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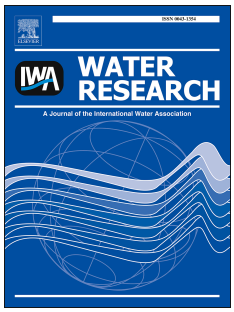

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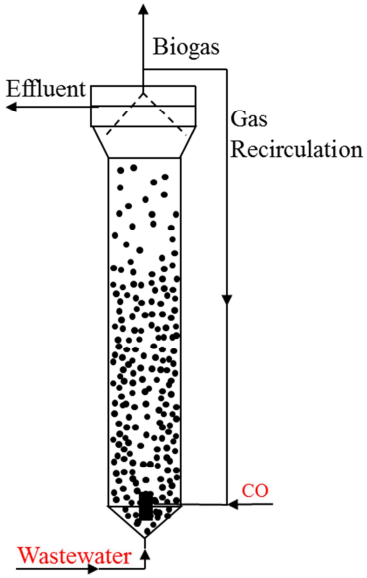

Responses of AGS to CO

- Enhanced hydrogenotrophic CO-oxidizing pathway
- Overproduction of EPS
- Changes in the appearance and microbial community compositions

Metagenomic analysis

- Reconstruction of 70 GBs
- 23 enriched GBs containing genes relating with CO conversion
- Reveal of more taxonomically diverse CO-converting microorganisms

Anaerobic granular sludge for simultaneous biomethanation of synthetic wastewater and CO with focus on the identification of CO-converting microorganisms

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Abstract:

CO is a main component of syngas, which can be produced from the gasification of organic wastes and biomass. CO can be converted to methane by anaerobic digestion (AD), however, it is still challenging due to its toxicity to microorganisms and limited knowledge about CO converting microorganisms. In the present study, anaerobic granular sludge (AGS) was used for the simultaneous biomethanation of wastewater and CO. Batch experiments showed that AGS tolerated CO partial pressure as high as 0.5 atm without affecting its ability for synthetic wastewater degradation, which had higher tolerance of CO compared to suspended sludge (less than 0.25 atm) as previously reported. Continuous experiments in upflow anaerobic sludge blanket (UASB) reactors showed AGS could efficiently convert synthetic wastewater and CO into methane by applying gas-recirculation. The addition of CO to UASB reactor enhanced the hydrogenotrophic CO-oxidizing pathway, resulted in the increase of extracellular polymeric substances, changed the morphology of AGS and significantly altered the microbial community compositions of AGS. The microbial species relating with CO conversion and their functions were revealed by metagenomic analysis. It showed that 23 of the 70 reconstructed genome bins (GBs), most of which were not previously characterized at genomic level, were enriched and contained genes involved in CO conversion upon CO addition. CO-converting microorganisms might be taxonomically more diverse than previously known and have multi-functions in the AD process. The reductive tricarboxylic acid (TCA) cycle in combination with the oxidation of the CO was probably crucial for CO utilization by the majority of the GBs in the present study.

Key words: Anaerobic granular sludge; Wastewater treatment; CO biomethanation; Metagenomic analysis; CO-converting microorganisms

1. Introduction

Biomass-based energy benefits society by providing clean fuels from renewable feedstocks. Although biomass can be biologically converted to biofuels, a significant portion is difficult to be biodegraded due to the refractory and polymeric characteristics (Demirbas 2007, Guo et al. 2015). Thermal gasification is an alternative method to convert biomass to syngas (Fytili and Zabaniotou 2008). Gasification is the process by which biomass is treated at high temperature (>700 °C) and pressure conditions, supplied with limited amounts of oxygen in order to only partially oxidize biomass. By this partial oxidation, the biomass is converted to syngas consisting mainly of CO, H_2 and CO_2 (Gaunt and Lehmann 2008). Syngas can also be produced from the gasification of municipal solid wastes (Arena 2012). In addition, there are also CO-rich industrial off-gases (E.g. steel mill off gases) (Köpke et al. 2011). Although syngas or CO-rich gases can directly be used as fuels, the volumetric energy densities were less than 50 % of natural gas (mainly CH_4) (Munasinghe and Khanal 2010a). The conversion of syngas to methane can meet the increasing demand for natural gas (Liu et al. 2012).

Anaerobic conversion of CO and H_2/CO_2 to CH_4 by mixed cultures is feasible and previously demonstrated (Luo et al. 2012, Munasinghe and Khanal 2010b). Since anaerobic conversion of H_2/CO_2 to CH_4 is a well-known biological reaction occurring in biogas reactors, and also H_2 can be separated from syngas to be used as fuel or chemical, the present study mainly focused on the biomethanation of CO. The biomethanation of CO in biogas reactor treating sewage sludge was demonstrated in our previous study (Luo et al. 2013). Two main challenges for bioconversion of CO in biogas reactors treating sewage sludge were identified. One is the toxicity of CO to methanogens at high CO partial pressures. It was found that the activity of

methanogens was suppressed with the increase of CO partial pressure to higher than 0.25 atm (Luo et al. 2013), while the activity of bacteria was not obviously affected. The other challenge was the identification of CO conversion bacteria. The known CO conversion bacteria were not identified by high-throughput sequencing (HTS) of 16S rRNA genes (Luo et al. 2013), indicating the possible presence of novel CO conversion bacteria.

