8. Summary and conclusions

The absence of oxygen in an anaerobic digester is the prerequisite to allow growth of the strict obligate anaerobic methanogens. However, anaerobiosis leads to a reduced possibility to conserve energy, and thus limits the growth of microorganisms. For compensation, microorganisms benefit from each other if they grow in close proximity to reduce diffusion rates. With a reduced distance the transport of intermediates is improved, as well as the spatial concentration of substrates and/or end products is in a favourable range. This leads to thermodynamically feasible conditions for both partners, which would not be able to grow alone. Therefore, a closer look at the thermodynamics in anaerobic digestion was drawn at the beginning of the present work. It needs to be stated that changes of the Gibbs free energy, calculated under standard conditions, are less convincing compared to those based on *in situ* conditions (Δ_rG) and should be interpreted with precaution when trying to assess or optimize fermenter conditions. If calculated with *in situ* concentrations, significantly different values are expected, which can turn reactions on either side, exergonic or endergonic (Lins & Illmer, 2009). For this purpose, medians of process values were taken to calculate Δ_rG, which were determined over two years of operation of a 900 m^3 thermophilic reactor of a biogas plant (Roppen, Austria). These conditions were 618 ppm H_2, 61.3% CH_4, 39.3% CO_2, 24.98 mM acetate, 10.93 mM propionate, 0.57 mM butyrate, and pH 8.44. Additionally, the actual temperature of 52 °C was used for calculations. The methanogenic as well as the acetogenic reactions have to be exergonic to allow a good overall performance. If these reactions are endergonic or the involved microorganisms are inhibited, volatile fatty acids (VFAs) accumulate. This leads to an acidification of the process, followed by a pH drop hampering the complete degradation cascade. In this study methanogenesis from H_2 and acetate was only slightly exergonic, indicated by a Δ_rG of -43.4 and -20.7 kJ mol^{-1}, respectively. Furthermore, the degradation of propionate and butyrate was endergonic but if combined with methanogenesis their degradation reactions got energetically feasible. Due to syntrophic degradations some reactions get exergonic but the energy needs to be split up by at least two organisms. The interpretation of Δ_rG should always be made with care since the sample is withdrawn from the liquid broth and not from the direct micro-habitat. There, syntrophs are associated in biofilms, granules, aggregates or are more or less loosely attached to each other where completely different conditions might prevail. In another study, these aggregates or co-localizations within 120 mL incubation flasks were severely affected by agitation of a horizontal shaker (Lins & Illmer, 2012). Agitation increases the liquid-gas transfer and

distributes the microorganisms and their substrates and end products. A significantly higher CH_4 and total gas production was determined for a moderate agitation (150 rpm) compared to the static incubated control. The improved distribution must have led to a more homogenous density of microorganisms and their nutrients, which might have had beneficial effects for growth. However, a lower agitation speed (100 rpm) or a further increase of the speed (≥200 rpm) led to a suppressed CH_4 production. Obviously, 100 rpm resulted in a too low shear force to allow a proper mixing, while ≥200 rpm most likely had an impact on physical disruption and shear stress of mutually associated syntrophic microbial consortia. However, this outcome might vary with the shape of the bottles used for cultivation. Interestingly, during the first day of incubation a gradual increase of H_2 production was measured with increasing agitation speed, indicating an interruption of the syntrophic H^+ transfer and a release of dissolved H_2.

Beside the pH value, whose direct and indirect impact was already described in the introduction section, the concentration of the individual VFAs is another relevant process parameter. To investigate the short-term effects caused by different concentrations of the important VFAs formate, acetate, propionate and butyrate, they were separately applied as sole carbon sources at concentrations of up to 360 mM (Lins & Illmer, 2012). The addition of formate led to a different effect on the CH_4 production compared to the other three VFAs. Formate did not result in an inhibition of the CH_4 production until a concentration of 120 mM. Only at the highest concentration (360 mM) no CH_4 production was detectable within 3 d but slightly started after additional 2 d of incubation. The positive effect of 120 mM formate might indicate a high number of active hydrogenotrophic, formate-utilizing methanogens present in the diluted fermenter sludge (DFS), which was confirmed by other investigations (Lins et al., 2010 & 2014). The application of acetate, the most important intermediate in the anaerobic digestion, led to an inhibition at >36 mM. Propionate and butyrate already showed inhibitions at 36 mM. However, a kind of adaptation might have taken place within these variants because a weak CH_4 production was detected after additional 2 d. Nevertheless, the incubation for 5 d was too short to produce large amounts of CH_4 from acetate, propionate and butyrate. Most probably the number of acetoclastic methanogens, syntrophic acetate, propionate and butyrate degraders was too low or more time for adaptation would have been required.

