3. Introduction

Anaerobic digestion – General notes

The European Commission is pushing the relevance of the energy household and usage of renewable sources with the strategy 'Energy 2020' into public´s spotlight (Anonymous, 2011a). Until 2020 each member state of the European Union is committed to reduce greenhouse gas emissions by 20% and to share 20% of the energy from renewable sources, which is a step towards the aims for 2050 (Anonymous, 2011b). Furthermore, 5% of the total energy budget should be derived from biogas production (De Vrieze et al., 2012). In Austria, the biogenic waste quantities from households increased between 1999 and 2009 by 57% to above 0.75 million t a^{-1}, however, this value is still low compared to the minimum capacity of 0.86 million t a^{-1} for biogas plants reported for 2010 (Anonymous, 2006, 2011c).To fulfil the prospective aims, the unused potential of the anaerobic waste treatment and the potential for overall process performance improvements should be considered.

During the last decades the anaerobic digestion has gained more and more popularity as an economic and ecological reasonable way for treating waste. This is also confirmed by the remarkably increased number of publications within this field (Fig. 1). In addition to the common keywords, which are generally used for literature research, '*Methanosarcina*' was added because it represents an important genus of methanogens. Their members are known to be all-round talents due to a high number of utilizable substrates and the potential to withstand perturbed conditions compared to other methanogens, like for example *Methanosaeta* spp. (De Vrieze et al., 2012). Furthermore, *Methanosarcina* spp. play a significant role in the present Doctoral Thesis.

The main aims of the anaerobic treatment are i) to significantly decrease the volume of the organic waste, ii) to reach a well-balanced process where neither products (intermediates) nor their reactants are able to accumulate, and iii) to keep the methane (CH_4) production rate as high as possible. The anaerobic digestion became more than just an alternative to composting, however, despite its advantages, it also has some drawbacks including low process stability (Illmer et al., 2009), and high maintenance costs of digestion plants, which can be relatively complex (Mata-Alvarez et al., 2000).

Although in the last decades good scientific research was performed and knowledge is significantly increasing, the anaerobic digestion is often called a 'black box' (Riviere et al., 2009; Supaphol et al., 2011) where insights into the microbial communities and key players are still incomplete. A step forward is to increase the knowledge about the trophic levels,

which might include up to several hundreds of different microorganisms. Thereof, a big stake might not be detectable or culturable, or even in a viable but nonculturable state. Nevertheless, as stable as a well-performing process might seem, as vulnerable it is if deteriorated conditions prevail. They potentially lead to an accumulation of intermediates if even a single key organism is inhibited, suppressed or washed out. The result is a suboptimal process or even a complete digestion failure with an accumulation of intermediates and a cessation of the CH_4 production.

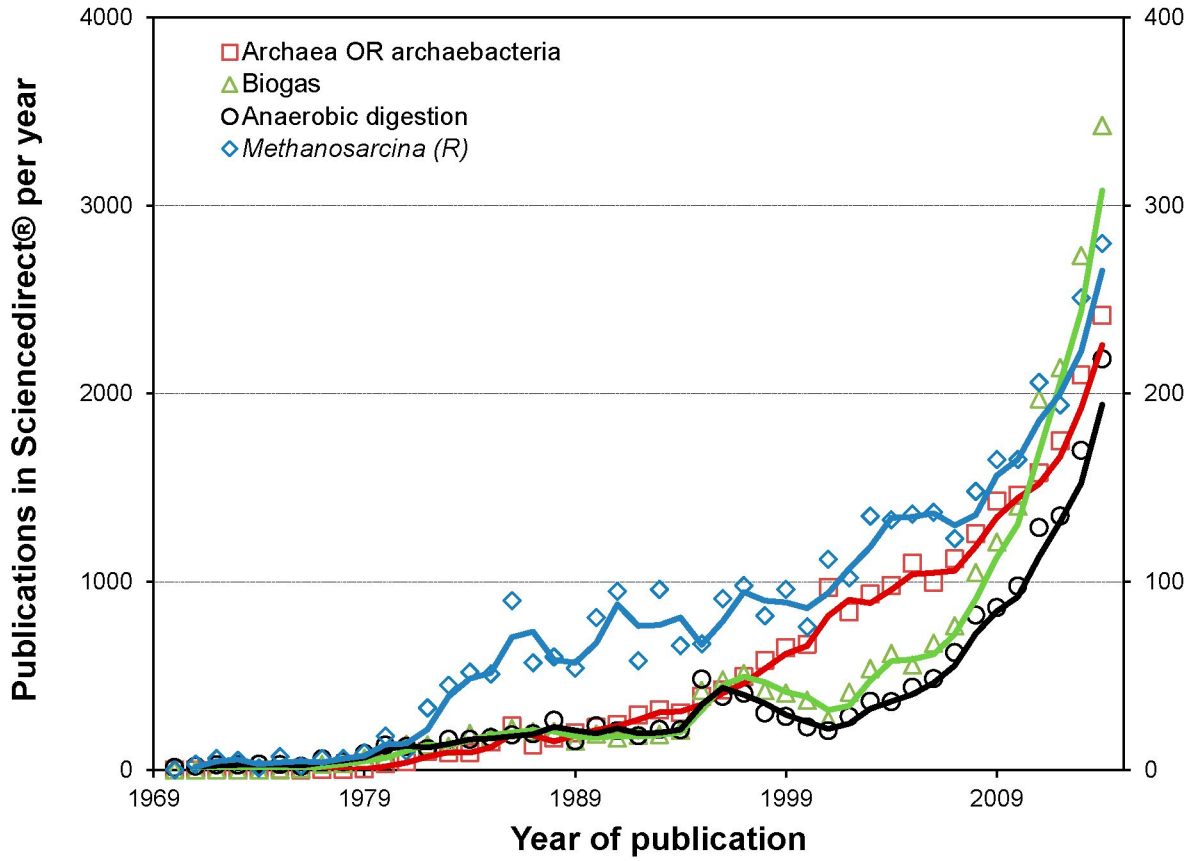

Figure 1: Numbers of annual publications found in Sciencedirect® (www.sciencedirect.com, accessed at 23.02.2015) for the keywords: Archaea OR archaebacteria, biogas, anaerobic digestion, and *Methanosarcina* (right y-axis).

