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1 Effects of CO₂ enrichment on the anaerobic digestion of sewage sludge in
2 continuously operated fermenters

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Abstract

The effect of CO₂ enrichment in sewage sludge anaerobic digestion (AD) as a potential strategy to increase the biogas yield was assessed at increasing organic loading rates (OLR). Effects on process performance and resilience were evaluated in long-term continuous AD experiments at lab-scale. The specific methane production (SMP) was sustainably enhanced in the test digester compared to a control at elevated OLRs, reaching an increase of 6±12% on average at the highest OLR tested (4.0 kg_{VS}/(m³·d)). The reduction of CO₂ via homoacetogenesis, facilitating acetoclastic CH₄ formation is proposed as the dominant conversion pathway. Results suggest that sufficient load of easily degradable substances is a prerequisite for intrinsic formation of the reduction equivalent H₂ via acidogenesis. The enhanced resilience of the process under CO₂-enriched conditions in response to acid accumulation further qualifies this approach as a viable option for improving AD performance by converting a waste stream into a valuable product.

Keywords

Anaerobic digestion, CO₂ enrichment, specific methane production, process stability, sewage sludge treatment

1. Introduction

Biogas production by anaerobic digestion (AD) has become one of the leading renewable energy technologies in recent decades, with an increasing importance in sewage sludge treatment as a means of combining sludge processing and on-site energy generation in sewage treatment plants (STP). Sewage treatment is regarded as an energy-intensive process, with its electrical energy usage accounting for up to 1 to 4 % of the total consumption in developed countries (Gude, 2015; Longo et al., 2016; Nghiem et al., 2017). In Germany, STPs are responsible for about 20 % of the energy demand at the community level, which equates to roughly 3 million tons of annual carbon dioxide (CO₂) emissions (Wang et al., 2016). Energy consumption of STPs is expected to further increase in order to meet more stringent effluent qualities before discharging to water bodies (Capodaglio and Olsson, 2019). Furthermore, a significant increase in sludge production is expected in the future due to more efficient wastewater treatment and population growth (Appels et al., 2013). As a consequence, anaerobic digesters might have to be operated at higher organic loading rates (OLR), which can cause process disturbances or even complete failure of the digestion process. Therefore, enhancing the efficiency and stability of the digestion process to generate biogas by reducing biogenic CO₂ emissions and the methane (CH₄) potential in the digestate, thereby improving carbon negative renewable energy production, would be of great benefit.

CO₂ can be both a byproduct of the AD process and a reactant of intermediate reactions, hence playing a vital role in the overall process. This implies a substantial stimulatory effect of CO₂ on certain anaerobic degradation sub-processes, particularly on acetogenesis and hydrogenotrophic methanogenesis, where it is reduced to acetic acid and CH₄, respectively, by reduction equivalents such as hydrogen (H₂) (Demirel and Scherer, 2008; Misoph and

Drake, 1996; Ragsdale and Pierce, 2008). The potential to use CO₂ originating from the biogas or other waste streams in combination with externally added H₂ to promote hydrogenotrophic methanogenesis, and thus increase the biogas CH₄ content, is already being exploited in the microbial methanation approach (Götz et al., 2016; Grimalt-Alemany et al., 2018). However, the generation of H₂ is energy-intensive, and the low water solubility of H₂ is a bottleneck for full-scale implementation.

Recent studies involving conventional digestion processes have reported an increased CH₄ yield by providing solely higher CO₂ concentrations, without any exogenous H₂ addition, either in the headspace gas (Koch et al., 2016) or by enriching the mixed liquor (Bajón Fernández et al., 2017). It has been demonstrated that in this context, volatile fatty acids (VFAs) can serve as a source of H₂ for the reduction of CO₂ to CH₄ (Alimahmoodi and Mulligan, 2008). Furthermore, the ability of anaerobic bacteria to produce H₂ from carbohydrates and other substrates was substantiated in various studies (Argun and Kargi, 2011; Davila-Vazquez et al., 2008; Demirel and Scherer, 2008). This indicates the essential role of the applied substrate (both in terms of quantity and quality), as a source of reduction equivalents, on the CO₂ to CH₄ bioconversion efficiency.

Reusing CO₂ waste streams to enhance the anaerobic substrate turnover and thus increase the CH₄ yields is therefore a novel and promising approach to reduce CO₂ emissions while providing a valuable product in the form of CH₄. Increases in the specific CH₄ production (SMP) of up to 13 % were reported for a batch AD experiment treating food waste (Bajón Fernández et al., 2014). For sewage sludge, it was demonstrated that an increased CO₂ share of 20 % and 100 % in the initial headspace volume led to significantly increased CH₄ production - by more than 20 % and 30 %, respectively, relative to pure nitrogen in batch tests (Koch et al., 2016, 2015a). However, batch tests can only contribute to evaluate the

potential of a certain treatment as a first approximation, as their interpretation tends to overestimate treatment effects (Holliger et al., 2017). To reliably assess the effects of a treatment on the performance of full-scale anaerobic digesters and exclude developments that lead to deterioration of the degradation process in the long term, batch tests are not sufficiently conclusive. Thus, to further drive this new approach of CO₂ to CH₄ conversion towards full-scale implementation, continuous long-term AD experiments are needed as a next step.