The concentration of ammonium (NH_4^+) is generally not determined on-line but, if protein-rich substrates are digested, ammonia (NH_3) (or NH_4^+) might reach toxic concentrations. To simulate these perturbations, a study was set up where NH_4^+ (as NH_4Cl) was supplemented at final concentrations of up to 720 mM (=10.08 g NH_4^+-N L^{-1}), and 36 mM formate was

chosen as sole carbon source since this concentration resulted in significant CH_4 production without a sign of stress in previous investigations (Lins & Illmer, 2012). An increase of CH_4 production along with increasing the concentration of NH_4^+ up to 120 mM (=1.68 g NH_4^+-N L^{-1}) could be detected after 3 d of incubation. This might rely on the fact that NH_4^+ is a favourable nitrogen source readily available for microorganisms. At first glance, the optimum in this study seems to be relatively high but the original biogas plant sludge also contained about 2.8 g NH_4^+-N kg^{-1}, and therefore the microorganisms already might have been adapted to high NH_4^+ concentrations. The addition of 720 mM NH_4^+, however, led to a complete inhibition until day 3. Although CH_4 production started 2 d later, it was still significantly suppressed.

In a further study, the methanogenic potential of formate during thermophilic anaerobic digestion was investigated in detail (Lins et al., 2012b). After appropriate conditions for methanogenesis (formate and inoculum concentration, pH, and duration of incubation) were assessed, an experiment was set up with 36 mM formate as sole carbon source with and without a 150 mM phosphate buffer provided. One might suppose that CH_4 was solely produced from formate because CH_4 production ceased after depletion of formate although H_2 was still present but until now no methanogenic organism is known that is able to utilize formate without the ability to utilize H_2. Based on dHPLC, DGGE, and additional subsequent sequencing approaches the hydrogenotrophic *Methanothermobacter wolfeii*, located within the order Methanobacteriales, was the dominant methanogen responsible for CH_4 production. Further confirmation was achieved with the detection of autofluorescing rods with a size of up to ~3 µm, which were often arranged in pairs and chains. *Methanoculleus wolfeii* grew well at the established conditions (temperature, pH, etc.), which are in accordance to the literature. An initial Δ_rG of about -145 kJ mol^{-1} was calculated for methanogenesis from formate but a dramatic decrease was followed with the degradation of formate. Although the reaction remained exergonic, methanogenesis from H_2/CO_2 was more favourable from hour 72 on. Additionally, the applied buffer had some minor effects on the pH, and CO_2 and H_2 in the gas phase, however, formate-utilization and CH_4 production were not considerably different. Due to the fast turnover rate of formate (1.5 mM h^{-1}), even high concentrations were spent within several hours, which might allow a decrease of the formate concentration close to or even under the detection limit. This fact could lead to an underestimation of both, the concentration and thus, the importance of formate during anaerobic digestion.

The anaerobic digestion process is susceptible to diverse stress factors. Antibiotics, as potential contaminants, are for example frequently found in slurry and manure of livestock animals and industrial wastewater, and can persist up to several months to years because

they are not completely and sometimes even scarcely degraded. The accumulation of antibiotics in different waste streams, which are subsequently used as input material for anaerobic digestion, might lead to detrimental effects of the overall performance if key microorganisms are inhibited or suppressed. Therefore, a study was performed where the focus was (i) on gaining an insight into the stability of a process that is the VFA degradation and CH_4 production capacity of an anaerobic digestion suffering from exposure to antibiotics and the methanogenic inhibitor 2-bromoethanesulfonate (BES). Another focus was laid (ii) on investigating the complex interactions of the bacterial and archaeal community during exposure of these compounds, and finally, (iii) on determining if it is possible to specifically inhibit a degradation pathway of a VFA with one of the added antimicrobial agents. For this purpose the effects of eleven antibiotics were investigated and compared to controls and BES (Lins et al., 2015). Among these compounds, only the three protein synthesis interfering antimicrobial agents neomycin, gentamicin, and rifampicin, and BES led to inhibitions of VFA degradation. This points to distinct interferences with important trophic degradation cascades. Interestingly, BES and neomycin completely inhibited the degradation of acetate while a complete inhibition of methanogenesis was only achieved by BES. If acetate-oxidizing bacteria were present, they were also susceptible towards neomycin. The CH_4 production within the propionate control flasks did not show any considerable lag-phase. Neomycin, gentamicin, and rifampicin significantly inhibited syntrophic propionate and butyrate degraders, and thus methanogenesis was negligible. The conversion of 1 mole butyrate to 2 moles acetate also produces 2 moles H_2, which might have been the source for the CH_4 production during the first 2 weeks of incubation within the control (Lins & Illmer, 2009). As a result of the acetate degradation, a second distinct CH_4 peak was detectable at a later time. Interestingly, considerable different effects of the four aminoglycosides neomycin, gentamicin, streptomycin and kanamycin were detected. Streptomycin is based upon a streptamine, while kanamycin and gentamicin belong to the group of 4,6-linked 2-deoxystreptamines, and neomycin to 4,5-linked 2-deoxystreptamines. Therefore, it is obvious that they have different specificities and also different targets for modifications. Additionally, antibiotics can also have multiple targets and probably some of them still need to be determined. The cell wall-affecting antibiotics (ampicillin, bacitracin, D-cycloserine and penicillin G) did not show significant degradation inhibitions or accumulations of VFAs. This might be due to the differing cell wall characteristics of Archaea compared to Bacteria, which did not pose a potential target for these antibiotics. Furthermore, a possibly too low concentration of these antibiotics and/or a too low stability at the incubation temperature of 52 °C were prevailing with respect to spontaneous decay and enzymatic degradation. In