The complete anaerobic degradation of organic matter to CH_4 and carbon dioxide (CO_2) requires a complex cascade of microorganisms (Fig. 2). Generally, four degradation steps are discriminated: (i) extracellular enzymatic breakdown (hydrolysis), (ii) fermentation of large organic molecules to organic acids, (iii) degradation of these fermentation intermediates to acetate, hydrogen (H_2) and CO_2, and subsequently as a final step (iv) acetoclastic (out of acetate) and hydrogenotrophic (out of H_2/CO_2 or C_1-methylated compounds) methanogenesis (Schink & Stams, 2006). It is assumed that methanogenesis from acetate is

responsible for more than two-thirds of the total CH_4 production in anaerobic digestion (Conrad, 1999), either directly via acetoclastic methanogenesis or indirectly via syntrophic acetate oxidation (SAO) followed by hydrogentrophic methanogenesis. During the last years the SAO was investigated in more detail, and it is emphasized that SAO might even be the main methanogenic pathway during anaerobic digestion (Hao et al., 2011; Hattori, 2008; Karakashev et al., 2006; Sun et al., 2014).

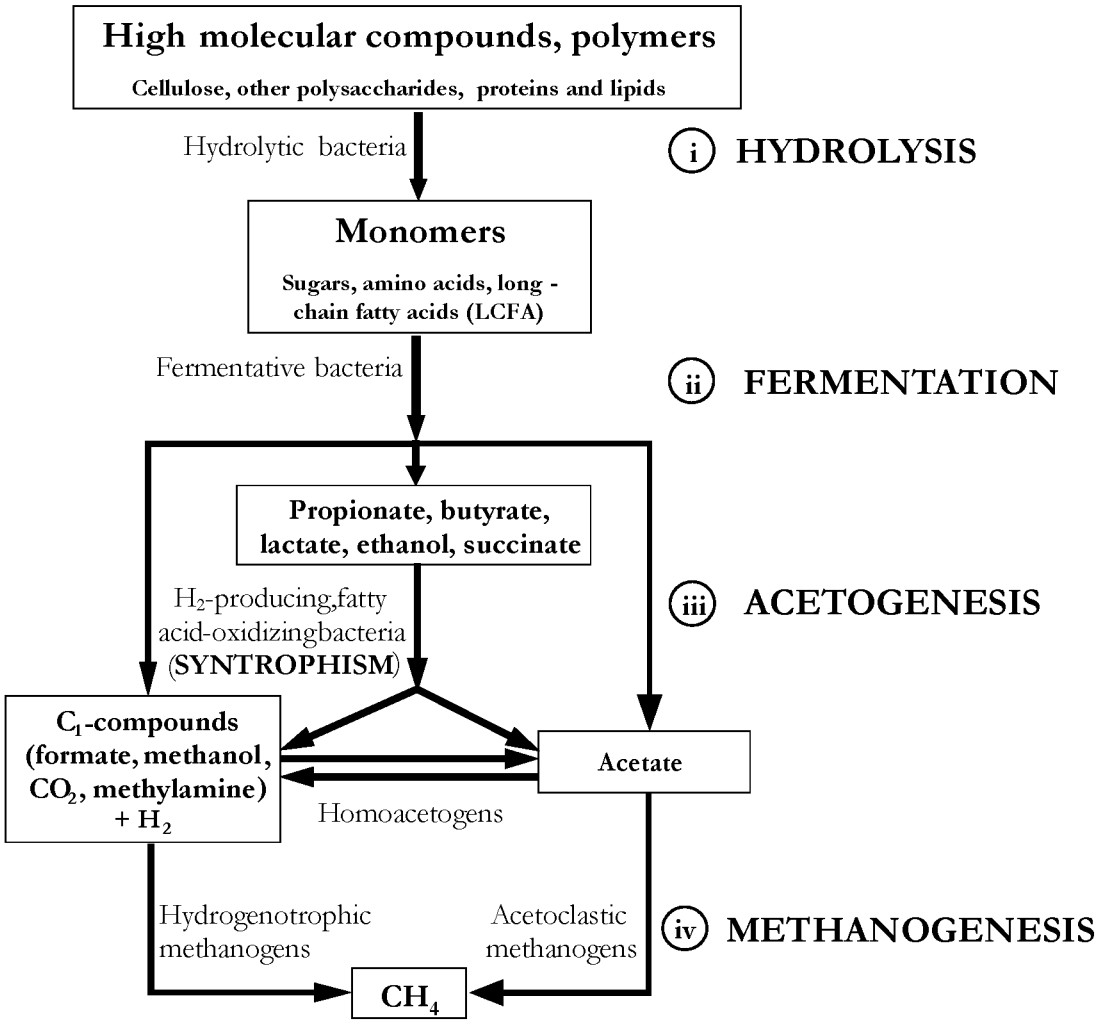

Figure 2: Trophic degradation cascade during anaerobic digestion of organic matter (modified after Conrad (1999) and Schink (1997)).