Until now, the biomethanation of CO in anaerobic granular sludge (AGS) based reactors treating organic wastewater has not been studied. Upflow anaerobic sludge blanket (UASB), expanded granular sludge bed (EGSB) and internal circulation (IC) reactors, using granular sludge as microbial catalyst, can withstand higher influent flows than the growth rates of the microorganisms. The microorganisms are not washed out due to the good settling abilities of AGS (Schmidt and Ahring 1996), and therefore the above rectors are widely used in the treatment of both high and low concentration organic wastewater (McHugh et al. 2003, Pereboom and Vereijken 1994). AGS, which has unique layered structure and properties (Grotenhuis et al. 1991), may reduce CO toxicity to methanogens. For instance, bacteria, which can degrade organics to H_2 and acetate, are mostly distributed in the outer layer of the granules, while methanogens are distributed in the inner layers of the granules which can further convert H_2 and acetate to CH_4 (Sekiguchi et al. 1999). Therefore, CO might be converted by bacteria in the outer of AGS into intermediates (H_2 or acetate) (Satoh et al. 2007), and then the intermediates could diffuse into the inner of AGS to produce methane by methanogens, which therefore avoid the direct contact between CO and methanogens.

The identification of species involved in CO biomethanation is needed in order to improve the process performance. It is well known that the microbial communities in

biogas reactors are commonly characterized by sequencing of 16S rRNA genes followed by sequence similarity search against reference genomes present in public databases (Campanaro et al. 2016, Luo et al. 2016, Sundberg et al. 2013). However, the majority of these genomes were isolated from different environments, and even if they belong to phylogenetically related groups, they might have different functional properties (Campanaro et al. 2016). Therefore, the exact identification and characterization of dominant microorganisms in biogas reactors are still challenging. Reconstructing the genomes of individual species from mixed cultures can be achieved by binning of the contigs obtained from metagenomic shotgun sequencing (Albertsen et al. 2013, Alneberg et al. 2014, Campanaro et al. 2016, Palomo et al. 2016), which would improve our knowledge about the diversity and functional properties of the microorganisms relating with CO conversion without relying on the culture-dependent technology (Kougias et al. 2016, Treu et al. 2016).

Based on the above considerations, the present study aimed to explore the potential of biomethanation of CO in AGS based reactors treating synthetic wastewater. The effect of CO addition on the synthetic wastewater degradation by AGS was elucidated, and also the long-term performance of the continuous reactor with the addition of CO was studied. The effect of CO addition on the microbial community composition of the AGS was revealed by metagenomic sequencing, focusing on the identification of the microbial species relating with CO conversion.

2. Material and Methods

2.1 Anaerobic granular sludge

The AGS used in this study was obtained from a mesophilic UASB reactor treating cassava stillage in an ethanol plant (Taicang, Suzhou, China). The characteristics of the AGS were as follows: Total solids (TS)= 21.3±0.7 g/L, Volatile solids (VS)=

16.9±1.1 g/L, pH = 7.2±0.5.

2.2 Effect of CO partial pressures on the methane production from glucose by AGS

Batch experiments were conducted to investigate the effect of CO partial pressures on the methane production from glucose by AGS. All the batch experiments were conducted in 118 mL serum bottles. Glucose was used as the substrate. AGS, glucose and basic anaerobic (BA) medium were added to the bottles, and the final liquid mixture was 50 mL with the concentrations of AGS 5 gVS/L and glucose 1 g/L. Bottles with BA medium and inoculum only, but without glucose and CO, were used as blanks. The detailed information of BA medium was described in a previous study (Angelidaki and Sanders 2004). The bottles were then sealed with butyl rubber stoppers and aluminum crimps. The headspaces of the bottles were purged with the mixture of CO and N_2 with different ratios to obtain the required initial partial pressures of CO (0, 0.1, 0.25, 0.5 and 1 atm). The above procedure was conducted in a fume hood to avoid the accumulation of CO in the lab. The bottles were then incubated in a 37 °C shaker (KS 3000 I, IKA, Germany) with 300 rpm to overcome the gas−liquid mass transfer limitation. All the tests were performed in triplicates. When the methane production ceased, a new batch was conducted by repeating the above procedure except keeping the AGS to test the long-term effects of CO on the degradation of glucose by AGS.

2.3 Continuous experiments

Two 1.2 L stainless steel UASB reactors with 1L working volume were set up. The temperature of the reactors was controlled at 37 °C by a water bath system. Microporous gas-diffusers were installed at the bottom of both reactors. $UASB_{CO}$ was fed with BA medium containing glucose (15 g/L) and CO, while $UASB_C$ was fed with

only BA medium containing glucose (15 g/L). Both reactors were inoculated with 300 mL AGS, and they were initially fed with only BA medium containing glucose (15 g/L). CO was started to be injected into $UASB_{CO}$ when both reactors achieved stable performances and were considered to be in steady-states. The inflow rate of CO was set initially at 2.5 L/day (also corresponding to 2.5 L/day/L-reactor), and then was increased to 5 L/day. The CO conversion was strongly limited by the gas-liquid mass transfer because of its low solubility (Munasinghe and Khanal 2010b). Therefore, the combination of microporous gas injection and gas recirculation (1.5 L/min) was applied in the present study to improve the gas-liquid mass transfer of CO. The hydraulic retention time of the two reactors were controlled at 3 days corresponding to the influent organic loading rate of 5 gCOD/L/day (COD, Chemical oxygen demand) during the whole operational periods. The operational parameters are shown in Table 1.