So far, only a few studies have investigated the effect of increased CO₂ to CH₄ conversion in (semi-)continuous AD experiments with various substrates and by applying different reactor types, such as two-phase anaerobic digesters (TPAD), single-phase anaerobic digesters (SPAD) and upflow anaerobic sludge blanket reactors (UASB) (Bajón Fernández et al., 2017). The degradation of food waste treated in a discontinuously fed pilot-scale anaerobic digester (106 L working volume) for 77 days resulted in a CO₂-induced CH₄ production rate enhancement of 20 % in comparison to a corresponding time period before CO₂ enrichment- (Bajón Fernández et al., 2015). Enhanced CH₄ yield of 30 % associated with an increased headspace CO₂ share of 50 – 60 % v/v was reported when waste activated sludge (WAS) was treated in semi-continuous SPAD (6 L liquid working volume) for 30 days (Sato and Ochi, 1994). Comparable improvements were stated by Salomoni et al. (2011), who investigated the effect of CO₂ enrichment in the acidogenic phase of a TPAD treating a mixture of primary and secondary sludge. An increase of the specific CH₄ yield by up to 25 % compared to the full-scale SPAD was reported (Salomoni et al., 2011). More recently, Alibardi et al. (2017) observed an average specific CH₄ production increase of approx. 12 % compared to an identical control unit by periodically injecting CO₂ via a bubble column into the test fermenter fed with sewage sludge. However, the effects of a CO₂-promoted increased CH₄

formation in sewage sludge-operated systems were reported for a maximum CO₂ enrichment period of 36 days corresponding to ~ 2 HRTs (Alibardi et al., 2017). In the context of continuous anaerobic digester operation, this is rather short and does not conform to the recommendations of the VDI 4630 guideline (VDI, 2016) and Schnürer et al. (2016), which both suggesting to conducting a specific treatment for at least 3 HRTs. Consequently, it cannot be ruled out that the previously reported effect of enhanced CH₄ formation is only a transient response of the microbial community to the increased CO₂ availability and that there will not be a readjustment of metabolic activity in the long term. Furthermore, fundamental knowledge about the underlying mechanisms of CO₂ to CH₄ bioconversion and its influence on the stability of the AD process is lacking. In this context, the influence of the used substrate, as a source of reduction equivalents, on the efficiency of CO₂-induced CH₄ increase also remains unclear.

In order to bridge the gap of missing long-term investigations to reliably appraise the valorization potential of CO₂ conversion and finally deduce essential preconditions for an increased CO₂ to CH₄ turnover from the perspective of full-scale implementation, the present study aimed to systematically investigate the potentials and limitations of long-term CO₂ enrichment (> 450 days) on the AD process. Further, the relevance of increasing reduction equivalent availability as a potential prerequisite for efficient bioconversion of CO₂ to CH₄ was examined by applying different OLRs. Therefore, a continuous lab-scale AD experiment (35 L) treating sewage sludge as the major feed substrate was established. SMP, process stability (VFA to total inorganic carbon (TIC) ratio), and VFA concentrations of a periodically CO₂-enriched test unit were evaluated and compared to a control unit without CO₂ addition operated in parallel under otherwise identical conditions.

2. Material and methods

2.1. Laboratory anaerobic digester and bubble column setup

A lab-scale continuous AD experiment treating sewage sludge as the major feed substrate for 453 days was established (Figure 1). The test system (purchased from Umwelt- und Ingenieurtechnik GmbH (UIT), Dresden, Germany) comprised two identical cylindrical anaerobic digesters with a liquid working volume of 35 L each. One was used as test digester with discontinuous CO₂ enrichment (R_{CO2}) and one served as control digester without any CO₂ addition (R₀). Each unit was continuously stirred with a propeller agitator set at 40 rpm. The digesters were maintained at mesophilic conditions (38 °C) by a heating jacket and were operated at an average hydraulic retention time (HRT) of 20.6 ± 2.3 days. Both digesters were equipped with a bubble column 1 m in height and 7 cm in diameter (1.2 L liquid working volume) placed in a recirculation loop. During the CO₂ enrichment of R_{CO2}, the fermentation broth of both digesters was conveyed in co-current mode through the respective columns by eccentric screw pumps (3NU 10, Netzsch Holding, Selb, Germany). To enhance the gas to liquid mass transfer and ensure an equal distribution of small diameter CO₂ gas bubbles into the liquid phase, ceramic membranes (Ø 50 mm) with an average pore size of 10 - 16 µm (ROBU® Glasfilter-Geräte GmbH, Hattert, Germany) were placed above the gas inlet at the column bottom. The bubble column of the test unit R_{CO2} was used for CO₂ gas to liquid mass transfer, while that of the control unit R₀ served as a dummy to ensure equal liquid working volume and to keep operation of the two experimental units as closely comparable as possible.

2.2. Feeding procedure

The digesters were fed semi-continuously every two hours with an automated feeding system comprising two identical syringes (one per digester) with a volume of 5.3 L each. The substrate volume per feeding event was adjusted according to the volatile solids (VS) content of the substrates (see section 2.3) to meet the intended OLR. To maintain a constant liquid level in the digesters, an equal volume to that added per feed was disposed by gravity-driven passive overflows. During start-up and experimental phase OLR 1.5, the digesters were fed with 1.5 kg VS per day and cubic meter of digester volume ($\text{kg}_{\text{VS}}/(\text{m}^3\cdot\text{d})$). To investigate the influence of different stress regimes on the bioconversion of CO_2 , the OLR was gradually increased to 2.0 $\text{kg}_{\text{VS}}/(\text{m}^3\cdot\text{d})$ and 4.0 $\text{kg}_{\text{VS}}/(\text{m}^3\cdot\text{d})$ in phase OLR 2.0 and OLR 4.0, respectively, while maintaining the HRT at about 20 days (detailed description in section 2.3.). Each phase was maintained for a minimum of 3 HRTs as recommended by VDI 4630 (2016). Thus, sufficient time was guaranteed for the digestion process to acclimate to the prevailing operational settings. In order to avoid shock instability of the digestion process due to the steep increase of organic loading from OLR 2.0 to OLR 4.0, a short adaptation phase (1.5 HRTs) with an OLR 3.0 $\text{kg}_{\text{VS}}/(\text{m}^3\cdot\text{d})$ (OLR 3.0) was intercalated.