comparison, even a low concentration of 1 mM BES inhibited the VFA degradation for 14 d. This is probably directed to the initial accumulation of H_2 and formate, which especially affected the propionate degradation. To completely prevent methanogenesis a high concentration of 50 mM BES was required. Based upon DGGE and sequencing approaches, members of the genus *Methanosarcina* were severely influenced by the treatments while hydrogentrophic methanogens as *M. wolfeii* and *Methanoculleus* spp. were less affected. Compared with the controls, it seems that members of the genus *Methanosarcina* were mandatory for the degradation of acetate at high rates. The most abundant bacteria belong to the order Clostridiales representing gram-positive bacteria. Among them apparently the most prominent bacteria were members of the genus *Desulfotomaculum*, whereof some species degrade C_3 and C_4 substrates in co-culture. The cluster analysis for archaea revealed that a variation in carbon sources (acetate, propionate and butyrate) had a higher impact on the community than the treatments with the antimicrobial compounds. Contrarily, the bacterial population clustered within the rifampicin and gentamicin treatments, and thus might have been more influenced by these agents than by the different carbon sources supplied. Last but not least, although surprisingly, there was neither an obvious clustering of archaea nor of bacteria with respect to the treatments resulting in high CH_4 production. That highly emphasizes the detrimental effects of antimicrobial compounds with the potential to significantly inhibit the anaerobic digestion. Nevertheless, further steps are required to identify the respective suppressed syntrophs in detail, to determine their sensitivity to the applied antibiotics, and to have a closer look to the inhibition of acetate degradation by neomycin.

In addition to abiotic effects, the composition of the microbial community is an important factor since a well performing interrelationship of the different trophic levels is the prerequisite that no intermediate accumulates and causes severe problems. In a study with a stagnating biogas production, due to accumulation of VFAs, the bioaugmentation of an acetate-degrading enrichment culture (ADEC) was investigated (Lins et al., 2010). Initially, a thermophilic batch operated lab-scale digester was inoculated with DFS. Within 4 d after inoculation, a distinct increase of the concentration of proteins, NH_4^+, NH_3, and VFAs was detected. The sum of the measured VFAs reached a maximum of almost 6000 mg L^{-1}, especially the accumulation of acetate and propionate was prominent, and the pH value and NH_4^+/NH_3 levels did not reach auspicious values. Therefore, most probably, the imbalance between acid-producers and acid-reducers might have been the driving force for the failure of the digestion, resulting in a drop of the pH below 7.0 within 4 d. Thus, the lack of active acetoclastic methanogens or other acetotrophic microorganisms, which would have been