2.4 CO conversion pathways

To determine the CO conversion pathways, batch experiments were conducted. Most of the procedures for the experiments were similar as described in part 2.2, and there were differences in the preparation of the bottles. For instance, granules retrieved from both reactors during steady-states were inoculated to the 118 mL bottles, and BA medium was added to adjust the liquid volume to 50 mL. The headspace was filled with the mixture of CO and N_2 with a ratio 20/80. Bottles without supplementation of CO were used as controls. 2-bromo-ethanesulfonic acid (BES) was added to all the bottles with a final concentration 25 mM to inhibit methanogenesis (Luo et al. 2013). CO consumption rates, acetate production rates and H_2 production rates were determined periodically.

2.5 High-throughput sequencing (HTS) of 16S rRNA genes

Samples from $UASB_{CO}$ and $UASB_{C}$ were collected when steady-states were achieved in phase IV. Two samples were obtained from each reactor (day 100 and 130, named as $UASB_{CO1}$ and $UASB_{CO2}$ for $UASB_{CO}$, $UASB_{C1}$ and $UASB_{C2}$ for $UASB_{C}$). The samples were then used for DNA extraction, polymerase chain reaction (PCR) and HTS. The obtained sequences (19074 sequences for $UASB_{CO1}$, 20344 sequences for $UASB_{CO2}$, 24232 sequences for $UASB_{C1}$ and 23520 sequences for $UASB_{C2}$,) were deposited to European Nucleotide Archive (ENA) with accession number PRJEB20130. Detailed information about the PCR and bioinformatic analysis can be found in supporting information.

2.6 Quantitative polymerase chain reaction (qPCR) analysis

Genomic DNA of each sample obtained in part 2.5 was also used for qPCR analysis. The abundances of bacteria, methanogens (*Methanobacteriales*, *Methanomicrobiales*, *Methanosarcina* and *Methanosaeta*), and acetogens were quantified as described in Supporting information.

2.7 Metagenomic sequencing and analysis

Genomic DNA of each sample obtained from both reactors on day 130 was also used for metagenomic sequencing. Libraries with insert size of 400 bp were constructed according to the manufacturer's instructions (Illumina). Sequencing was performed on Illumina Hiseq 2500 platform (Illumina Inc., USA) by applying 2×150 bp paired-end strategy. Metagenomic sequencing generated 63546838 and 56060032 paired-end reads for samples $UASB_{CO}$ and $UASB_{C}$, respectively. All the metagenomic datasets were submitted to European Nucleotide Archive (ENA) with accession number PRJEB19890. Sequence assembly, reconstruction of the genome bins (GBs), and taxonomic and functional analysis of the GBs were described in Supporting information.

2.8 Analytical methods

COD was analyzed according to APHA (APHA 1995). Gas chromatography (GC-2014, Shimadzu, Japan) and HPLC (Agilent 1100, Agilent, USA) were used to analyze the gas compositions and volatile fatty acids (VFAs), respectively (Supporting information). The scanning electron microscopy (SEM) for microbial observation of the AGS was conducted using an electron microscope (SUPERSCAN SSX-550, Shimadzu, Japan) (Supporting information). Microbial extracellular polymeric substances (EPS) were also analyzed (Supporting information). All the chemicals used in the present study were purchased from Sinopharm Chemical Reagent Co., Ltd.

3. Results and Discussion

3.1 Effect of CO partial pressures on the AD of glucose based on anaerobic granular sludge

The cumulative CH_4 production from glucose at different CO partial pressures by AGS is shown in Fig 1. In the first batch, obvious inhibition of CO on the methane production rate was found at partial pressure of 0.5 atm. It was different from our previous study (Luo et al. 2013), where digested sewage sludge was used and obvious inhibition was found at CO partial pressure 0.25 atm. This proved the hypothesis that AGS could tolerate higher CO partial pressure compared to suspended sludge. The produced methane at CO partial pressures 0.1, 0.25 and 0.5 atm at the end of the batch experiments were all higher than that without CO, which could be due to the conversion of CO to methane since the consumption of CO in the reactor was detected (Fig 1). After six batch cultivation, the methane production was faster at all CO partial pressures compared to the first batch cultivation as seen in Fig 1. In addition, the inhibition of CO partial pressure at 0.5 atm on the methane production was not obvious, which indicated that the tolerance of AGS on CO could be improved after

long-term cultivation. Higher cumulative CH_4 production at CO partial pressures of 0.5 and 1.0 atm at the end of the sixth batch experiments were obtained compared to the first batch experiments, which was due to the more consumed glucose as shown in Fig S1. The above results clearly showed that AGS could tolerate higher CO partial pressure and the tolerance could be improved by long-term cultivation. Therefore, the addition of CO to AGS based reactors treating wastewater is promising since efficient and simultaneous conversion of CO and synthetic wastewater could be achieved.

