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# Reduction of the hydraulic retention time at constant high organic loading rate to reach the microbial limits of anaerobic digestion in various reactor systems

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## HIGHLIGHTS

- Anaerobic digestion at extreme short HRT was investigated in various reactors.
- Functional markers for $H_2$ and $CH_4$ producing pathways were analyzed on mRNA level.
- Stable isotope analysis of the biogas was applied to assess methanogenic pathways.
- Depending on HRT *Clostridiales* and *Spirochaetales* were the most active in reactors.
- Increased activity of *Methanosaeta* was found during HRT decrease in CSTR and ASBR.

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## ABSTRACT

The effects of hydraulic retention time (HRT) reduction at constant high organic loading rate on the activity of hydrogen-producing bacteria and methanogens were investigated in reactors digesting thin stillage. Stable isotope fingerprinting was additionally applied to assess methanogenic pathways. Based on *hydA* gene transcripts, *Clostridiales* was the most active hydrogen-producing order in continuous stirred tank reactor (CSTR), fixed-bed reactor (FBR) and anaerobic sequencing batch reactor (ASBR), but shorter HRT stimulated the activity of *Spirochaetales*. Further decreasing HRT diminished *Spirochaetales* activity in systems with biomass retention. Based on *mcrA* gene transcripts, *Methanoculleus* and *Methanosarcina* were the predominantly active in CSTR and ASBR, whereas *Methanosaeta* and *Methanospirillum* activity was more significant in stably performing FBR. Isotope values indicated the predominance of aceticlastic pathway in FBR. Interestingly, an increased activity of *Methanosaeta* was observed during shortening HRT in CSTR and ASBR despite high organic acids concentrations, what was supported by stable isotope data.
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## 1. Introduction

One of the rapidly advancing areas of modern biotechnology is the development of sustainable waste treatment technologies. Much attention is paid to the efficient anaerobic digestion of organic substances, including agricultural, industrial, and household wastes, with the production and utilization of energy rich biogas. Anaerobic conversion of waste organic matter into biogas in engineered systems is also a promising way of reducing uncontrolled greenhouse gas emissions. However, it is also recommended to improve the digestate storage to reduce further methane emissions (Mata-Alvarez et al., 2000; Holm-Nielsen et al., 2009).

Among the main operational conditions that need to be periodically monitored in anaerobic digesters, hydraulic retention time (HRT) is one of the key process parameters. Assuming volume constancy this parameter is calculated from the working volume of the reactor that is divided by the daily feeding volume of the substrate; therefore, it is closely linked to the organic loading rate (OLR) and defines the average residence time of the substrate in the system. Current biogas plants in the agricultural sector usually apply long

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HRT in order to allow enough time for the effective degradation of complex compounds (e.g. lignocellulosic materials) and to prevent the wash out of slow growing microorganisms. Economical biogas production aims to operate the system effectively at high throughput, which consequently results in the decrease of the HRT or requires larger digesters. Therefore, shortening the HRT of high water content substrates would enable to design smaller digesters that can process the same amount of feed material, which would decrease the investment cost (Gerardi, 2003).

HRT reduction affects the microbial community composition as shown in previous works (Krakat et al., 2010; Ziganshin et al., 2013; Moestedt et al., 2014), but these studies achieved HRT reduction by increasing the OLR. Krakat et al. (2010) observed a shift of the methanogenic community from hydrogenotrophic *Methanobacteriales* towards hydrogenotrophic *Methanospirillum* and strict aceticlastic *Methanosaeta* representatives during a rapid decrease of HRT and an increase of OLR in a mesophilic continuous stirred tank reactor (CSTR) digesting sugar beet silage. The genus name *Methanosaeta* was recently rejected in favor of the previously used notation *Methanothrix* (Tindall, 2014). However, the *Methanosaeta* name is used in this study to avoid confusion when comparing the present results to recently published works. Ziganshin et al. (2013) showed that the methanogenic community in a mesophilic CSTR digesting chicken and cattle manure and operating at high levels of volatile fatty acids (VFA) and $NH_4^+$–N was very stable during HRT decrease and OLR increase and consisted almost completely of hydrogenotrophic *Methanoculleus* phylotypes. This indicates that anaerobic digestion of chicken/cattle manure relied on syntrophic acetate oxidation (SAO) as the dominant acetate-consuming process. In the work of Moestedt et al. (2014) a significant increase of the syntrophic acetate oxidizing bacterium (SAOB) *Tepidanaerobacter acetatoxydans* with the methanogenic taxa *Methanomicrobiales* and *Methanosarcinaceae* were observed during HRT decrease and OLR increase phases in a CSTR digesting thin stillage at 38 °C. The relative abundances of another SAOB, *Clostridium ultunese*, and the methanogenic order *Methanobacteriales* were relatively stable. In the next study, thermophilic anaerobic digestion of waste-activated sludge with HRT reduction from 15–20 to 3–2 days was investigated (Ho et al., 2013). While at 3-day HRT the process was relatively stable with the predominance of *Methanosarcina* spp., the further reduction of HRT to 2 days resulted in reduction of methanogenesis and loss of methanogens. Stable isotope analysis of the produced biogas suggested that methane was mainly produced by the hydrogenotrophic pathway.