Abiotic factors affecting the anaerobic digestion

In general, the concentration of **volatile fatty acids (VFAs)** is among approved process parameters (Ahring et al., 1995; Boe et al., 2010; Pind et al., 2003) and should be monitored and controlled during anaerobic digestion. Among them are for example acetate and propionate. Many investigations have shown that an accumulation of VFAs is usually accompanied by a drop in pH (Illmer et al., 2009). This results in an increase of the

undissociated VFA species, which are well known to be easily membrane permeable and cause significant intracellular effects (Kadam & Boone, 1996; Lay et al., 1997).

Although several investigations on formate and H_2 regarding their solubility, diffusibility and role as intermediates have already been published in the 80s and 90s (Boone et al., 1989; Goodwin et al., 1991; Schauer et al., 1982; Stams, 1994; Thiele & Zeikus, 1988), the importance of formate is sometimes neglected, whereas **H_2** has become a common process monitoring tool in anaerobic digestion systems (Huang et al., 2000; Lins et al., 2012a). In contrast, Boe et al. (2010) investigated several state indicators during the anaerobic digestion process where the dissolved and gaseous H_2 concentration was not an appropriate indicator parameter. In their investigation the concentration of dissolved H_2 was also affected by disturbances not influencing the process performance, and the determined gas composition lacked of sensitivity for a fast response on starting instabilities. This discrepancy within the scientific literature points to the necessity for further investigations.

In digesters where the substrate consists of protein- and nitrogen-rich content, the level of **ammonia (NH_3) and/or ammonium (NH_4^+)** might be of special interest because at high concentrations they might show toxic effects (Illmer et al., 2009; Lins & Illmer, 2012; Wagner et al., 2012). It is well known that the thermophilic anaerobic digestion has a higher potential for biogas production compared to mesophilic conditions, for example due to increased conversion rates and lower generation times of the engaged microorganisms (Ho et al., 2013).

However, with increasing the temperature, the process gets more vulnerable to stress conditions like toxic concentrations of the freely diffusible species of ammonia and VFAs (Bayr et al., 2012). Especially during thermophilic digestion NH_3 might reach higher concentrations because with a rise in temperature and pH the level of undissociated NH_3 will increase (Scherer, 2001) (Fig 3.).

To conclude, an adequate monitoring is crucial because an accumulation of a single intermediate might hamper the complete degradation cascade and should therefore be detected as soon as possible.

Apart from the above mentioned process parameters, the mode of **stirring and agitation** is an important tool, which affects the complete anaerobic digestion, and attracts civil and process engineers as well as applied microbiologists. Generally, it is accepted that gentle or intermittent mixing is beneficial for the process compared to vigorous or continuous mixing, which might inhibit interactions of syntrophic consortia (Kaparaju et al., 2008; Lindmark et al., 2014). The effect of hydrodynamic shear forces as well as other forces, which microbial communities have to compete with for cell immobilization, is reviewed by Liu & Tay (2002).

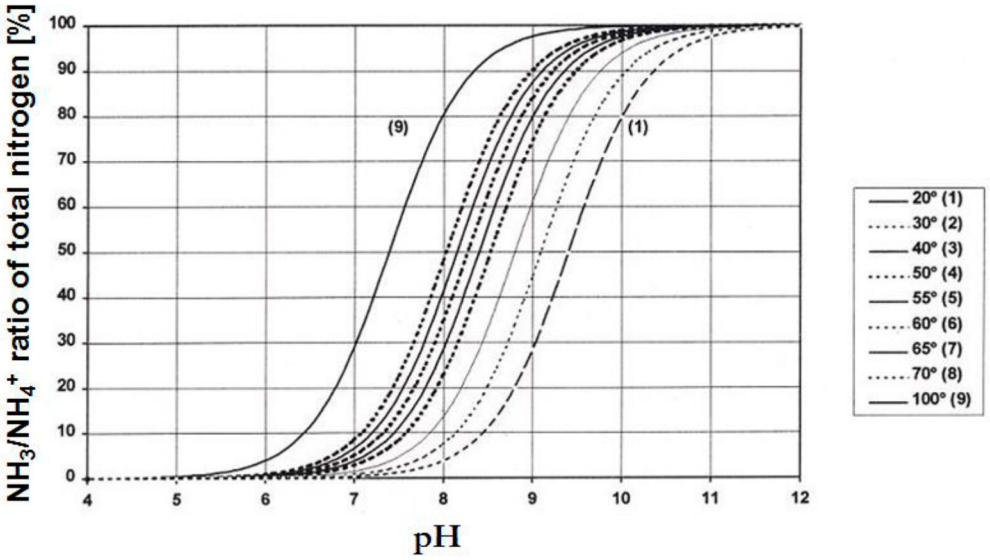

Figure 3: Dependence of the NH_3/NH_4^+ ratio on the pH value and temperature (modified after Scherer (2001)).

However, there are also investigations where a non-agitated status of the process was beneficial (Tian, et al., 2013). With increasing the mixing or agitation speed the hydrolysis rate of the polymeric substrates is improved (Yuan et al., 2011), which might pose a problem if the VFA degradation with subsequent methanogenesis cannot catch up. For further details regarding the effects of mixing in anaerobic digestion it is referred to the review of Lindmark et al. (2014).