3.2 Performances of UASB reactor treating synthetic wastewater with the addition of CO

The feasibility of the addition of CO to AGS based reactor treating synthetic wastewater was then tested in UASB reactors. The operational parameters and reactor performances were summarized in Table 1. During phase I, both reactors were operated with glucose as the substrate, but without the addition of CO. During the steady-states of phase I, similar reactor performances (methane production rate, COD removal rate et al.) were obtained from the two reactors. In phase II, CO was then injected into $UASB_{CO}$ with the flow rate of 2.5 L/d. Higher CH_4 production rate was observed in $UASB_{CO}$ (1709 mL/(L·d)) compared to $UASB_C$ (1532mL/(L·d)). In this phase only around 37% of the injected CO was consumed (Table 1). A high concentration of CO (34.2%) was detected in the produced biogas. Gas recirculation through the microporous diffusers installed at the bottom of the reactors with the flow rate of 1.5 L/min was introduced in phase III. The gas recirculation was applied to both reactors in order to ensure similar operational conditions. Subsequently only minor amounts of CO (2.8%) was detected in the biogas of $UASB_{CO}$, indicating the efficient consumption of the added CO. Simultaneously, the methane production rate was increased to 2121 mL/(L·d). The above results showed that gas recirculation was

an effective method to increase the CO conversion rate, which might be resulted from the increased residence time and concentration of CO in the liquid of the reactor. In phase IV, the CO flow rate was further increased to 5 L/d, and higher CO consumption rate was obtained. It should be noted that the CO in the biogas of $UASB_{CO}$ was increased from 2.8% in phase III to 5.1% in phase IV, which was much lower compared to that in phased II. The addition of CO did not obviously affect the degradation of glucose since there was no significant difference in COD removal efficiency of both reactors. Besides, the concentrations of acetate were also kept at very low levels in both reactors during the whole experimental period, while no other volatile fatty acids were detected. The only obvious effect of CO on the degradation of glucose was the low methane content (36.7-43.1%) in the biogas, which was much lower than that in $UASB_C$. This could be due to the residual CO in the biogas and also the production of CO_2 during the anaerobic conversion of CO (equation 1). Stoichiometrically, the conversion of glucose and CO to methane results in 50% and 25% of CH_4 in the biogas, respectively (Equations 1 and 2). Assuming that the methane produced from glucose was the same from both reactors, the methane coming from CO could be calculated by subtracting the methane originating from glucose. As shown in Table 1, the conversion efficiencies of CO in $UASB_{CO}$ were between 91.4% and 97% in phase III and IV, which showed that the consumed CO was almost fully converted to methane.

$$4CO+2H_2O \rightarrow CH_4+3CO_2 \qquad \text{(Equation 1)}$$

$$C_6H_{12}O_6+2H_2O \rightarrow 3CH_4+3CO_2 \qquad \text{(Equation 2)}$$

3.3 The pathways of CO conversion by AGS

The pathways of CO conversion catalyzed by AGS from both reactors in phase IV were determined. As shown in Fig S2, the concentration of CO in the bottles

decreased faster with the AGS obtained from $UASB_{CO}$ compared to that from $UASB_C$, which was expected since the granules from $UASB_{CO}$ had been acclimatized to CO. For the AGS from $UASB_{CO}$, H_2 was temporarily detected during the batch experiments, which could be due to the consumption of H_2 for acetate production via homoacetogenesis. This assumption was further supported by the observed gradual increase of acetate in the batch reactors. Previous studies showed that CO was converted via acetogenic CO-oxidizing pathway at mesophilic conditions, while it was via hydrogenotrophic CO-oxidizing pathway at thermophilic condition (Navarro et al. 2014, Oelgeschlager and Rother 2009, Sipma et al. 2003). From our study, it seems that both pathways were active when AGS were used for the CO conversion at mesophilic condition. The maximum accumulated H_2 contents in the headspace of the bottles were around 6 %, corresponding to around 30 % CO conversion. It should be noted that H_2 might be produced and consumed simultaneously in the batch experiments, and therefore the actual value of produced H_2 from CO might be higher than the above value, indicating that hydrogenotrophic CO-oxidizing pathway might be an important pathway in our study. For AGS from $UASB_C$, the accumulated H_2 (around 1.5 %) was much lower compared to that from $UASB_{CO}$, indicating a possible dominance of acetogenic CO-oxidizing pathway. It was consistent with a previous study that CO was found to be converted via acetogenic CO-oxidizing pathway in mesophilic condition, when the sludge was not acclimatized to CO (Luo et al. 2013). From the present study, it seems that the acclimatization of the sludge to CO might promote the hydrogenotrophic CO-oxidizing pathway.

3.4 Extracellular polymeric substances (EPS) of the anaerobic granular sludge

EPS is an important component of granules, playing an important role in maintaining the matrix structure and stability of AGS and protecting the inner microorganisms

297 against toxicity (Henriques and Love 2007, Luongo and Zhang 2010). Thus, the effect
298 of CO on EPS of AGS was also investigated. Table S1 shows the concentrations of
299 both polysaccharides and proteins from $UASB_{CO}$ were higher than that in $UASB_{C}$.
300 The reason could be that the stress caused by the presence of CO led to the
301 overproduction of EPS in $UASB_{CO}$, which was used to maintain the functional
302 integrity of the microorganisms based on the defensive mechanism considering the
303 toxicity of CO to microorganisms (Pasquini et al. 2013, Yadav et al. 2016). The
304 overproduction of EPS also ensured the stable glucose conversion efficiency in
305 $UASB_{CO}$. Fourier transform infrared spectroscopy was also used to characterize the
306 major functional groups of organic matters in EPS. It can be seen from Figure S3 that
307 there were some changes (decrease and shift) in the bands of 1635 and 1540 cm^{-1},
308 which were lower in $UASB_{CO}$ than $UASB_{C}$. They were assigned to the amido-I and
309 -II bands of proteins, indicating the changes closely related with the proteins of EPS.
310 The presence of band of 1100 cm^{-1}, representing C−O−C of polysaccharides, showed
311 that polysaccharides were one of the main component of EPS, which agreed with the
312 above phenomenon that the polysaccharides increased after the addition of CO. In
313 summary, the above results showed that the addition of CO to the UASB reactor
314 induced the overproduction of EPS of AGS.