The direct effect of increasing OLR is usually an enhanced overall activity of primary fermenting bacteria with increased VFA production, with which the acid utilizing processes (syntrophic VFA oxidation, methanogenesis) cannot keep up (Xu et al., 2014; Belostotskiy et al., 2015). The process imbalances result in VFA accumulation that frequently leads to complete reactors failure. Therefore, simply increasing the OLR is not appropriate to investigate the direct effect of HRT, which is presumed to be wash out of slow growing microorganisms. Since not just methanogens but also SAOB and syntrophic VFA degraders are considered as slow growing microorganisms compared to other trophic groups of the anaerobic digestion (Schnürer et al., 1999), immobilization of microorganisms as an alternative of increased retention times has been suggested in anaerobic bioreactors to stay under optimal conditions. Immobilization preventing the wash out of microorganisms can be achieved by granulation (upflow anaerobic sludge blanket (UASB) or expanded granular sludge bed (EGSB) reactors) or by biofilm attachment on specific carriers (fixed-bed or fluidized-bed reactors) (Gerardi, 2003).

In a previous study of our group, the effect of increasing OLR and decreasing HRT on anaerobic process stability, biogas quantity and quality in three distinct reactor systems with biomass retention (anaerobic sequencing batch reactor, ASBR; and fixed-bed reactor, FBR) and without it (CSTR) utilizing simulated thin stillage (STS) was investigated (Schmidt et al., 2013). In following experiments the OLR was kept at constant high level, but the HRT was further decreased from 6 to 1.5 days by diluting the substrate (STS) (Schmidt et al., 2014). The effect of decreasing HRT on the methanogenic community structure was investigated based on the analysis of archaeal 16S rRNA genes. It was found that all reactors were run stably at HRT of 6–4 days, and members of the genera *Methanoculleus* and *Methanosarcina* were the dominant methanogens under stable running conditions in the CSTR and ASBR. Surprisingly, representatives of *Methanosaeta* appeared in the digesters at HRT of 4 days and lower. In the FBR, *Methanosarcina* species were not found, but *Methanosaeta* representatives were more abundant than in the two other reactors (CSTR and ASBR).

Despite the fact that methanogenic communities involved in anaerobic digestion of STS were described in our previous work, we could not satisfactorily clarify whether methanogenic community shifts were caused by the different generation times of the methanogens or by other factors such as attachment properties, pH, accumulating VFA, nutrient, and trace element concentrations. Moreover, it remained questionable that the shift towards the predominance of *Methanosaeta* also resulted in a shift towards the aceticlastic pathway. The methanogenic community structure detected by ribosomal RNA approach does not completely reflect the actual physiological activity status of methanogens (Nikolausz et al., 2013). Therefore, further experiments were conducted on the identification of the active key microorganisms over the course of the HRT decrease from 6 to 1.5 days. The methanogenic activity was assessed by the expressed *mcrA* genes encoding the alpha subunit of methyl-CoM reductase (Luton et al., 2002) comparing results also with the 16S rRNA data obtained previously. In addition, stable isotope fingerprinting of the produced biogas was used for the investigation of the predominant methanogenic pathways (Lv et al., 2014).

Within the anaerobic food chain, $H_2$-producing bacteria form trophic links with $H_2$-consuming methanogenic archaea, acetogenic bacteria (Drake et al., 2006), sulfate-reducing bacteria (Loy et al., 2004), and Fe(III)-reducing bacteria (Lovley et al., 1995). Fe–Fe-hydrogenases catalyze the reduction of protons as terminal acceptors of electrons to produce $H_2$, therefore, genes encoding Fe–Fe-hydrogenases (*hyd*) can be used as specific biomarkers of $H_2$-producing bacteria (Vignais et al., 2001). To assess the activity of hydrogen-producing bacteria, expression of the *hydA* genes encoding the large subunit of Fe–Fe-hydrogenases were also investigated in the present study.

## 2. Methods

### 2.1. Biogas reactors and operating conditions

Three laboratory-scale biogas reactors, CSTR, FBR and ASBR, with the working volume of 5.0 L, 12.9 L, 13.0 L, respectively were operated for 137 days at 38 °C as described previously (Schmidt et al., 2014). Between 110 and 131 days of reactors operation, $NH_4HCO_3$ (5 g $L^{-1}$) and $KHCO_3$ (5 g $L^{-1}$) were added to reactors from day 110 to day 120, $CH_4N_2O$ (3.32 g $L^{-1}$) and $KHCO_3$ (3.32 g $L^{-1}$) from day 121 to day 123, $CH_4N_2O$ (2 g $L^{-1}$) and $KHCO_3$ (2 g $L^{-1}$) from day 124 to day 130 to increase the pH in all reactors. As a feeding substrate, simulated thin stillage (STS) produced by dilution and separation of dried distillers grains with solubles was used instead of fresh stillage to guarantee a constant composition. Table S1 (Supporting Information, SI) shows the process

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