2.3. Source of inoculum and feedstock

Both digesters were initially inoculated with digested sludge (DS) of a full-scale digester located at the Garching STP (approximately 20 kilometers north of Munich, Germany), which has a design capacity of 45,000 population equivalents. The digester is fed with a mixture of primary and WAS, and is operated at mesophilic conditions (approx. 38 °C) at an HRT of 30 days and an associated OLR of 1.0 $\text{kg}_{\text{VS}}/(\text{m}^3\cdot\text{d})$.

The digesters were fed with thickened WAS (FS1) from Freising STP, with a design capacity of 110,000 population equivalents. The sludge was stored at 4 °C for a maximum of two weeks until use. From experimental day 261 on, the WAS was supplemented with dog food pellets

(Nature plus Junior, GoRaw LLC) as a surrogate of food waste (Vinnerås et al., 2003) to increase the VS content of the feedstock (FS2). The dog food contained 89.9 % total solids (TS) and, according to the producer, was composed of 28.0 % protein, 11.5 % lipid, 3.1 % fiber, 6.5 % ash, and the remains (including carbohydrates) accounted for 50.9 % on a dry-weight basis. In order to maintain a constant HRT of approximately 20 days at different OLRs (FS2-OLR 2.0; FS2-OLR 3.0; FS2-OLR 4.0), a corresponding amount of tap water was added to the dried dog food pellets.

The TS and VS concentrations of the inoculum and feedstocks (FS1; FS2-OLR 2.0; FS2-OLR 3.0; FS2-OLR 4.0) were determined according to standard methods (Baird et al., 2017) and are reported in Table 1.

2.4. CO₂ enrichment of the digester content

Discontinuous CO₂ enrichment was commenced on day 158 after both digesters reached equal steady-state conditions (see section 2.6). From that day on, CO₂ gas was injected from the bottom of the R_{CO2} bubble column at a gas flow rate of 1 L per minute three times a week for three hours each. The gas flow rate was controlled by a gas mixing system (QCAL Messtechnik GmbH, Munich, Germany) supplied by a gas cylinder (Air Liquide, Düsseldorf, Germany). Excess CO₂, which did not dissolve during passage across the bubble column, was discharged through the column outlet at the upper top (Figure 1). During each enrichment period, the eccentric screw pumps of both reactors were operated at a flow rate of 1.25 L/minute achieving a complete recirculation of the liquid working volume every 30 minutes. To ensure homogeneity of the fermentation broth, also for sampling, the pumps were already activated two hours before the CO₂ enrichment.

2.5. Analytical methods

The biogas volume was measured continuously using drum-type gas flow meters (TG-05, Dr.-Ing. RITTER Apparatebau GmbH & Co. KG, Bochum, Germany) connected to each unit and normalized to standard conditions (0 °C, 101,325 Pa). Before composition analysis, the biogas was temporarily stored in a 10 L gas bag. The biogas composition was automatically analyzed every two hours for CH₄ (%), CO₂ (%), O₂ (%) and H₂ (ppm) concentration by a gas analyzer (SSM-6000, Pronova Analysetchnik GmbH & Co. KG, Berlin, Germany). Based on the gas analyzes the SMP was calculated referring to kg of VS added per day [$SMP = L_{CH_4}/(kg_{VS} \cdot d)$].

The fermentation broth was constantly monitored for pH value, oxidation reduction potential (ORP) and temperature by probes installed on the lid of each digestion unit.

Before the CO₂ enrichment was commenced in the R_{CO₂}, the digestate of both digesters was analyzed three times a week for VFA/TIC ratio, TS and VS. In addition, samples for total and individual VFA (acetic acid, propionic acid, n-butyric acid, iso-butyric acid, n-valeric acid, iso-valeric acid) determination were collected on a regular basis. Samples were taken from a tap placed in the recirculation loop downstream of the bubble column. To ensure sufficient mixing of the fermentation broth and thus representative samples, the screw pumps were started at least one hour before sampling. Sampling was conducted 30 minutes before the next feeding to obtain representative samples of the continuous AD experiment.

Digestate sampling was done three times a week twice a day throughout the discontinuous CO₂ enrichment period. The samples were taken one hour before and directly after the CO₂ injection in R_{CO₂}. Digestate samples were subsequently analyzed in triplicate for VFA/TIC ratio, TS and VS. To determine the VFA/TIC ratio, samples were diluted 1:5 with MilliQ water to 50 mL and titrated with 0.1 N sulfuric acid (Titrisol, Merck, Darmstadt, Germany) to pH 5.0 and pH 4.4 using the TitraLab AT 1000 (Hach, Düsseldorf, Germany). The VS removal was determined according to Koch (2015b). Samples for VFA analysis were frozen at -20 °C and

stored until analysis. Individual VFAs were quantified by head space-gas chromatography-flame ionization detection. Further details are given within the supplementary information. To evaluate the gas to liquid CO₂ transfer under the prevailing anaerobic conditions, digestate samples of both reactors were analyzed for dissolved CO₂ concentration via end-point titration (according to DIN 38409-H7-4-1) with 0.05 M sodium hydroxide (Titrisol, Merck, Darmstadt, Germany) before and after CO₂ enrichment.

