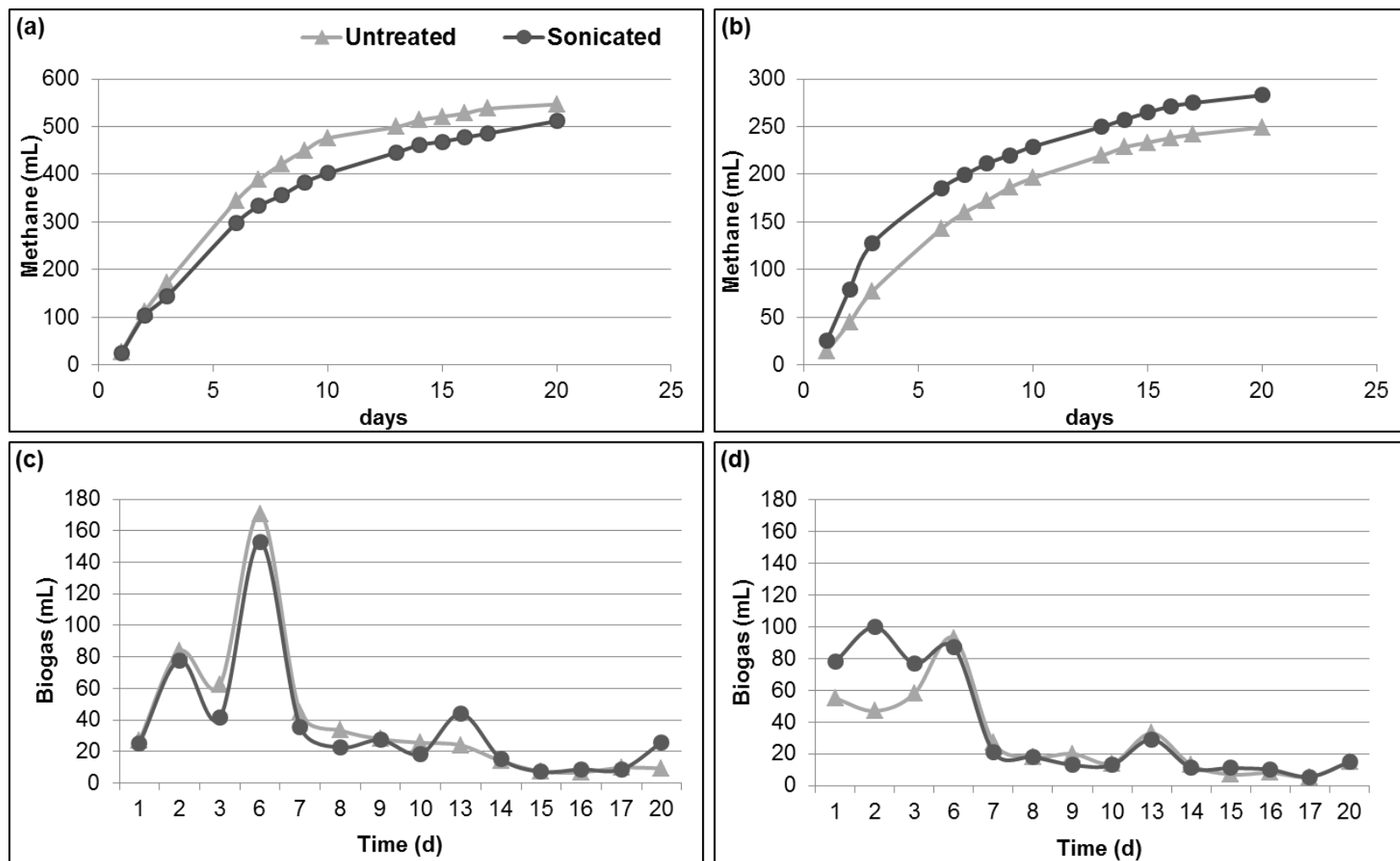


Figure 2 - Cumulative methane production for test 1 (a) and test 2 (b) and daily biogas production for test 1 (c) and test 2 (d).