315 **3.5 SEM analysis of AGS**

316 The granules in both reactors were also used for SEM analysis (Fig S4). As shown in
317 Figure S4A and S4D, there were distinct differences in the appearance and diameter
318 of the AGS obtained from both reactors. The AGS cultured with CO were irregular in
319 shape, porous in surface and small in size, while the AGS cultured without CO were
320 more regular in shape, more smooth in surface and bigger in size than the ones from
321 the $UASB_{CO}$. Figure S4B and S4E show the observation on the surface of AGS. In

322 $UASB_C$, the surface of AGS was colonized by a large amount of microcolony of cocci,
323 rods, and thin filaments, whereas the surface of AGS from $UASB_{CO}$ was more
324 uniform mainly containing cocci, and only a very small numbers of filaments. In
325 comparison, the interior granule principally contained *Methanosaeta*-like rods with
326 flat ends (Figure S4C and S4F) (Araujo et al. 2003, Shin et al. 2001). The dominance
327 of *Methanosaeta* in both reactors was also demonstrated by microbial community
328 composition analysis, which was discussed in the following parts. The above results
329 showed that CO only affected the morphology and distribution of microorganisms on
330 the surface of AGS, while inner granules were almost the same.

331 **3.6 Microbial community compositions as revealed by HTS of 16S rRNA genes**

332 Taxonomic analysis of the obtained sequences by HTS of 16S rRNA genes is shown
333 in Fig 2. The samples obtained from $UASB_C$ were clustered together, and they were
334 well separated from the samples obtained from $UASB_{CO}$, which indicated that the
335 addition of CO to the biogas reactor significantly determined the composition of the
336 microbial communities. Similar distribution of microbial communities at both phylum
337 and genus levels were observed for $UASB_{C1}$ and $UASB_{C2}$, as well as $UASB_{CO1}$ and
338 $UASB_{CO2}$, showing that relatively stable microbial community compositions were
339 formed in both reactors during the steady-states.

340 The phyla *Chloroflexi* and *Proteobacteria* were dominant in all the samples, which
341 was in agreement with previous studies in biogas reactors (Nelson et al. 2011, Pope et
342 al. 2013). The phylum *Candidate division TM7* was found to be dominant only in CO
343 samples, which could be attributed to the addition of CO. The phylum *Candidate*
344 *division TM7* is a highly ubiquitous phylum present in soils, sediments, wastewater,
345 biogas reactors, animals etc., and it is only known from environmental 16S ribosomal
346 DNA sequence data (Ariesyady et al. 2007, Ferrari et al. 2014). Therefore, the

physiological characterics of the microorganisms belonging to this phylum are still unknown due to the lack of cultured isolates. It could be hypothesized that the microorganisms belonging to the phylum *Candidate division TM7* could utilize CO as substrate since they were only enriched in the reactor that was supplemented with CO. Further investigations are needed in order to fully verify this assumption. Another interesting finding is that none of the known mesophilic anaerobic CO-converting bacteria (Table S2) were identified even at genus level in the samples (Fig 2). Nevertheless, the results from the batch assays demonstrated that CO was utilized by bacteria in the reactors (Fig S2). The results show the lack of knowledge about the microbial CO conversion and the necessity to discover additional bacterial species that are able to utilize CO.

3.7 qPCR analysis

The bacterial concentration in $UASB_{CO}$ (2.8×10^{11} copies/mL) was significantly higher than that in $UASB_C$ (1.5×10^{11} copies/mL), which was due to the addition of CO in $UASB_{CO}$ (Fig 3). The concentrations of methanogens were at least 10 times lower than that of bacteria in both $UASB_{CO}$ and $UASB_C$. *Methanosaeta* was the most abundant methanogen, which was consistent with the results from HTS of 16S rRNA genes. Increased concentrations of *Methanobacteriales*, *Methanosarcina* and *Methanosaeta* in $UASB_{CO}$ compared to $UASB_C$ were observed, which could be related with the addition of CO. As previously mentioned, both H_2 and acetate were demonstrated to be the intermediates in the anaerobic conversion of CO. Therefore, it was expectable that both hydrogenotrophic and acetoclastic methanogens were enriched. It should be noted that *Methanomicrobiales* was not enriched, and the operational conditions were more favorable for other hydrogenotrophic methanogens. Several species belonging to *Methanosarcina* were reported to convert CO