2.6. Steady-state and performance data analysis

To assess the impact of enhanced CO₂ availability on the AD process at varying stress regimes, equal performance of R_{CO2} and R₀ was required before starting the CO₂ injection. In general, no obligatory criteria to determine equal performance and steady-state conditions of continuous AD experiments exist. Therefore, minimum steady-state criteria for the start-up phase, which should prevail for at least one HRT, were defined. These criteria were developed partially based on recommendations by Lippert et al. (2020):

(1) A maximum of 15 % relative standard deviation (RSD) of all measured parameters for both reactors individually, and

(2) An average daily percentage difference between test and control of less than 10 % for all measured parameters.

(3) The defined criteria were tested for all investigated measured variables, being ORP, pH value, VS removal, VFA/TIC ratio, CH₄ content in the biogas and SMP.

Analysis of disparity between the data sets obtained from control and test digester were assessed using a student's *t*-test as described by Lippert et al. (2020). All measurements of one individual parameter obtained during one individual HRT were defined as one data set. Differences were considered as significant at $p < 0.05$ and as highly significant at $p < 0.01$,

which will be indicated in the following figures with the asterisk symbols * and **, respectively.

3. Results and discussion

3.1. Stable continuous anaerobic digestion experiment – start-up phase

Stable operation of reactors employed in continuous AD experiments is crucial to assess the impact of an applied treatment. In this study, to evaluate the effects of enhanced CO₂ availability on the AD process, the performance of both digesters during the start-up phase was compared as a first step. Steady-state conditions according to the predefined criteria (section 2.6) were reached after 114 days, and maintained for 1.9 HRTs (40 days) before CO₂ injection was commenced in the test reactor R_{CO2} on day 154. Digestate characteristics, SMP and the CH₄ content of the produced biogas before CO₂ enrichment are summarized in Table 2 for both digesters. The maximum percentage difference between both fermenters during this initial period was determined for the VFA/TIC ratio with 9.2 ± 11.5 %. Thus, the criterion (2) of less than 10 % average difference between test and control unit was met for all parameters investigated. The RSD of all measured values was below < 15 % with a maximum RSD of 11.9 % for the SMP of the control unit R₀ and thus fulfilled criterion (1) of the steady-state analysis.

3.2. Assessment of semi-continuous CO₂ enrichment on digestion performance

The SMP is a highly sensitive parameter and has been successfully applied to assess the overall impact on the AD process of pre-treatments, such as ultrasonication (Lippert et al., 2018), microwave (Appels et al., 2013), thermal, chemical and biological treatment (Carrere

et al., 2016; Souza et al., 2013) or an increase in CO₂ availability during the digestion process (Bajón Fernández et al., 2017). The long-term effect of CO₂ enrichment on the digestion performance at different OLRs was therefore evaluated based on the SMP in this study. Furthermore, to appraise potential negative impacts, the trends in pH value, total and individual VFA concentrations were monitored.

Performance parameters of the control unit R₀ and test digesters R_{CO2} were measured at the different OLRs operated throughout the CO₂ enrichment period and are summarized in Table 3.

CO₂ can engage in several biochemical reactions of the AD process, such as the carbonic acid equilibrium. Observing the evolution of the pH value over the whole experimental period did not reveal significant deviations between R_{CO2} and R₀ (Table 3). Thus, acidification caused by increased dissolved CO₂ concentrations can be excluded due to the buffering properties of the sludge. This is in accordance with findings previously reported by Bajón Fernández et al. (2015), Al-mashhadani et al. (2016) and Alibardi et al. (2017) where CO₂ enrichment did not lead to long-term impacts on the pH value in semi-continuous SPAD treating food waste, kitchen waste and sewage sludge, respectively.

3.2.1. CO₂ treatment effects at low organic loading rates

After the start of the semi-continuous CO₂ injection, the average daily percentage difference in SMP increased significantly by 6.6 ± 10.1 % compared to the control unit R₀ within the first HRT (20 days) of the test phase OLR 1.5 (R_{CO2}: 179 ± 17 L_{CH4}/(kg_{VS}·d) vs. R₀: 169 ± 18 L_{CH4}/(kg_{VS}·d); student's t-test, $p < 0.05$) (Figure 2 B). However, the initial enhancement was not sustained and the SMP dropped to even slightly negative percentage differences in the second and third HRT.

Limitation of the CO₂ gas to liquid mass transfer was stated as a potential reason for an insufficient CO₂ to CH₄ bioconversion when bubble columns were applied to enhance the dissolved CO₂ concentration in the fermentation broth (Alibardi et al., 2017; Bajón Fernández et al., 2015). With the experimental setup applied in this study, the average dissolved CO₂ concentration after each CO₂ enrichment (1 liter (L) per minute for 180 minutes) was 962 ± 140 mg/L, equivalent to an increase of 28.3 ± 7.4 % and 40.0 ± 12.1 % compared to the concentrations of the test digester R_{CO2} before injection and the control digester R₀, respectively. Thus, 4.92 ± 1.70 L per CO₂ injection, corresponding to 2.7 ± 1.0 % of the injected CO₂ volume, was dissolved in the liquid phase of R_{CO2} with each injection event. The obtained enrichment of the digester content with a surplus of 9.7 ± 3.4 g CO₂ with each CO₂ injection was below a previously reported CO₂ input of 18.445 g CO₂ per enrichment when a comparable experimental setup was applied (Bajón Fernández et al., 2015). However, even though an improved gas to liquid mass transfer would be desirable, a significant increase in the dissolved CO₂ concentration was achieved allowing for the observation of the previously reported CO₂ to CH₄ bioconversion mechanism. The CO₂ content surveyed in the biogas of the test digester R_{CO2} was 40.1 ± 3.1 % and thus increased by 3.8 ± 0.2 % compared to the control digester R₀ throughout the whole enrichment trial.