This issue was underlined also looking at the daily biogas production, reported in figure 1 c and d. During test 2 the reactors fed with sonicated sludge produced more biogas than the untreated one in the first days of digestion, which corresponded to the hydrolysis phase. So, the original aim of the ultrasound pretreatment was encountered only in the digestion fed with a lower organic load (Tab. 2). The rate of conversion of organic matter was evaluated by means of VS destruction and specific methane production, reported in in Figure 3.

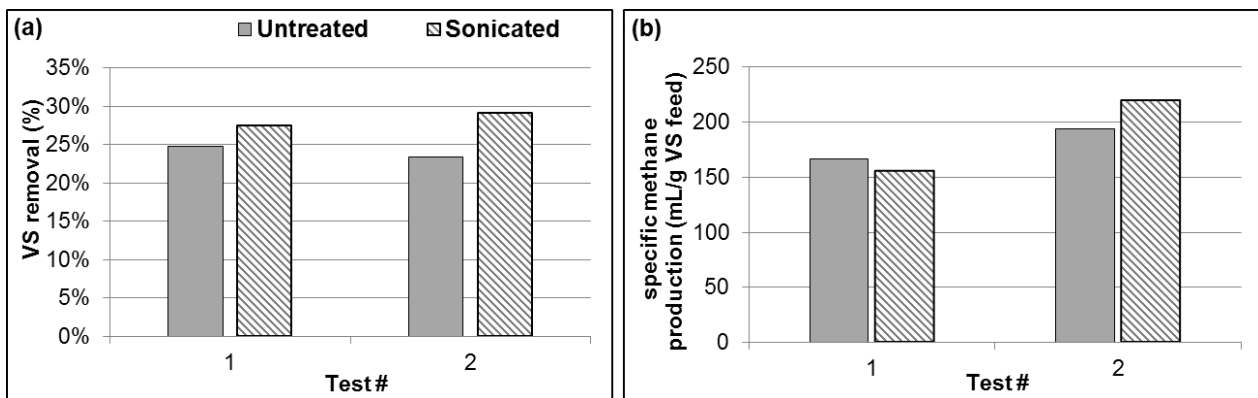


Figure 3 - Conversion rate of organic matter during both test was evaluated by VS removal (a) and specific methane production (b).

VS removal (Fig.3 a) was higher for the reactors fed with sonicated sludge, in particular for test 2 confirming previous data. This result is associated with sCOD trends in the reactors during the anaerobic process (Tab.2). During the pretreated sludge digestion a significant removal of the soluble substances, previously released because of the disintegration pretreatment, occurred while during the untreated sludge digestion no removal was observed, and sCOD increased due to hydrolysis. The same trend was observed for EPS components, protein and carbohydrates (Tab.2). Comparing the conversion rate of VS fed into methane (Fig.3 b), best performance was evidenced by test 2, with a relevant positive effect of sonication, too.

During test 2, the percentage of methane with respect to the total biogas composition was around 75%, while during test 1 was significantly lower, around 60%.

Since aceticlastic methanogenesis is the main pathway of methane production in mesophilic conditions, acetate concentration was monitored, too. As seen in Figure 3 a and b, there was an increase in the second day for both tests, in correspondence with hydrolysis phase, confirmed also by the increase of sCOD (Tab.2). As expected, acetate concentration the 2nd day was always higher for the reactors fed with sonicated sludge. After this peak, the amount of acetate became negligible (Fig.4 b) in the case of test 2, while during the digestion of test 1 persisted

Table 2 - Characterization of sludges and anaerobic supernatants during both tests, along time.