There are also some specific factors, which demands attention: For example chemical compounds, pathogens, and yet unknown substances present in waste streams. **Antibiotics**, as potential contaminants are also frequently found in slurry and manure of livestock animals and industrial wastewater, which concern the public health (Hirsch et al., 1999; Homem & Santos, 2011; Joy et al., 2014; Pan et al., 2011). They are among the most dangerous compounds and are posing increasing problems due to the increased misuse and overuse in human as well as veterinary medicine (Schwarz et al., 2013; USFDA, 2012; Wise, 2002). This inevitably leads to an accumulation of antibiotics and their metabolites in the environment because most antibiotics are not completely and sometimes even scarcely degraded, and can persist up to several months to years (Álvarez et al., 2010; Jechalke et al., 2014; Jjemba, 2002). The accumulation of antibiotics in different wastes, which are subsequently used as input material for anaerobic digestion, might lead to detrimental effects of the overall performance if relevant and significant microorganisms are inhibited or suppressed (Aydin et al., 2015; Cetecioglu et al., 2013; Cetecioglu et al., 2012; Ke et al. 2014; Sanz et al., 1996). The recovery attempts afterwards bring along heavy economic and

ecological losses. Antibiotics are often overlooked or underestimated in the environment, and their influence on the methanization process has received justified increasing attention (Beneragama et al., 2013; Du et al., 2015; Lins et al., 2015; Mitchell et al., 2013; Shi et al., 2011). However, Poels et al. (1984) stated that under real-world scenarios a significant reduction in biogas production or disturbance of the process stability will be unlikely. To this end, the controversially discussed literature points out the need for increasing research.

The methanogenic inhibitor **2-bromoethanesulfonate (BES)** was discovered in the 1970s as a structural analogue of coenzyme M (Taylor & Wolfe, 1974), and from the end of this decade it was specifically used as a potent inhibitor of the terminal reductive step during formation of CH_4 (Wolfe & Higgins, 1979). After the establishment of adequate radio-tracer methods, BES was used for example for characterization of methanogens (Krzycki et al., 1985). Although plenty studies have been performed with the application of BES, questions regarding its specificity, its direct effects beside its indirect ones by influencing syntrophic partners, and its fate during anaerobic digestion are still open yet.

To complete this, there are several other causes for digestion failures and decreased process performances (cf. Chen et al. (2008), Illmer & Gstraunthaler (2009) and Illmer et al. (2009)).

Biotic factors affecting the anaerobic digestion

An overview of the most relevant **methanogens** with respect to anaerobic digestion is provided by Tab. 1. For completeness, the order Methanocellales, located within the class Methanomicrobia, needs to be mentioned, which contains mesophilic rods utilizing H_2/CO_2 and formate for methanogenesis and growth (Sakai et al., 2008). Recently, the seventh methanogenic order 'Methanomassiliicoccales' was proposed, which consists of methylotrophic methanogens (Borrel et al., 2013; Castelle et al. 2015). All orders are placed within the phylum Euryarchaeota of the domain Archaea. However, due to an increase in sophisticated phylogenetic and phylogenomic techniques and new insights, the up-to-date taxonomic trees might have only a limited period of validity (Forterre, 2015; Petitjean et al., 2015).

Acetate is converted either via the acetoclastic pathway or via the SAO to formate or H_2/CO_2 with subsequent conversion to CH_4 (Tabatabaei et al., 2010). Methanogens capable to utilize acetate via the acetoclastic pathway belong to the genus *Methanosaeta* or *Methanosarcina* (Fig. 4), whereof especially the latter is prominent in anaerobic digestion due to high acetate turnover rates and high resistance towards deteriorating conditions. Thus, *Methanosarcina* spp. are even stated to be responsible for heavy duty biomethanation during anaerobic digestion (De Vrieze et al., 2012).

Table 1: Taxonomic list and basal characteristics of the most relevant methanogenic archaea (according to Hedderich & Whitman (2006)). Substrates in brackets do not apply to all assigned species. $MeNH_2$, methylamine (Mono-, di- and trimethylamine); DMS, dimethylsulfide; MT, methanethiol; Ac, acetate; HPS, heteropolysaccharides; ND, not determined.