These observations suggest that after an initial positive stimulation of microbial activity, the microbial community and its metabolism adapted to the changed conditions. An initial but non-sustained boost of CH₄ formation has previously been reported by Lippert et al. (2020) after ultrasound was used as a method of increasing the AD efficiency. Alibardi et al. (2017) investigated the effect of CO₂ enrichment on sewage sludge AD in a comparable experimental setup. The enrichment of the fermentation broth via a bubble column three

times a week did not result in a notable improvement of the SMP in the test digester. An intensified CO₂ enrichment, five times a week with a packed column to enhance the gas to liquid mass transfer, was needed to obtain a 12 % increment of the SMP, which was sustained over an observation period of 37 days (corresponding to ~ 2 HRTs). However, since the experiment was terminated at this point, it remains unclear whether the SMP increase was of a permanent nature or would have reverted to the initial yields.

No significant long-term effects of the CO₂ enrichment on the AD process were observed during the remaining operation at OLR of 1.5 kg_{VS}/(m³·d). The performance of R_{CO2} was slightly lower (by 0.2 % on average) than R₀ for the following 4 HRTs. With an increase of the OLR to 2.0 kg_{VS}/(m³·d) on day 248, still no distinct difference between R_{CO2} and R₀ was observed due to CO₂ enrichment. Nevertheless, the SMPs increased by 52 % in R₀ and by 56 % in R_{CO2} (Figure 2 A), probably due to the alteration of the feedstock on day 261. A protein-rich food waste was added to the sewage sludge in the ratio of 20:80 in order to increase the VS content of the feed substrate and thus maintain the associated HRT at about 20 days (see section 2.3.). Analysis of the digestate of R_{CO2} and R₀ revealed acetic acid concentrations of 29.3 ± 6.1 mg/L and 30.6 ± 10.4 mg/L, respectively, in phase OLR 1.5 and of 12.1 ± 5.7 mg/L and 8.5 ± 6.8 mg/L in phase OLR 2.0. In the respective time periods, none of the longer-chained VFAs investigated were detectable (Figure 3). This shows that the food additive had no measurable negative impact in terms of reduced microbial degradation rates. As the acetic acid concentrations were extremely low in both test phases OLR 1.5 and OLR 2.0, and even below the detection limit on some days, it can conclusively be stated that the upstream microbial conversion rates did not exceed the metabolic capacities of the methanogens. This can be explained by the degradation characteristics of sewage sludge, which is known to be limited primarily by hydrolysis (Ponsá et al., 2008) and hence does not

overburden the assimilatory capacities of microbes involved in the subsequent degradation stages at low OLRs.

3.2.2. CO₂ treatment effects at increased organic loading rates

Starting with an OLR of 3.0 kg_{VS}/(m³·d), the CH₄ production in the test digester R_{CO2} was consistently higher than in the control fermenter R₀. In the progressing time, a slightly increasing benefit in SMP of 3.2 ± 6.3 % and 6.3 ± 12.2 % was observed on average for OLR 3.0 kg_{VS}/(m³·d) and 4.0 kg_{VS}/(m³·d), respectively, with significant differences in the last two HRTs (student's t-test, $p < 0.05$ and $p < 0.01$) at the highest tested OLR (Figure 2 B). An increase in VFA occurred at an OLR of 4.0 kg_{VS}/(m³·d) in both digesters compared to values at the lower loading rates (Figure 3). Between days 369 and 380, significantly higher acetic acid concentrations were present in both reactors, which can be attributed to an enhanced rate of hydrolytic-fermentative turnover compared to methanogenic degradation. On day 399, considerable acid accumulation was detected in both fermenters. For the first time during the trials, propionic, iso-valeric, iso-butyric and n-butyric acids were also measured in higher concentrations (Figure 3). After the peak of acids accumulation was exceeded on day 418, VFA analysis still identified elevated concentrations of n-butyric and iso-valeric acid with acetic acid remaining the dominant species (Figure 3). Interestingly, the concentration of acetic acid was 5.2 % higher on average in R_{CO2}, possibly due to activation of the Wood-Ljungdahl and/or the glycine cleavage pathway of homoacetogenesis by the addition of CO₂. This agrees with the results previously reported by Mohd Yasin et al. (2015), who also observed increased acetic acid formation in a methanogen-enriched culture from WAS in response to CO₂ sparging with subsequent acetic acid degradation and CH₄ production. A positive stimulatory effect of CO₂ on the Wood-Ljungdahl pathway was also suggested by Francioso et al. (2010), who observed a remarkable reduction of CO₂ introduced into the first

(fermentation) phase of a TPAD with a concomitant substantial increase in VFAs. Simultaneously with the comparatively higher acetic acid concentrations (after day 390), the SMP of R_{CO_2} exceeded that of the control unit R_0 with a highly significant benefit of $14.3 \pm 18.9 \%$ (R_{CO_2} : $352 \pm 38 \text{ L}_{CH_4}/(\text{kg}_{VS} \cdot \text{d})$ vs. R_0 : $314 \pm 55 \text{ L}_{CH_4}/(\text{kg}_{VS} \cdot \text{d})$; $p < 0.01$) (Figure 2 A). This might be attributed to more efficient degradation of the accumulated VFAs (and presumably also of other intermediates) as adaptation to CO_2 -induced increased acetate formation improving substrate supply for acetoclastic methanogens. However, this CO_2 -stimulated increased degradation efficiency due to either adaptation of the microbial community composition and/or higher metabolic activity needs to be investigated further.