Test 1 (VS fed 3.3 g)										
<i>days</i>										
	TS (g/L)		VS (g/L)		sCOD(mg/L)		Proteins (mg/L)		Carbohydrates (mg/L)	
	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>
0	26.8	27.4	13.7	13.8	269	1235	269.8	732.3	34.3	105.2
2	25.5	24.8	12.8	12.2	1018	1575	458.5	662.3	39.7	76.8
6	25.2	24.3	12	11.1	313	734	329.8	534.8	37.5	57.0
9	23.9	23.5	10.7	10.4	316	357	284.8	499.8	31.6	51.9
14	23.6	23.1	10.4	10	315	355	309.8	349.8	30.8	43.0
20	23.5	23.2	10.3	10	300	319	329.1	384.1	32.6	42.4
Test 2 (VS fed 1.3 g)										
<i>days</i>										
	TS (g/L)		VS (g/L)		sCOD(mg/L)		Proteins (mg/L)		Carbohydrates (mg/L)	
	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>	<i>Untreated</i>	<i>Sonicated</i>
0	14.9	15.4	10.7	11.3	301	717	216.4	549.3	27.9	71.3
2	14.3	14	9.3	9.1	338	210	273.6	349.3	34.3	41.3
6	14	13.5	8.7	8.3	290	412	267.9	412.1	28.7	31.7
9	13.9	13.5	8.5	8.3	294	278	252.3	232.3	25.7	32.6
14	13.6	13.1	8.4	8	271	430	295.7	254.6	32.6	29.3
20	13.1	14.2	8.2	8	390	572	239.3	271.6	26.9	33.7

until the end of the digestion (around 70 mg/L, Fig.4 a). The persistence of acetate in batch and aceticlastic conditions, may indicate a low rate of methanogenesis. On the other hand, in test 2 the complete conversion of acetate into methane was observed, a first indication of a better methanogenic community in this system, as seen in the next section. The biogas gain for the reactor fed with sonicated sludge could be easily explained by the higher acetate present in the system (95 mg/L vs 49 mg/L), due to the high hydrolyzed organic matter released after mechanical pretreatment step and fed into the system. The possible reason for these differences in digestion performances could be mainly due to the different inocula used to start-up the two test. Indeed, in test 1, the inoculum was taken from a semi-continuous mesophilic reactor in acidogenic conditions, while in test 2, the inoculum was anaerobic sludge after 35 days of mesophilic batch acclimation. These observation underscored the importance of an acclimation step for methanogenic community, as seen in next section.

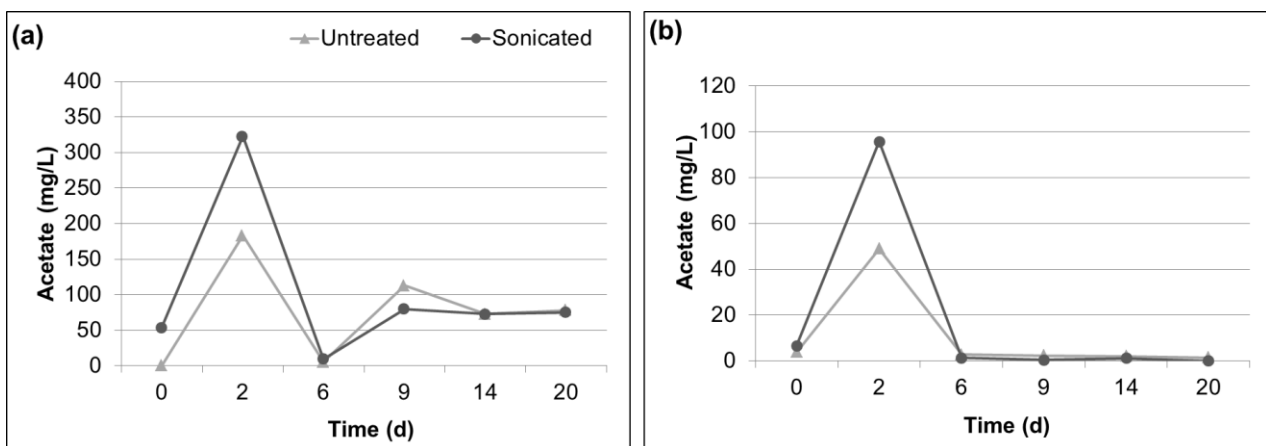


Figure 4 - Acetate concentration trends during test 1 (a) and test 2 (b).