Order, family and genus	Morphology	Major energy substrates	Temperature optimum (°C)	Cell wall
Order Methanobacteriales				
Family Methanobacteriaceae				
Genus *Methanobacterium*	Rod	H_2, (formate, alcohols)	37–45	Pseudomurein
Methanothermobacter	Rod	H_2, (formate)	55–65	Pseudomurein
Methanobrevibacter	Short rod	H_2, (formate)	37–40	Pseudomurein
Methanosphaera	Coccus	H_2 + methanol	37	Pseudomurein
Family Methanothermaceae				
Genus *Methanothermus*	Rod	H_2	80–88	Pseudomurein + protein
Order Methanococcales				
Family Methanococcaceae				
Genus *Methanococcus*	Coccus	H_2, formate	35–40	Protein
Methanothermococcus	Coccus	H_2, formate	60–65	Protein
Family Methanocaldococcaceae				
Genus *Methanocaldococcus*	Coccus	H_2	80–85	Protein
Methanotorrts	Coccus	H_2	88	Protein
Order Methanomicrobiales				
Family Methanomicrobiaceae				
Genus *Methanomicrobium*	Rod	H_2, formate	40	Protein
Methanoculleus	Irregular coccus	H_2, formate (alcohols)	20–55	Glycoprotein
Methanofollis	Irregular coccus	H_2, formate (alcohols)	37–40	Glycoprotein
Methanogenium	Irregular coccus	H_2, formate (alcohols)	15–57	Protein
Methanolactnia	Rod	H_2 (alcohols)	40	Glycoprotein
Methanoplama	Plate or disc	H_2, formate (alcohols)	32–40	Glycoprotein
Family Methanospirillaceae				
Methanospirillum	Spirillum	H_2, formate (alcohols)	30–37	Protein + sheath
Family Methanocorpusculaceae				
Genus *Methanocorpusculum*	Small coccus	H_2, formate (alcohols)	30–40	Glycoprotein
Methanocalculus	Irregular coccus	H_2, formate	30–40	ND
Order Methanosarcinales				
Family Methanosarcinaceae				
Genus *Methanosarcina*	Coccus, packets	Methanol, $MeNH_2$, (H_2, Ac, DMS)	35–60	Protein + HPS
Methanococcoides	Coccus	Methanol, $MeNH_2$	23–35	Protein
Methanohalophilus	Irregular coccus	Methanol, $MeNH_2$	35–40	Protein
Methanohalobium	Flat polygons	Methanol, $MeNH_2$	40–55	ND
Methanolobus	Irregular coccus	Methanol, $MeNH_2$ (DMS)	37	Glycoprotein
Methanomethylovorans	coccus, packets	Methanol, $MeNH_2$ DMS, MT	34–37	ND
Methanomicrococcus	Flat polygons	H_2 + Methanol, H_2 + $MeNH_2$	39	ND
Methanosalsum	Irregular coccus	Methanol, MeH_2, DMS	35–45	ND
Family Methanosaetaceae				
Genus *Methanosaeta (Methanothrix)*	Rod	Ac	35–60	Protein + sheath
Order Methanopyrales				
Family Methanopyraceae				
Genus *Methanopyrus*	Rod	H_2	98	Pseudormurein

During thermophilic digestion, and at high levels of salts, ammonium and VFAs, it is considered that the SAO could be dominant due to the higher sensitivity of acetoclastic compared to hydrogenotrophic methanogens (Demirel & Scherer, 2008; Malin & Illmer,

2008). However, from an energetic point of view, two organisms need to share the free energy during SAO in contrast to conversion by acetoclastic methanogenesis (Hattori, 2008).