The phases operated at the higher OLRs of $3.0 \text{ kg}_{VS}/(\text{m}^3 \cdot \text{d})$ and $4.0 \text{ kg}_{VS}/(\text{m}^3 \cdot \text{d})$ were characterized by a depletion of the biogas H_2 concentration in R_{CO_2} compared to R_0 (Figure 2 C). At OLR 4.0, the H_2 concentration detected in R_{CO_2} was on average significantly lower, by $162 \pm 658 \text{ ppm}$ (student's t-test, $p < 0.05$), than that in R_0 . The lower H_2 concentration can be attributed to a boosted assimilation by hydrogenotrophic methanogens but could also suggest an increased consumption by homoacetogens, reducing CO_2 and forming acetic acid. It is more likely that the observation of higher acetic acid concentrations in R_{CO_2} (days 422 – 448) described previously may reveal an enhanced acetogenic assimilation of the introduced CO_2 . The improved SMP in the test reactor R_{CO_2} in phase OLR 4.0 can thus potentially be traced back to an increased activity of acetoclastic methanogens under CO_2 enrichment, as was also proposed by other authors (Alibardi et al., 2017; Bajón Fernández et al., 2019; Salomoni et al., 2011). However, an activation of the hydrogenotrophic methanogenesis cannot be entirely ruled out.

Previously reported enhancements of the SMP in CO_2 -enriched semi-continuously operated anaerobic digesters treating sewage sludge ranged from 12 % to 30 %, which is higher than

in the present study. Salomoni et al. (2011) reported 25 % enhanced CH_4 formation when a TPAD was supplemented with CO_2 . The control, however, was a full-scale SPAD, which makes it impossible to distinguish between benefits of CO_2 and two-stage digestion. A 12 %-increased CH_4 yield was reported by Alibardi et al. (2017) when the performance of the digester exposed to CO_2 injection was compared to an identical control unit operated in parallel. The reported improvement was twice as high compared to the present study, but was also associated with higher VFA concentrations in the digestate. As VFA can serve as an alternative source for H_2 , these compared with the results of the present study suggest that the bioconversion of CO_2 to CH_4 is increased if more easily degradable partially reduced substrates, e.g., high VFA concentrations in the fermentation broth, are present, which is typically the case at higher OLRs. This further emphasizes the essential role of the quantity and quality of the substrate used to sustainably improve CO_2 to CH_4 bioconversion.

It can conclusively be stated that CO_2 enrichment did not adversely affect the process performance, based on the parameters SMP (Figure 2 A), pH value (Table 3) and the evolution of individual VFA concentrations (Figure 3). At elevated OLRs, an increasing trend in CH_4 production prevailing for 7 HRTs was observed. Thus, long-term improvement of the digestion performance can be achieved by CO_2 replenishment. In this context, the production of the degradation-intermediate H_2 appears to be crucial, which can be facilitated by either elevated OLRs or the use of more easily degradable substrates (such as food waste compared to sewage sludge). Even if only minor gains in CH_4 yields are obtained, as in this study, the CO_2 enrichment benefits greatly from its rather simple implementation and low investment costs in cases where digester content is mixed by means of biogas recirculation and a system for CO_2 removal for biogas enrichment already exists.

3.3. Effect of enhanced CO_2 availability on process stability

Stable process performance is characterized by equilibrated steady production and consumption of degradation intermediates such as H_2 and VFAs. Digestion stability was therefore assessed by the VFA/TIC ratio, a simple and robust indicator of process disturbances such as acidification events, e.g., as a result of organic overload (Wilches et al., 2019). In general, VFA/TIC ratios < 0.3 are considered for a stable digestion process (Ripley and Boyle, 1986). However, VFA/TIC ratios are highly dependent on the analyzed system. Stable operation can also be assumed if constantly elevated values are measured over time without deterioration of the biogas volume and quality (Lebuhn et al., 2014).

In the course of the continuous digestion experiment, the VFA/TIC ratio showed steady operation even at a high OLR of $4.0 \text{ kg}_{VS}/(\text{m}^3 \cdot \text{d})$ (except HRT 3 in OLR 4.0) with maximum values of 0.38 and 0.43 in R_{CO_2} and R_0 , respectively (Figure 4). In HRT 3 of test phase OLR 4.0, the VFA/TIC ratio increased rapidly in both digesters, but more pronouncedly in R_0 (Figure 4). On day 408, maximum values of 0.87 and 0.60 were measured in R_0 and R_{CO_2} , respectively. This phase was further characterized by lower pH values (Figure 5 B) and substantially increased total VFA (tVFA) concentration in R_0 . On day 410, the tVFA concentration in R_0 reached 4,616 mg/L, which was 38 % above the value measured in R_{CO_2} (Figure 3). The observed higher tVFA concentration between experimental days 399 and 415 probably derived from a feedstock batch used for the indicated feeding interval, which was exposed to a prolonged storage time (during the Christmas holidays), and might have been spoiled, causing the accumulation of VFAs.