3.2 Archaeal community analysis

Several studies revealed the importance of the methanogenic community composition of the biomass utilized for the start-up of anaerobic digesters (McMahon et al., 2004; Lee et al., 2008; Ghanimeh et al., 2013). In this study, the composition of the anaerobic inoculum as well as the archaeal population dynamics were evaluated by FISH during the reactor operation. The amount of total *Archaea* out of total DAPI stained cells in untreated and sonicated sludge samples is shown in Figure 5. The starting relative abundance of archaea (Fig.5 a) was lower in test 1 with respect to test 2 (Fig.5 b). As described in Stroot et al. (2001), the progress of the anaerobic digestion is strictly dependent on the abundance of the methanogenic microorganisms initially present. In test 1, the relative abundances of archaea ranged from 10 to 6% and showed a quite stable trend during the

process. The same stability was also observed during test 2, with an oscillation in relative abundances from 25% to 18-20% of methanogens out of total cells.

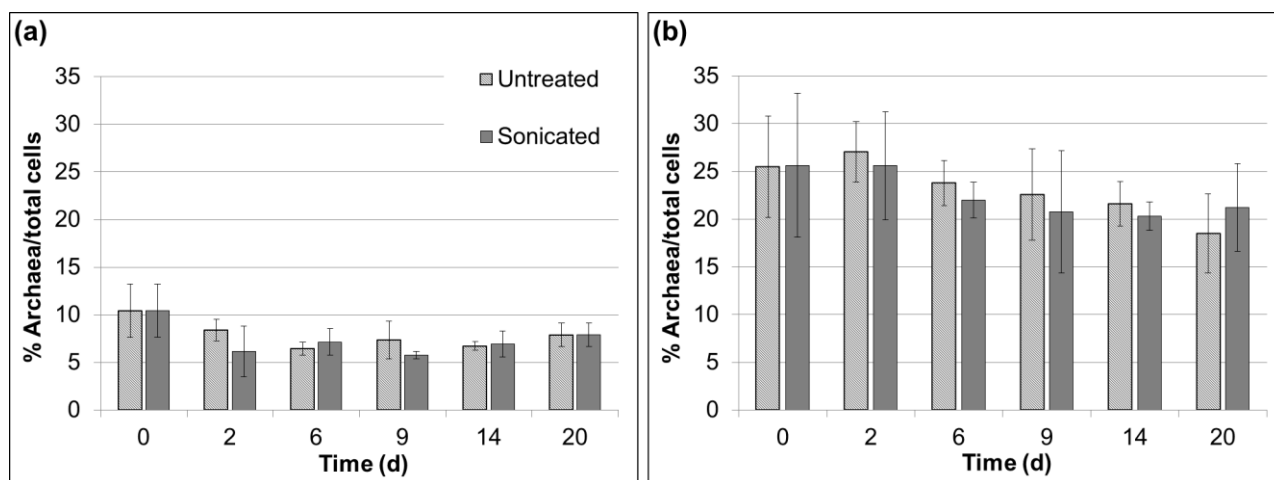


Figure 5 - Amount of total Archaea out of total DAPI stained cells in test 1 (a) and test 2 (b) for untreated and sonicated sludge samples during reactor operation.

Therefore, as archaeal populations in both reactors were similar, the higher biogas production observed for the reactor fed with sonicated sludge in test 2 can be explained by the higher available substrate, as discussed previously.

Overall, FISH analysis explained the worst performances of the system during test 1 despite the higher organic load fed into the system. These data highlighted that the original features of the inoculum are important for the reactor performances, especially in batch conditions, in which there is a short time for acclimation. It should be emphasized that the start-up inoculum of test 1 was sampled from a semi-continuous reactor maintained under acidogenic conditions (Gagliano et al., 2014), instead the inoculum of test 2 was a mixture of a mesophilic anaerobic sludge and WAS subjected to 35 days of acclimation with the same conditions used for the two digestion tests described in this study. Importance of acclimation was also explained by the observation of morphologies of *Archaea* highlighted with specific hybridizations using the readily available FISH probes targeting members of the different archaeal orders (Fig.6). Archaeal cells identified in test 1 belonged to *Methanosaeta* occurring as single rods (identified with MX825 mix probes) together with few unidentified coccoid cells (Fig.6 a). On the other hand, in test 2 filamentous *Methanosaeta* (Fig.6 b) and *Methanosarcina* occurring as coccoid clusters (Fig.6 c) were found (identified with MSMX860 probe). Only the genera *Methanosarcina* and *Methanosaeta* are able to transform acetate into methane (Smith and Ingram Smith 2007). These two genera were found to be the dominant aceticlastic methanogens in a variety of anaerobic reactors (Vavilin et al., 2008), mainly under methanogenic conditions, as reviewed in Demirel and Scherer (2008).