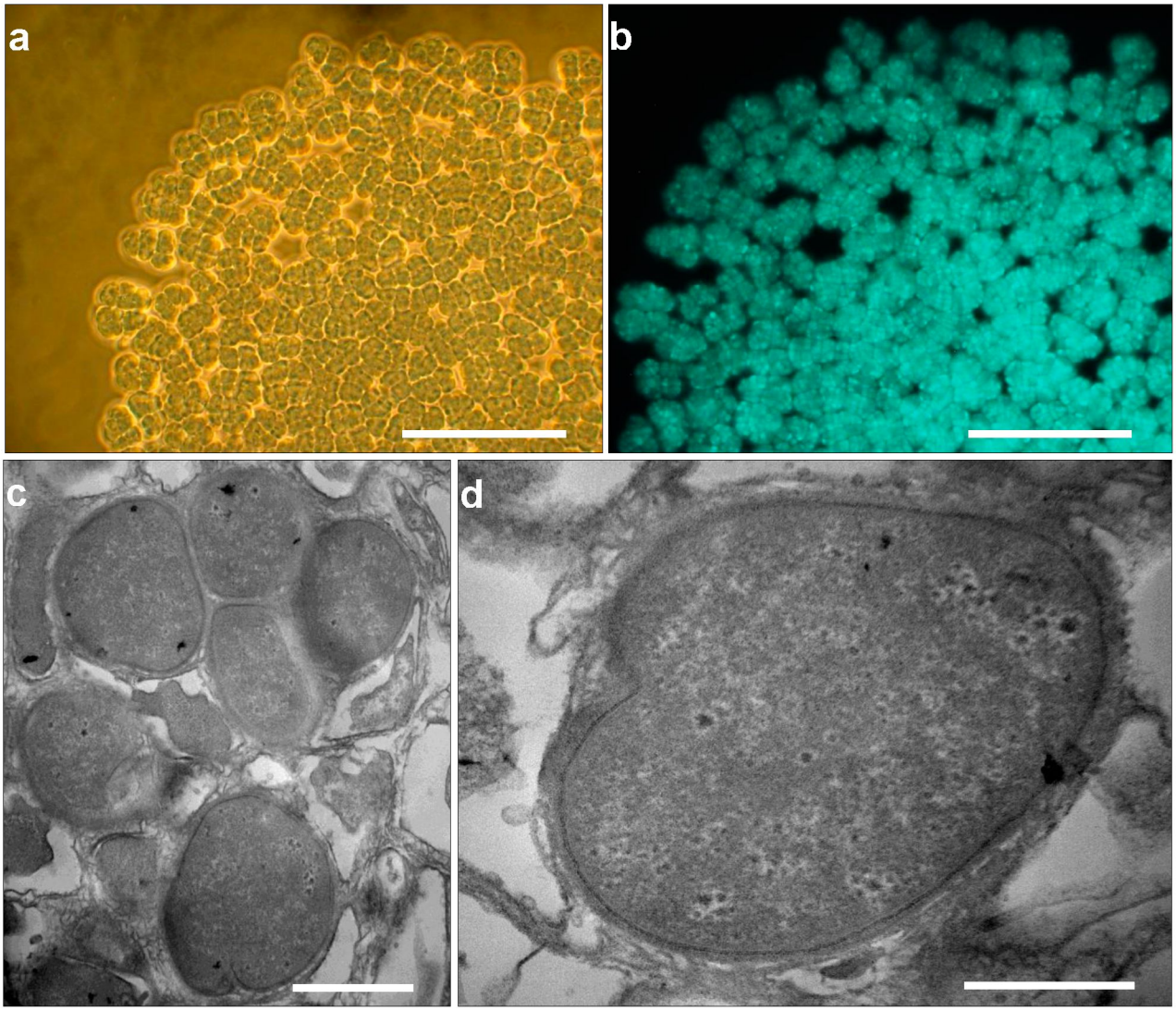

Figure 4: *Methanosarcina* spp. observed with a) phase-contrast, b) detection of the autofluorescating co-factor F_{420}, specific for methanogens, and c) and d) with transmission electron microscopy after ultrathin sectioning. The above figures derive from an axenic *Methanosarcina thermophila* culture, and the lower from an acetate-degrading enrichment culture (ADEC). Bars indicate: 50 µm (a & b), 1 µm (c), or 500 nm (d), respectively. All pictures by Lins P.

The **start-up** of an anaerobic digestion is often thought to be the most sensitive and challenging phase in anaerobic digestion because several different microorganisms are introduced, which should always be in good balance, e.g. hydrolytic, fermentative acid-producers and methanogens (Pandey et al., 2011). These groups of microorganisms are very diverse with respect to their growth rates, pH optima, inhibitors and substrates. Therefore, the start-up process especially under overload and stress operations has gained increasing

interest during the last few years, and several start-up procedures were established and evaluated. Among them, the pre-aeration of the substrates with the aim to reduce easily degradable organic carbons, which otherwise might lead to distinct production of VFAs with a subsequent drop in pH, seems to be an adequate treatment (Botheju & Bakke, 2011).

Charles et al. (2009) demonstrated that the pre-aeration for five days was sufficient to improve the start-up process significantly. However, attention should be paid to avoid an over aeration, and thus affecting the strict anaerobic methanogens directly or by an excess reduction of substrates limiting the biochemical methane potential. Additional impact on the start-up process has the substrate-to-inoculum ratio (Elbeshbishy et al., 2012; Zhou et al., 2011), the source of the inoculum (Pandey et al., 2011; Quintero et al., 2012), the initial mode of operation of the digesters as for example the mixing/stirring intensity (McMahon et al., 2004; Suwannoppadol et al., 2011), the organic loading rate and hydraulic and solid retention time (Fernandez-Güelfo et al., 2010; Le Hyaric et al., 2010). A relative new approach for start-up improvements is the adaptation and pre-incubation of the biomass prior the anaerobic digestion process (Elbeshbishy et al., 2012; Fernandez-Güelfo et al., 2010; Lins et al., 2012b; Lins et al., 2014; Silvestre et al., 2011).

Under well-performing conditions many different microorganisms are efficiently working together as a trophic cascade and no intermediate is accumulating, thus the biogas process proceeds near to its optimum (Schmitz et al., 2006). Nevertheless, if a trophic level or even a single **key microorganism** is disturbed, it inevitably leads to digestion failures, resulting in a cessation of the CH_4 production. Previously, it was determined that increased acetate concentrations within the input material have led to a suppressed biogas production in a 900 m^3 fermenter (Illmer et al., 2009). Therefore, it was necessary to search for a solution to combat high acetate loads by establishing specific inocula (Lins et al., 2012b; Lins et al., 2014). Hence, it is important to know the engaged key players to be able to provide them an optimal environment or to allow **bioaugmentation** with specific inocula to counteract deteriorating conditions (Fernandez-Güelfo et al., 2010). Furthermore, it was shown that the application of an enrichment culture to a suppressed anaerobic digestion process led to a reduction of VFAs, and a restart of methanogenesis (Lins et al., 2010). Despite these promising results, the knowledge about microbial community changes during transitional phases (e.g. start-up) is incomplete or controversially discussed (Shin et al., 2010). The start-up is the most crucial phase of an anaerobic digestion especially because a proper microbial community has not been established yet, which makes it susceptible to imbalances (Pandey et al., 2011).

It is generally accepted that several microbes participating in the anaerobic digestion of organic matter live near the **thermodynamic limit**. Because oxygen is missing as high energy-yielding terminal electron acceptor, the degradation for example of glucose yields only about a tenth of the energy (ca. -240 kJ mol^{-1}) compared to aerobic conditions (Aiyuk et al., 2006; Scherer, 2001). As shown in Tab. 2 the degradation of the central intermediate acetic acid also yields only -34.3 kJ mol^{-1} at 52 °C, and a big stake of the synthesized ATP is used to activate acetic acid to acetyl-CoA (Buckel, 1999).

Table 2: Changes of the Gibbs's free energy of relevant acetogenic and methanogenic reactions (rct) at the thermophilic temperature of 52 °C. Reactions 9-12 describe syntrophic degradation pathways (modified after Lins & Illmer (2009)).