(Oelgeschlager and Rother 2008), and therefore the enrichment of *Methanosarcina* might be related with the conversion of CO. However, the concentration of *Methanosarcina* was extremely low compared to the other detected microorganisms, which might indicate that they did not play an important role during the anaerobic conversion of CO. It was reported that most of the acetogens can grow on CO (Drake et al. 2006), and therefore acetogens were quantified. Acetogens were quantified by targeting the gene encoding for the formyltetrahydrofolate synthetase (FTHFS), a conserved gene involved in their CO_2 fixation pathway, based on literatures (Parameswaran et al. 2011, Ruiz et al. 2014, Xu et al. 2009). Indeed, the increased concentration of acetogens was recorded in the present research, indicating a potential role of these microorganisms in the anaerobic conversion of CO. It should be noted that the concentration of acetogens was also very low compared to the concentration of total bacteria (Fig 3). The $UASB_{CO}$ was highly loaded with CO, and therefore, it would be expected that the microorganisms responsible for CO conversion would be present in high abundances. Since the known potential CO converting microorganisms (*Methanosarcinaceae* and acetogens) were found in low concentrations, it could be assumed that the major players in the anaerobic conversion of CO were still unknown. It should be noted that both *Methanosarcinaceae* and acetogens were not detected by HTS of 16S rRNA genes (Fig 2), and it might be due to the higher sensitivity of qPCR compared to HTS of 16S rRNA genes at current sequencing depth, the PCR bias for the amplification of 16S rRNA genes and the presence of unclassified sequences in genus level for HTS of 16S rRNA genes (Becker et al. 2000, Ye and Zhang 2011).

3.8 Genome reconstruction and metabolic potentials by metagenomic analysis

The high-quality paired-end reads of the samples obtained from $UASB_{CO}$ and $UASB_C$ were assembled, resulting in 127587 scaffolds with length ranging from 1000 to

896694 bp (N50 5666 bp). The total length of the assembly was around 458.45 Mbp. The scaffolds were then binned into genomes, which generated 70 GBs as shown in Table S3. The quality of the binning was very high with nearly 80 % of the GBs having completeness higher than 70 % and very low contamination level with the average redundancy to be 5.6 % (Table S3). PhyloPhlAn is a software used for both phylogenetic assessment and taxonomic quality control of newly-sequenced genomes (Segata et al. 2013), and taxonomic assignment by Phylophlan showed that none of the GBs was assigned to species level, and only 5 GBs were assigned to genus level (Table S4). The result was also confirmed by calculating the average nucleotide identity (ANI) level of each GB in comparison with all the microbial genomes deposited in national center for biotechnology information (NCBI) database. Only *Flexilinea flocculi* FUN15 satisfied the parameters for the assignment at species level (>95 % ANI on more than 70 % of the genes) (Konstantinidis and Tiedje 2007). The results indicated that most of the GBs in the present study were not previously characterized at genomic level and might be related with new microbial species. This is consistent with our assumption that unknown microorganisms were the key players responsible for CO conversion, as the known CO converting bacteria were not found neither in the present study nor in our previous study based on high-throughput sequencing of the 16S rRNA genes (Luo et al. 2013).

The applied metagenomic analysis allowed the identification of the gene contents of the reconstructed GBs and thereby the assignment of potential functional properties to the microbial species. Exploring this opportunity, the presence of specific key genes for the process was investigated. CO dehydrogenase (CODH) carries out the primary oxidation of CO to CO_2 (Bonam and Ludden 1987) and passes the resulting reducing equivalents through a ferredoxin-like subunit. The ferredoxin passes the electrons to

uncharacterized electron carriers and then eventually to a tightly membrane bound hydrogenase, where protons are reduced to hydrogen (Ensign and Ludden 1991). Therefore, CODH is essential for H_2 production from CO. Moreover, acetyl-CoA synthase (ACS) is a NiFeS enzyme that condenses Coenzyme A and CO to form acetyl-CoA (Ragsdale 2004), which is essential for acetate production from CO. Both H_2 and acetate were detected to be intermediates for methane production from CO in the present study. Therefore, the presence of genes encoding CODH and ACS in the GBs was searched in order to identify the GBs that could convert CO (Results are shown in Table S3). In total, 38 out of the 70 GBs contained genes encoding CODH and/or ACS, which could be potential microorganisms converting CO. Particularly, the GBs *Methanosaeta sp.* FUN43, *Peptococcaceae sp.* FUN46, *Syntrophobacterales sp.* FUN60 and *Syntrophobacterales sp.* FUN62 were those harboring a more complete ACS protein subunit complex since genes acsA, acsB, acsC and acsD were identified. It should be noted that some other GBs might also contain the above mentioned genes without being identified due to the incompleteness of the genomes (ranging from 30% to 100%). Table S3 also shows that not all the GBs containing genes encoding CODH and/or ACS, were enriched in $UASB_{CO}$ as reflected by the coverage value ratios of $UASB_{CO}$ to $UASB_{C}$, which could be due to genes inactivity. In addition, both CODH and ACS are also essential to the Wood-Ljungdahl pathway for acetate production, which is active during the heterotrophic growth for recycling of generated reducing equivalents and might be inactivated during autotrophic growth resulting in a growth defect (Drake et al. 2006, Liew et al. 2016, Marcellin et al. 2016). Only the 23 GBs enriched in $UASB_{CO}$, were considered as the most probable candidates for CO conversion in the present study (Table 2). Currently, the known CO-converting microorganisms belong to the bacteria *Firmicutes* and *Proteobacteria*,