The tVFA concentration, with acetic acid being the dominant species, reached substantially higher levels in the control unit R_0 (Figure 3). As CO_2 can be reduced by acetogens via the Wood-Ljungdahl and/or glycine cleavage pathway to acetic acid (Ragsdale and Pierce, 2008; Ruiz-Sánchez et al., 2018), the lower acetic acid concentrations measured in R_{CO_2} may be the

result of a more efficient methanogenic acetic acid catabolism, as compared to R_0 , possibly due to a CO_2 -induced adaptation of acetoclastic methanogens to a higher substrate availability. Improved methanogenic activity in R_{CO_2} was further confirmed by the fact that the percentage difference showed a 4.4 ± 7.6 % increased SMP in R_{CO_2} (Figure 5 A).

Isochronous with the feeding of a new batch on day 416, the VFA/TIC ratio and the VFA concentrations started to decrease in both fermenters (Figure 4), while the ORP in R_0 remained at a high level, indicating that stronger reducing conditions had been established in the test unit R_{CO_2} (Figure 5 C). The HRT 4 in OLR 4.0 (days 418 – 437) was further characterized by lower tVFA and, especially, acetic acid concentrations in R_0 , while the SMP was outperformed by more than 14 % by the test unit R_{CO_2} (Figure 5 A). The observations made for the test unit R_{CO_2} of (1) increased acetic acid degradation during acid accumulation and, after recovery from acid accumulation, (2) elevated SMP and (3) increased acetic acid availability, suggest that CO_2 enrichment stimulates acetate generation via homoacetogenesis, leading to activation of acetoclastic CH_4 formation.

The difference in the stress response between R_0 and R_{CO_2} can be associated with an improved microbial resistance in CO_2 -enriched anaerobic digesters. A comparable improved response to transiently unbalanced process conditions, namely lower peaks in stability parameters, less pronounced acetic acid accumulation, and faster recovery was also reported by Alibardi et al. (2017) for a SPAD subjected to CO_2 enrichment. CO_2 -induced ameliorated process resilience was also observed by Bajón Fernández et al. (2015), where the test and control anaerobic digesters treating food waste were subjected to failure of the temperature control, leading to similar temperature drops. Both digesters faced a recession in biogas production and decreased pH values, but the impact was significantly lower and no VFA accumulation occurred, as well as a faster recovery being observed for the test reactor,

while the control unit needed to be partially reseeded. Microbial community investigations during start-up of co-digesting fermenters treating municipal solid waste and sewage sludge revealed that digesters that experienced a history of high acetic acid concentrations had selected methanogenic populations with faster acetate assimilatory capacity (McMahon et al., 2004). Thus, CO₂-supported enhanced acetic acid formation via homoacetogenesis might facilitate the establishment of acetoclastic methanogens capable of high acetate turnover.

The potential benefit of CO₂ injection in process resilience is an argument in favor of the application of CO₂ enrichment as a technology to improve the digestion efficiency and hence increase biogas yields. Particularly for digestion processes frequently facing operational problems such as food waste digesters, CO₂ augmentation could be a promising option to encourage stable operation and avoid CH₄ potentials in the digestate. With regard to further development in capacity utilization of existing AD plants by means of co-digestion and the expansion of the STP infrastructure, which may confer increased sludge volumes, CO₂ enrichment may make it possible to deal with these challenges more effectively.

4. Conclusions

CO₂ enrichment of AD for more than 450 days proved to be a promising approach to a) increase the production of renewable energy while reducing CO₂ emissions and b) improve resilience after process disturbance. A sufficient supply of organic substances as a source of the co-substrate H₂ deriving from VFA degradation appeared to be a crucial prerequisite to facilitate CO₂ to CH₄ bioconversion in long-term trials. Even a minor increase in SMP can be

economically relevant for full-scale digesters with existing infrastructure for biogas circulation and can help reduce the CH₄ potential in the digestate.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at “XXX”.

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Figure captions

Figure 1: Schematic of the lab-scale digester system, including anaerobic digesters (control R_0 and test R_{CO_2}), recirculation loops and bubble column reactors.

Figure 2: Evolution of A) the specific methane production (SMP) of the test fermenter R_{CO_2} (red) and control fermenter R_0 (black) (dotted lines show the moving average; the mean values ($n = 20$) of one hydraulic retention time (HRT) are indicated as symbols), and the average deviation in B) SMP and C) hydrogen concentration per HRT from R_{CO_2} compared to R_0 . The vertical red line marks the start of the discontinuous CO_2 injection. Vertical dark solid lines indicate changes in organic loading rates (OLR in $kg_{VS}/(m^3 \cdot d)$). The distance between lighter solid lines indicates one HRT. Equal performance of both reactors is represented by the

horizontal line (0% deviation). Significant differences between R_{CO_2} and R_0 within the respective HRT are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$).

Figure 3: Total volatile fatty acid (tvFA) concentrations in test digester R_{CO_2} (red bars) and control digester R_0 (black bars). The concentrations of the individual VFA acetic acid (AA), iso-butyric acid (i-BA), n-butyric acid (n-BA), iso-valerianoic acid (i-VA) and propionic acid (PA) are indicated as symbols in red and black for R_{CO_2} and R_0 , respectively.

Figure 4: Trend of the volatile fatty acids to total inorganic carbon (VFA/TIC) ratio in test digester R_{CO_2} (red) and control digester R_0 (black). The vertical red line marks the start of the discontinuous CO_2 injection. Dark solid lines indicate altered organic loading rates (OLR, $kg_{VS}/(m^3 \cdot d)$). The distance between lighter solid lines indicates one HRT. The period of high VFA accumulation is highlighted with a light red box.