#	Reaction equations[a)]			$\Delta_r G^0$ [kJ rct^{-1}]
1	$4H_2 + HCO_3^- + H^+$	→	$CH_4 + 3H_2O$	-128.5
2	Formic $acid^- + H_2O$	→	$HCO_3^- + H_2$	-0.3
3	4 Formic $acid^- + H_2O + H^+$	→	$CH_4 + 3HCO_3^-$	-129.6
4	Acetic $acid^- + H_2O$	→	$CH_4 + HCO_3^-$	-34.3
5	Acetic $acid^- + 4H_2O$	→	$2HCO_3^- + 4H_2 + H^+$	94.3
6	Propionic $acid^- + 3H_2O$	→	Acetic $acid^- + HCO_3^- + 3H_2 + H^+$	73.2
7	Butyric $acid^- + 2H_2O$	→	2 Acetic $acid^- + H^+ + 2H_2$	42.9
8	Butyric $acid^- + 3H_2O$	→	Propionic $acid^- + HCO_3^- + H_2 + H^+$	63.9
9	Reaction 6 + Reaction 4			
	Propionic $acid^- + 4H_2O$	→	$CH_4 + 2HCO_3^- + 3H_2 + H^+$	38.9
10	Reaction 9 + 0.75 Reaction 1			
	Propionic $acid^- + 1.75H_2O$	→	$1.75CH_4 + 1.25HCO_3^- + 0.25H^+$	-57.5
11	Reaction 7 + 2 Reaction 4			
	Butyric $acid^- + 4H_2O$	→	$2CH_4 + 2HCO_3^- + H^+ + 2H_2$	-25.7
12	Reaction 11 + 0.5 Reaction 1			
	Butyric $acid^- + 2.5H_2O$	→	$2.5CH_4 + 1.5HCO_3^- + 0.5H^+$	-89.9

[a)] All VFAs and HCO_3^- in the dissolved state at 1 mole L^{-1}, H_2 and CH_4 in the gaseous state at 1 atm and H_2O in the liquid sate and corrected for pH neutrality.

To counteract the hurdles of low energy yields, these microorganisms establish **syntrophic microbial communities**, which are mostly located close to each other to further reduce the way for transport and/or diffusion. Hence, spatially higher concentrations of intermediates might be reached, which on the other hand might lead to thermodynamic feasible reactions (Tab. 2) (Jackson & McInerney, 2002; Schink & Stams, 2006). An example for such an interaction is the syntrophic degradation of propionate, which releases 3 mole H_2 per mole propionate. If propionate-oxidizing (and thus H_2-producing) bacteria are associated with H_2-consumers, both partners take a profit out of it. Firstly, the H_2 partial pressure will be reduced, thus counteracting an end product inhibition of the propionate degradation, and secondly the syntrophic partner might use H_2 either to reduce CO_2 to CH_4 (hydrogenotrophic methanogenesis) (Fig. 5, reaction 3 combined with reaction 4), or to reduce sulfate- and sulfur-compounds to H_2S (sulfate- or sulfur-reducing microorganisms) to gain energy (Scherer, 2001; Schink & Stams, 2006).

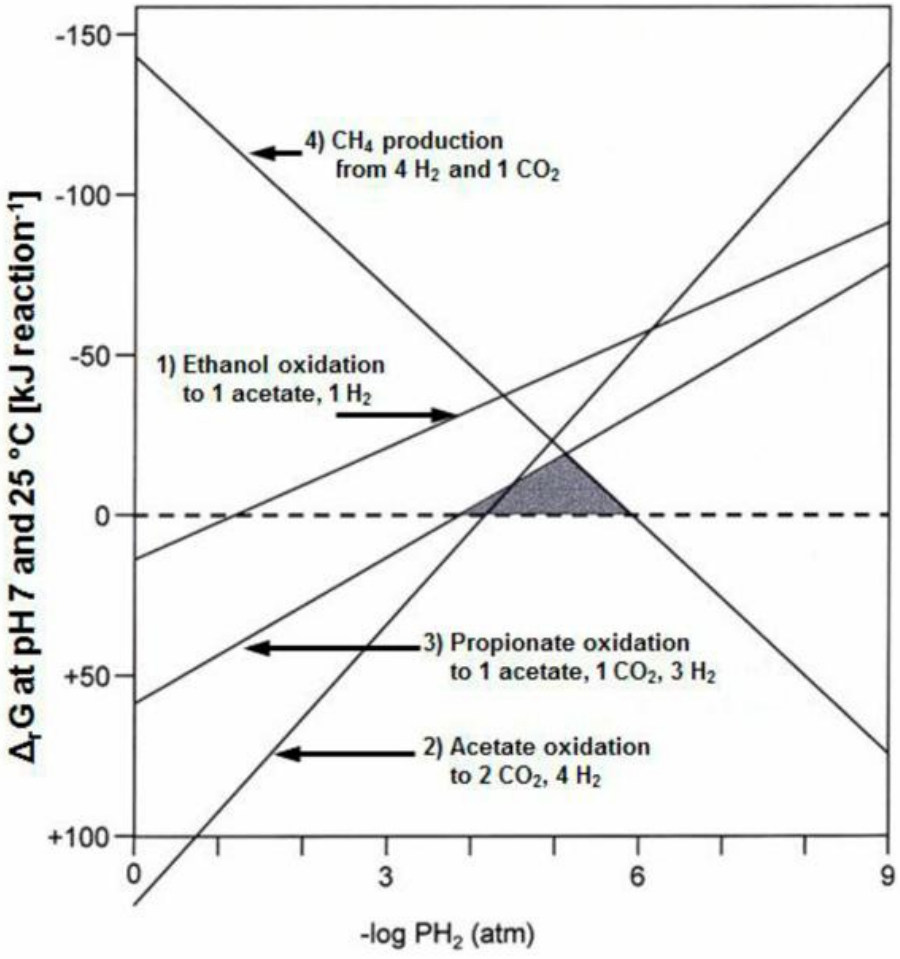

Figure 5: 'Thermodynamic window' representing the change of the Gibbs free energy of H_2-producing reactions (Reaction 1-3) and the H_2-consuming reaction of hydrogenotrophic methanogenesis (Reaction 4) in dependence on the H_2 partial pressure (PH_2) at standard conditions (modified after Scherer (2001)).