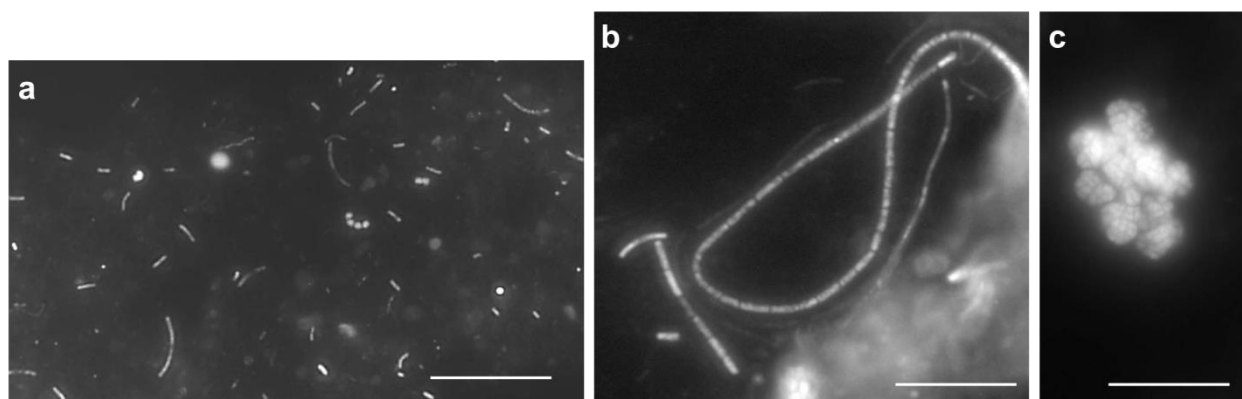


Figure 6 - Archeal cells highlighted with ARC915 probe in test 1 (a) and test 2 (b and c). In a), only *Methanosaeta* single rods and unidentified coccoid cells were highlighted. In b) and c), filaments of *Methanosaeta* and *Methanosarcina* coccoid clusters were shown. Bars are 10 μm .

Methanosaeta is a specialist in using acetate and grows only at low acetate concentrations. *Methanosarcina* is certainly the most versatile known methanogen, since it can use almost all substrates for methanogenesis, with the exception of formate (Roest, 2007; Thauer et al., 2008). Despite different kinetic peculiarities, their simultaneous presence can benefit the process (Braguglia et al., 2012; Ros et al., 2013). These two microorganisms benefit by cells aggregation and are characterized by increased methanogenic activity.

Methanogens grow much-slower than acidogenic bacteria, and unbalance in the process in favour of acidogens can lead to acidification, which can in turn inhibits methanogens. However, methanogens require aggregation to protect them from rapid acidogenesis (Vavilin and Angelidaki, 2005).

Vavilin et al. (2008) have shown that *Methanosarcina* sp. shaped multicellular aggregates containing about 500 cells. Aggregates may resist to inhibition of various compounds (especially VFA and H^+), because of the decreasing of potential inhibiting compounds inside the aggregate. Moreover, K_s for a microbial aggregate is proportional to the aggregate's square of radius (Vavilin et al., 2008). Concerning *Methanosaeta*, Ma et al. (2006) demonstrated that it is rod-shaped, non-motile, and usually found singly or in pairs, but when grown on acetate with high cell density, the cells form long filaments. Zhang et al. (2012), highlighted that filaments were generated through a quorum sensing pathway. The filamentous form can better compete with other microorganisms for resources in the environment, such as for acetate, and as well they can divide quicker due to faster metabolism and the lower biomass required, and outgrow any competitors (Zhang et al., 2012). Vavilin and Angelidaki (2005) described that archaeal aggregates made initial methanogenic centers, which dissipation negatively affected methane production. However, worse organic conversion rate and the persistence of acetate during test 1 is likely because of the presence of the sole rod-shaped *Methanosaeta* in archaeal biomass, whose cell density was too low to promote

aggregation. On the other hand, probably the long acclimation step carried out for the inoculum of test 2 allowed to the simultaneous presence of both aggregated *Methanosaeta* and *Methanosarcina*, resulting in better conversion of organics into methane.