able to convert acetate to CH_4 and CO_2, seemed to be the main reason for the stagnation. Most probably the accumulating acetate hampered the degradation of other VFAs since these reactions proceed close to thermodynamic limits (Lins & Illmer, 2009). After a stagnation of biogas production for 30 d it was hypothesized that the addition of an ADEC might reduce the acetate concentration, and thus, the degradation of the other VFAs might be stimulated and restarted again. In fact, after application most of the measured parameters showed significant changes within a few days. The most distinct changes were a sharp decrease in the acetate concentration, a rise in pH, and thus a prompt increase in the CH_4 production. During this time the methanogens doubled within 19.8 h, which was calculated on the basis of the CH_4 production. An archaeon with a similarity of 99% to the hydrogenotrophic *Methanoculleus thermophilus* was detected. Its intensity increased during the first days after inoculation but did not change significantly throughout the remaining experiment. The only observed significant change of the microbial community was the appearance of a methanogen with a similarity of 98% to *Methanosarcina barkeri*. Potentially it was the driving factor, as a key microorganism, for reducing the concentration of the accumulated VFAs and restarting significant CH_4 production almost immediately after application. Additionally, another *Methanosarcina* species (98% to *Methanosarcina thermophila*) was detectable. Obviously, representatives of the genus *Methanosarcina* successfully adapted to the high acetate levels in the digester. Within the ADEC sarcina-typical packets, arranged to aggregates with a size of about 30 µm, were detected, which confirm the presence of *Methanosarcina* spp.. Consequently, they were successful competitors at the high acetate concentrations during the VFA accumulation period.

The fundamental possibility to produce inocula to combat specific process failures of anaerobic digestion seemed to be promising and was further followed (Lins et al., 2010, 2012a & 2014). During previous investigations dealing with the performance of a full-scale anaerobic fermenter, a concentration of about 150 mM acetate within the organic input material was critical. A reverse course of the acetate concentration and the biogas production was determined, which indicated a stress at elevated concentrations. Therefore, this concentration was chosen for this study to investigate the start-up of an anaerobic digestion under high acetate load. Two strategies were followed (i) a gradual adaptation (A-variants) of the engaged microorganisms within 1, 2, 4 or 6 weeks (A1-A6), each at increasing acetate concentrations of 50, 100, and finally 150 mM, and (ii) shock (S-variants), meaning a direct exposure to 150 mM for the same durations (S1-S6). During the enrichment phase no significant differences between the A- and S-variants with 4 and 6 weeks could be determined. As it could be observed for the A6 variant, 6 weeks were necessary to degrade

the 50 mM acetate applied in the first adaptation step. However, 6 weeks were still too less to completely reduce 100 and 150 mM during the next two steps but a higher degrading capacity with subsequent steps and time for adaptation was obvious. Representatives of the hydrogenotrophic order Methanobacteriales were present in a relative high number in the DFS, however, they were significantly reduced in all treatments. Thus, they might have had only a limited contribution to the degradation of acetate, e.g. via syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis. *Methanoculleus* was the most abundant methanogenic group in the DFS and showed significantly increased numbers at treatment A4 and A6, while representatives of the order Methanosarcinales were significantly reduced at A1 but significantly increased at A2, A4 and A6. According to changes of the methanogenic copy numbers, generation times can roughly be estimated to about 2-3 d, which are consistent with those determined based on CH_4 production (Lins et al., 2012a). The shortest generation time of methanogens was 40 h at the A4 and S4 variants based on the CH_4 production. Indicated by autofluorescence, methanogens with an irregular cocci- and sarcina-typical structure were detected. This is in accordance to a previous study dealing with the reduction of accumulated VFAs by an ADEC, run with the same initial inoculum (Lins et al., 2010). Therefore, not only the presence but also the relevance of representatives of the order Methanosarcinales for the acetate degradation is undoubtedly.

After the adaptation and shock phase, the resulting inocula were evaluated for 11 weeks regarding their potential for CH_4 production under high acetate concentrations (150 mM). Among all, the inocula stepwise adapted for 4 and 6 weeks to increasing acetate concentrations led to a significant faster start-up compared to controls and shocked variants. Good agreements of the abundance of Methanosarcinales and *Methanoculleus* spp. with total DNA content and CH_4 production rate were apparent. In addition, a gradual adaptation of the inoculum for at least 4 weeks led to a significant increase of *Methanosarcina* spp. during the subsequent fermentation. The change of the acetate concentration correlated well with the CH_4 production, which is in agreement with the theoretical conversion of 1 mole acetate to 1 mole CH_4 resulting in a change of the Gibbs free energy of -34.3 kJ per reaction at 52 °C (Lins & Illmer, 2009). Remarkably, the control showed a relatively long lag-phase up to 14 d until relevant CH_4 production started. Whereas, A4, A6, S4 and S6 started to produce CH_4 without noticeable lag-phase. At approximately the half of the incubation duration at day 42, the variants A4 and A6 were not noticeable different to each other but significant differences to the control, shocked and shorter adapted variants were present. A relative low diversity of the archaeal community was apparent, and a predominance of representatives of *Methanosarcina* spp. and *Methanoculleus* spp. was detected. It seems that the adaptation