and the archaea *Euryarchaeota* and *Crenarchaeota* (Table S2). Besides *Firmicutes* and *Proteobacteria*, in the present study the GBs containing genes related to CO conversion also belonged to the phyla *Elusimicrobia, Actinobacteria, Chloroflexi, Spirochaetes,* and *Synergistetes,* showing that the CO-converting microorganisms might be taxonomically more diverse than expected. Eight GBs of Table 2 were highly enriched with a coverage ratio of $UASB_{CO}$ to $UASB_{C}$ higher than 10. The highly enriched *Chloroflexi sp.* FUN12 and *Chloroflexi sp.* FUN13 were only assigned to phylum level (*Chloroflexi*). Although 16S rRNA genes analysis did not show the enrichment of the phylum *Chloroflexi* in $UASB_{CO}$ (Fig 2), metagenomic analysis revealed that two new potential CO-converting microbial species (*Chloroflexi sp.* FUN12 and *Chloroflexi sp.* FUN13) were enriched. Only one highly enriched GB *Propionibacterium sp.* FUN51 was assigned to genus level (*Propionibacterium*). The microorganisms belonging to genus *Propionibacterium* could produce propionic acid from carbohydrates (Paik and Glatz 1994). Metagenomic analysis indicated that the genus *Propionibacterium* might also convert CO since it contained CODH genes. Enrichment of the genus *Propionibacterium* was also supported by the HTS of 16S rRNA genes analysis (Fig 2). The phylum *Candidate division TM7* was significantly enriched as revealed by the HTS of 16S rRNA genes analysis, however, none of the selected GBs (even all the reconstructed GBs) belonged to that phylum. It should be noted that 4 GBs were unclassified. RDP classifier has been widely used in 16s rRNA gene-based microbial community analysis for taxonomic assignment (Luo et al. 2013). Taxonomic assignment by RDP classifier of 16S genes of these 4 GBs suggests that two of them (*Unclassified bacterium sp.* FUN31 and *Unclassified bacterium sp.* FUN53) belong to phylum *Candidate division TM7* (also known as *Candidatus Saccharibacteria*), both of which were also enriched. However, CODH and ACS

genes were not detected in the above two GBs. On the one hand, their enrichment might be due to their high tolerance to CO. On the other hand, they might also contain CODH and ACS genes without being identified due to the incompleteness of the genomes as previously mentioned.

The GBs were functionally classified considering the general organization of the AD process (i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis) (Fig. 4). Some GBs have multiple functional roles, due to their complex metabolic potential, and for this reason it is difficult to assign them to one specific "layer" of the food chain. As expected, the methanogenesis "layer" is very simple because only one methanogenic archaea was very abundant and successfully recovered from the binning process (i.e. *Methanosaeta* sp. FUN43). This finding is in agreement with HTS of 16S rRNA genes analysis (Fig 2), where *Metanosaeta* was the most abundant genus of the phylum *Euryarchaeota*. All the other layers are extremely complex in terms of numbers of GBs, and in particular "hydrolysis" step is populated by 24 GBs potentially involved in degradation of many different polysaccharides (Table S5). Intermediate steps are populated by 7 GBs potentially involved in butyrate degradation and 7 in propionate degradation (acetogenesis). As expected, some of these GBs (*Deltaproteobacteria* sp. FUN36) can potentially degrade polysaccharides to generate butyrate, which can be subsequently degraded to CO_2 and H_2. It should be noticed that in Fig 4, only the GBs having the highest potential to perform a specific function are marked with asterisks, but also other GBs (e.g. *Syntrophobacterales* sp. FUN60 and *Syntrophobacterales* sp. FUN61) could be potentially involved in fatty acids degradation and butyrate/propionate utilization (despite having a lower number of predicted proteins). A more detailed representation of the functions is presented in Table S5.

497 It is clearly visible that the microbes which increased after CO addition were assigned
498 to all the possible layers except methanogenesis (Fig 4). This finding suggests that the
499 ability in CO fixation is not restricted to specific sub-populations into the AD food
500 chain. Additionally, it was not feasible to assign a specific functional role to some of
501 the GBs which highly increased after CO addition (e.g. *Elusimicrobia* sp. FUN39 and
502 *Chloroflexi* sp. FUN31). The main difficulty in the assignment of these GBs (marked
503 as "Undefined" in Fig. 4) was the impossibility to annotate a large majority of the
504 encoded genes, which resulted in a low completeness level of the predicted pathways.
505 This was independent on the method or the database used for gene prediction and
506 annotation. An explanation for the failure to annotate a large part of the encoded genes
507 could be that some of the unclassified GBs were very phylogenetically distant from
508 the well-known and functionally annotated species present in public databases.
509 Nevertheless, the significant increase in the abundance after CO addition (>40 fold)
510 and the finding of the novel genomes of *Elusimicrobia* sp. FUN39 and *Chloroflexi* sp.
511 FUN31 suggest that there is still a lot of undiscovered knowledge concerning the
512 conversion of CO underlining the need for a future deep investigation of their
513 functions.
514 The presence of the CODH subunits and ACS genes in the GBs was evaluated
515 together with the number of genes present in metabolic pathways involved in CO_2
516 fixation. According to Hügler and colleagues there are three main pathways
517 performing this process and working in anaerobic conditions, the reductive
518 tricarboxylic acid (TCA) cycle, the reductive acetyl coenzyme A (CoA) pathway (also
519 known as Wood-Ljungdahl pathway), and the 3-hydroxypropionate cycle (Hügler et al.
520 2005). Other pathways such as the "Calvin-Benson-Bassham cycle (Calvin cycle)" are
521 restricted to organisms with high-energy yield from a chemotrophic or phototrophic