These observations showed the importance of inoculum acclimation before digestion and highlighted primary presence of aggregated *Methanosarcina* in the context of mesophilic anaerobic digestion to obtain a good methane yield, which appear to be of crucial importance (Braguglia et al., 2012; De Vrieze et al., 2012).

4. Conclusions

Differences in microbial composition of the startup inocula strongly influenced the overall performances of the anaerobic digestion processes. The presence of well-acclimated archaeal community resulted in higher methane yield. The simultaneous presence of *Methanosarcina* and *Methanosaeta* in the archaeal biomass, the higher initial archaeal cells relative abundance and their occurrence in the aggregated forms were the main factors positively affecting the conversion of organic matter into methane. The better physiological state of methanogens permitted to appreciate the effect of hydrolysis improvement by ultrasounds pretreatment. These results revealed the importance of the composition of methanogenic community in the inoculum and the influence of physiological state of archaeal cells, which should be used as “biological marker” to monitor methanogenic potential of the mesophilic anaerobic inocula.

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Concluding remarks

Overall, the study allowed to better understand the performances of three advanced sludge stabilization systems, highlighting the key role of the elucidation of the microbial structure and metabolic potentialities to better operating biological processes like anaerobic digestion of activated sludge.

- The investigated sludge pretreatments, ultrasounds and thermal hydrolysis, had an overall positive effect on AD performances in all the lab-scale experiments.
- Thermophilic anaerobic digestion tests showed the feasible establishment of a well-studied syntrophic association driven by electron- transfer through hydrogen. The process was mainly driven by syntrophic protein fermentation due to high protein hydrolysis, related both to the high temperature of the process and the thermal pretreatment.
- Study of microbial populations during the innovative two-stage mesophilic/thermophilic process showed that thermophilic digestion was mainly driven by syntrophic acetate oxidation, due to high acetate availability, directly related to the performances of the mesophilic stage.
- Mesophilic batch digestion tests with untreated and sonicated sludge showed the impact of microbial composition of the inoculum on biogas production. Acetate conversion into methane was improved in the system with an acclimated microbial community, whose physiological conditions are crucial for the methane yield.

Finally, the microbiological results highlighted that FISH analysis still represents an indispensable molecular screening tool due to the unique ability i) to identify the metabolically active cells and their dynamics in relation to operational parameters during the digestion process, and ii) to highlight possible changes in cell morphology and physiology that can be very useful for the understanding of the entire system.

Future recommendations and research needs

Agricultural use of sewage sludge on land depends largely on the possibility to improve the sludge stabilization as well as to increase public confidence about sludge quality and safety. This implies the prevention of wastewater pollution at the source, e.g. by reducing the possibilities for heavy metals and organic compounds to enter wastewater treatment plants (WWTPs), and improving sludge treatment.

Among sludge treatment options, anaerobic digestion has been recognized to be the most appropriate stabilization technology to approach the problem of sludge reuse, also because of the added value of methane production.

In this prospect, future activity will be addressed to optimize new sludge treatments with the aim to preparing sludge for agricultural utilization by producing a very clean and stabilized sludge with specific concern to hygienic aspects and properties that can have impact on soil and crops.

The understanding and prediction of microbial community structure – function relationships is still challenging and needs integrated research efforts for resolution. Molecular ecology and physiological data can be obtained together with newly developed technologies, and the combination of classical and modern microbiological methods will definitely improve ecophysiological knowledge of sludge treatment and other environmental systems.

Many challenges were associated to the microbiological analysis of anaerobic microorganisms, in particular thermophilic ones. Drawbacks related to the in situ identification of thermophilic microorganisms and the successful strategies applied to solve them, suggested the need to a cautious characterization approach in analyzing such complex environments.

Only the cross-use of in situ detection methods and PCR-based approaches permit to describe the biodiversity of the biomass involved in the anaerobic digestion process, highlighting the need of multiple lines of evidence when the structure and the function of complex environments are analysed.

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