Two reactions give the limits for a so-called 'thermodynamic window' (Fig. 5, grey triangle) wherein exergonic conditions are prevailing as it is the case for syntrophic degradation pathways. This means that within the specific range of the H_2 partial pressure both reactions are feasible at the same time.

In Fig. 6 the available energy through methanogenesis from acetic acid is shown for different conditions (A, B and C). Additionally, the effects of different pH values are added. The courses indicate that, although significantly different concentrations of CH_4, CO_2 and acetic acid are simulated, the energy available does not change dramatically. However, as mentioned above, due to the proximate life near the thermodynamic threshold and limits, several kilojoules difference might determine if a complete degradation cascade is feasible or not.

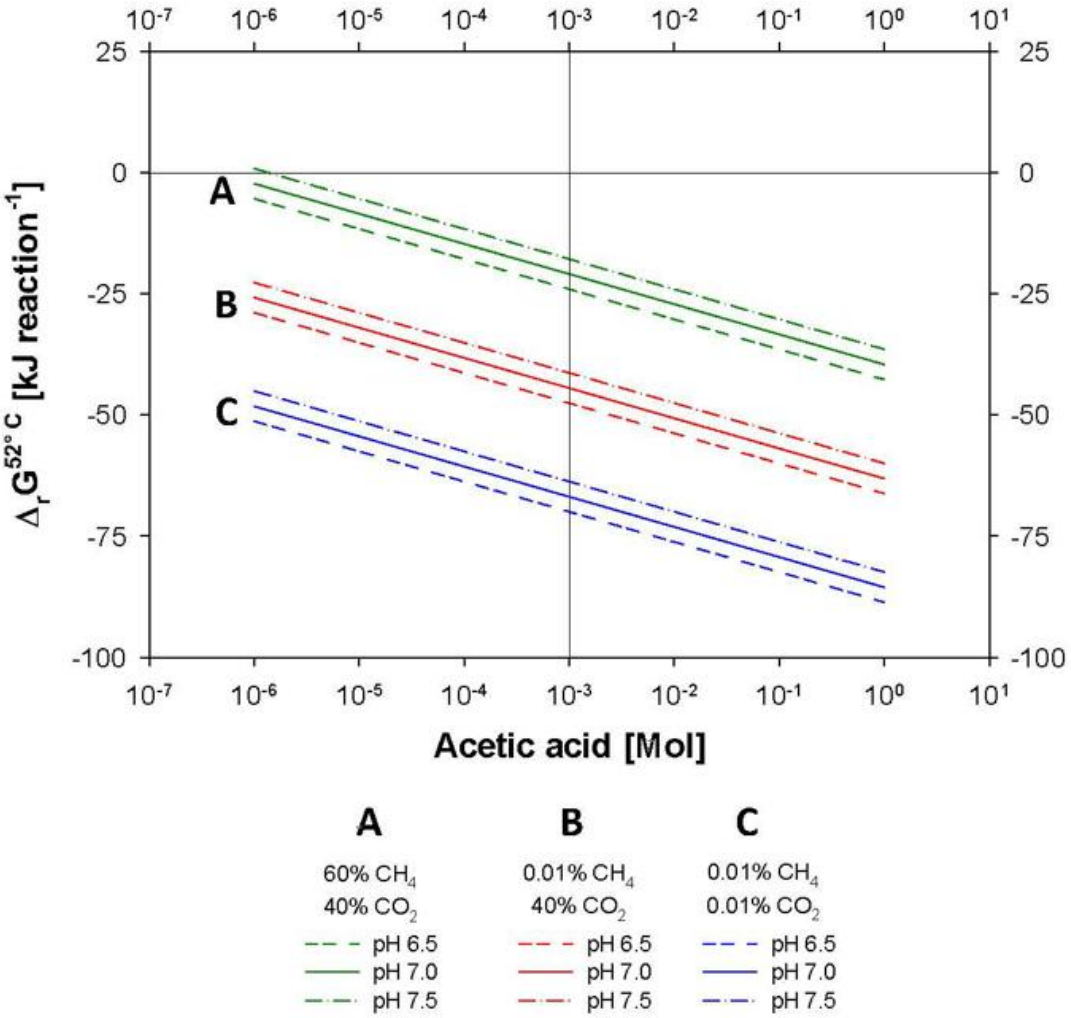

Figure 6: Available energy for acetoclastic methanogenesis (Tab. 2, reaction 4) under different conditions (concentration of CH_4 and CO_2, and pH values) for different acetic acid concentrations at 52 °C. Calculations were done according to Lins & Illmer, 2009.

In conclusion, insights into the microbial community might open the potential to provide an optimal environment or for bioaugmentation of specific inocula to counteract deteriorating conditions (Elbeshbishy et al., 2012; Fernandez-Güelfo et al., 2010; Lins et al. 2012b; Lins et al. 2014), which could further improve the stability and efficiency of the anaerobic digestion.

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