with 1 week steps (A1) was too short for *Methanosarcina thermophila* and *Methanoculleus thermophilus* to allow significant growth. Apparently, the adaptation for 4 weeks was preferred for the proliferation of an uncultured *Methanosarcina* sp.. A relevant abundance of *Methanoculleus thermophilus* was only detected at A4 and A6, which showed an opposite behaviour with another representative of *Methanoculleus*. Compared with the adaptation, the shock treatments showed hardly any differences between the individual enrichment durations. The shock treatments were dominated by *Methanoculleus* spp., while during the adaptations *Methanosarcina* sp. was most dominant in co-occurrence with at least two different *Methanoculleus* species. *Clostridium acetireducens* (93%), *Clostridium sporogenes* (99%), *Clostridium ultunense* (97%), and an uncultured clostridium (97%) were the dominant bacteria. Generally, the bacterial community structure remained remarkably constant during incubation; however, differences between adaptation and shock were apparent. Summed up, within the adaptation treatments *C. acetireducens* was predominant while within the shock treatments *C. sporogenes* was the most abundant bacterium. During the evaluation phase of the most stressed treatments, i.e. S1, A1 and S2, *C. ultunense* was a dominant bacterium. Its proliferation might have been favoured by the relatively low numbers of Methanosarcinales, which are potential competitors for acetate when present at significant numbers. *Clostridium ultunense* is one of so far few described bacteria that degrades acetate in cooperation with H_2-utilising organisms. This might be an explanation for the high abundance of *Methanoculleus* spp. during the present investigation. Therefore, acetate might have been degraded via acetoclastic methanogenesis and SAO with subsequent hydrogenotrophic methanogenesis simultaneously. The abundance of *Methanoculleus* spp. remained relatively stable, independent of the treatment and sampling day, which is in accordance to results from DGGE. Therefore, *Methanoculleus* spp. might not have functioned as sole driving force for acetate degradation and subsequent methanogenesis. Interestingly, a low abundance of Methanosarcinales was most likely the reason for the hampered CH_4 production. In a previous study it was pointed out that a relevant number of Methanosarcinales was required to be able to cope with accumulated VFAs during thermophilic anaerobic digestion (Lins et al., 2010). That highlights the potential prerequisite role of Methanosarcinales to allow fast and significant acetate degradation even under deteriorated conditions. The approach of combating start-up problems with specific inocula is an up-to-date topic in anaerobic digestion. Even if a lower concentration of the inoculum is applied as the generally suggested inoculum concentration of 10%, it might result in a positive boost of the overall performance. Additionally, this approach might be combined with other start-up procedures as it was stated in the introduction section. However, before the developed and evaluated inocula of the

present study might be applied to full-scale biogas plants, further research is required where the effects of upscaling, different fermenter designs and cultivation conditions need to be investigated. Furthermore, other recently established detection methods, as for example next-generation sequencing, could provide additional information with respect to the involved microorganisms.

References

Lins, P. & Illmer, P. (2009). Thermodynamics of anaerobic digestion (Thermodynamische Aspekte der anaeroben Vergärung). *Waste and Residues (Müll und Abfall)* 12:614-618.

Lins, P., Malin, C., Wagner, A.O. & Illmer, P. (2010). Reduction of accumulated volatile fatty acids by an acetate-degrading enrichment culture. *FEMS Microbiology Ecology* 71:469-478.

Lins, P. & Illmer, P. (2012). Effects of volatile fatty acids, ammonium and agitation on thermophilic methane production from biogas plant sludge in lab-scale experiments. *Folia Microbiologica* 57:313-316.

Lins, P., Reitschuler, C. & Illmer, P. (2012a). Development and evaluation of inocula combating high acetate concentrations during the start-up of an anaerobic digestion. *Bioresource Technology* 110:167-173.

Lins, P., Schwarzenauer, T., Reitschuler, C., Wagner, A.O. & Illmer, P. (2012b). Methanogenic potential of formate in thermophilic anaerobic digestion. *Waste Management and Research* 30:1031-1040.

Lins, P., Reitschuler, C. & Illmer, P. (2014). *Methanosarcina* spp., the key to relieve the start-up of a thermophilic anaerobic digestion suffering from high acetic acid loads. *Bioresource Technology* 152:347-354.

Lins, P., Reitschuler, C. & Illmer, P. (2015). Impact of several antibiotics and 2-bromoethanesulfonate on the volatile fatty acid degradation, methanogenesis and community structure during thermophilic anaerobic digestion. *Bioresource Technology* 190:148-158.