Figure 5: Trends of A) specific methane production (SMP), B) pH value and C) oxidation reduction potential (ORP) in the test digester R_{CO_2} and control digester R_0 at the organic loading rate (OLR) of $4.0 kg_{VS}/(m^3 \cdot d)$. The distance between dark solid lines indicates one hydraulic retention time (HRT). The period of high volatile fatty acids (VFA) accumulation is highlighted with a light red box.

- Long-term continuous anaerobic digestion experiments treating sewage sludge (450 d)
- Effects of CO₂ enrichment on the anaerobic digestion process
- Significantly increased methane production as immediate response to CO₂ enrichment
- Sustainably enhanced specific methane production at higher organic loading rates
- Improved process resilience after disturbance in a CO₂-enriched environment

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Tables

Table 1: Characteristics of inoculum and feedstocks (average $\pm$ standard deviation (SD)).

	Inoculum (sieved)	Feedstock			
		FS1	FS2-OLR 2.0	FS2-OLR 3.0	FS2-OLR 4.0
n	1	53	25	9	27
Feeding period (days of experiment)		110 - 262	263 - 334	335 - 357	358 - 453
TS [% of FM]	2.49 $\pm$ 0.09	4.38 $\pm$ 0.49	6.03 $\pm$ 0.30	7.04 $\pm$ 0.42	9.60 $\pm$ 0.49
VS [% of TS]	64.2 $\pm$ 0.7	71.3 $\pm$ 2.5	77.3 $\pm$ 2.4	79.4 $\pm$ 0.2	83.5 $\pm$ 1.5

n = Number of samples, FM = Fresh matter, FS = Feedstock, TS = Total solids, VS = Volatile solids

Table 2: Digestate and biogas characteristics (average $\pm$ SD) and mean daily percentage differences between the control (R_0) and test (R_{CO_2}) reactors during start-up phase before CO_2 enrichment.

		Control R_0		Test R_{CO_2}		R_{CO_2}/R_0 [%]
Before CO_2 enrichment	ORP [mV]	-498 $\pm$ 4	(n=40)	-503 $\pm$ 3	(n=40)	0.9 $\pm$ 0.6
	pH value [-]	7.5 $\pm$ 0.1	(n=37)	7.4 $\pm$ 0.0	(n=40)	1.8 $\pm$ 1.2
	VFA/TIC [-]	0.39 $\pm$ 0.04	(n=12)	0.43 $\pm$ 0.03	(n=12)	9.2 $\pm$ 11.4
	VS removal [%]	46.0 $\pm$ 2.9	(n=13)	45.5 $\pm$ 4.0	(n=13)	3.7 $\pm$ 3.0
	SMP [$L_{CH_4}/(kg_{VS}\cdot d)$]	190 $\pm$ 23	(n=38)	191 $\pm$ 19	(n=38)	6.2 $\pm$ 6.2
	CH_4 [%]	64.2 $\pm$ 0.7	(n=40)	64.3 $\pm$ 1.1	(n=40)	1.1 $\pm$ 1.5

ORP = Oxidation reduction potential, VFA/TIC = Volatile fatty acids to total inorganic carbon ratio, VS = Volatile solids, SMP = Specific methane production

Table 3: Average digestate and headspace characteristics (average $\pm$ SD) of the control (R_0) and test (R_{CO_2}) digesters at different organic loading rates (OLR) throughout the CO_2 enrichment period.

	OLR 1.5		OLR 2.0		OLR 3.0		
	R_0	R_{CO_2}	R_0	R_{CO_2}	R_0	R_{CO_2}	
ORP [mV]	-488 ± 6	-486 ± 8	-494 ± 8	-498 ± 11	-510 ± 3	-504 ± 3	-5
pH value [-]	7.4 ± 0.1	7.4 ± 0.1	7.3 ± 0.1	7.3 ± 0.1	7.4 ± 0.0	7.5 ± 0.0	7
CH_4 [Vol%]	63.4 ± 1.9	55.1 ± 3.9	59.0 ± 2.8	55.7 ± 3.1	59.2 ± 1.5	56.6 ± 1.9	58
CO_2 [Vol%]	33.0 ± 1.3	40.6 ± 4.0	37.5 ± 2.6	40.2 ± 3.0	37.6 ± 1.1	39.8 ± 1.5	38
H_2 [ppm]	299 ± 64	280.1 ± 63	$971 \pm 1,097$	$951 \pm 1,023$	$2,150 \pm 1,391$	$2,075 \pm 1,326$	3,7
SMP [$L_{CH_4}/(kg_{VS} \cdot d)$]	192 ± 35	188 ± 30	267 ± 75	267 ± 78	364 ± 85	376 ± 82	3
VFA/TIC [-]	0.44 ± 0.1	0.48 ± 0.0	0.43 ± 0.1	0.44 ± 0.1	0.35 ± 0.0	0.37 ± 0.0	0.
VS removal [%]	45.3 ± 2.7	44.6 ± 2.4	54.8 ± 7.9	54.6 ± 8.1	60.2 ± 1.2	60.9 ± 1.1	67

ORP = Oxidation reduction potential, SMP = Specific methane production, VFA/TIC = Volatile fatty acids/total inorganic carbon, VS = Volatile solids

45.



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Effects of CO₂ enrichment on the anaerobic digestion of sewage sludge in continuously operated fermenters

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