lifestyle and these metabolic pathways cannot work at high temperature or in anaerobic conditions. Considering the GBs identified in the present study, the reductive TCA cycle presented high completeness (9 or more unique genes) in 9 out of the 22 GBs (41%) that increased more than 4-fold after CO addition. All of them except *Bacteroidales* sp. FUN09 also encode one or more subunits of the CODH. On contrary after the CO addition, 18 GBs were found to decrease in abundance. Only 4 GBs out of these had 9 or more genes in the reductive TCA cycle and only one GB out of these 4 could encode also the CODH (Table S5). Additionally, some GBs enriched in sample $UASB_{CO}$ have a complete or nearly complete Wood-Ljungdahl pathway (8 or more univocal genes) (in particular *Peptococcaceae* sp. FUN46, *Syntrophobacterales* sp. FUN60 and *Syntrophobacterales* sp. FUN62, Table S6), while all the GBs enriched in $UASB_C$ have 3 or less genes belonging to this pathway. The 3-hydroxypropionate cycle seems to be incomplete in all the PGs. It has also to be considered that some of the GBs that were positively affected by the CO addition (e.g. *Unclassified bacterium* sp. FUN66 and *Chloroflexi* sp. FUN16) were incomplete and belonged to the group of species with a high percentage of unassigned genes (potentially novel). As they are unknown, an accurate identification of genes belonging to CO utilization in these GBs was not possible. The reductive TCA cycle can be used by some bacteria to produce carbon compounds from CO_2 and water, and this pathway in combination with the oxidation of the CO determined by CODH and with Wood–Ljungdahl pathway is probably crucial for CO utilization by the majority of the GBs in the present study.

4. Conclusions

CO has toxicity to microorganisms, and the present study clearly showed that AGS could tolerate the CO partial pressure as high as 0.5 atm after the accumulation

without affecting its ability for synthetic wastewater treatment by batch experiments. Gas-recirculation is crucial to increase the conversion efficiency of CO in UASB reactor treating synthetic wastewater in continuous experiments. The addition of CO to UASB reactor enhanced the hydrogenotrophic CO-oxidizing pathway and also resulted in the increase of EPS of AGS. The microbial community compositions of the AGS was also changed upon CO addition by the analysis of HTS of 16S rRNA genes, however, the known bacteria involved in CO conversion were not identified. In order to identify the CO-converting microorganisms and their potential functions, metagenomic analysis was conducted by reconstructing the genomes of individual species. The results revealed 23 of the 70 reconstructed genome bins (GBs), were potential CO-converting bacteria since they contained genes related to CO conversion, and most of the GBs were not previously characterized at genomic level. Further functional analysis also showed that the GBs that could potentially convert CO had multi-functions in the AD process.

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Supporting information

Supporting Information includes Fig S1-S4 and Table S1-S9 as noted in the main text and supporting information.

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730

Table 1. Summary of the performances of both reactors $UASB_C$ and $UASB_{CO}$ during steady-states

	I (1-21)		II (22-49)		III (50-84)		IV (85-147)	
	$UASB_{CO}$	$UASB_{Control}$	$UASB_{CO}$	$UASB_{Control}$	$UASB_{CO}$	$UASB_{Control}$	$UASB_{CO}$	$UASB_{Control}$
CO flow rate (mL/d)	0	0	2500±102.5	0	2500±97.5	0	5000±145	0
biogas backflow rate (mL/min)	0	0	0	0	1500	1500	1500	1500
biogas production rate (mL/(L·d))	2067.5±122.5	2082.5±125	4632.5±207.5	2072.5±112.5	4920±235	2120±105	7307.5±292.5	2100±100
CH_4 (%)	73.5±1.6	71.9±1.7	36.9±1.5	73.9±1.6	43.1±1.5	74.5±1.4	36.7±1.3	74.2±1.2
CO_2 (%)	24.9±1.1	26.7±1.2	23.8±1.1	25±1.1	51.7±0.9	24.6±0.8	55.3±0.9	24.5±0.6
CO (%)	0	–	34.2±2.1	–	2.8±0.6	–	5.1±0.8	–
CH_4 production rate (mL/(L·d))	1520±77.5	1497±82.5	1709±115	1532±75	2121±127.5	1579±77.5	2682±165	1558±72.5
CO consumption rate (mL/d)	–	–	915.7±47.4	–	2362.2±89.3	–	4627.3±129.7	–
measured CH_4 from CO/theoretical CH_4 from CO (%)	–	–	76.4	–	91.4	–	97	–
pH	7.51±0.04	7.56±0.05	7.41±0.02	7.54±0.03	7.32±0.04	7.65±0.05	7.28±0.04	7.67±0.05
acetate (mmol/L)	0.52±0.02	0.57±0.06	1.85±0.13	0.55±0.09	0.11±0.03	0.08±0.01	0.14±0.03	0.06±0.01
COD removal rate (%)	88.9±2.9	88.3±2.6	84.9±3.2	88.2±2.4	88.1±3.1	91.1±2.2	87.8±3.4	90.2±2.1

Table 2 The genome bins (GBs) enriched (with coverage ratio of $UASB_{CO}$ to $UASB_{C}$ higher than 1) in $UASB_{CO}$ containing CODH and/or ACS genes (The GB names with bold characters are highly enriched GBs in $UASB_{CO}$)

GB name	Phylum	Completeness estimated considering taxonomic assignment	Coverage ratio of $UASB_{CO}$ to $UASB_{C}$	CODH genes	ACS genes
Brachyspira sp. FUN11	*Spirochaetes*	84.62%	2.81	Y	N
***Chloroflexi* sp. FUN12**	*Chloroflexi*	97.09%	41.92	Y	N
***Chloroflexi* sp. FUN13**	*Chloroflexi*	82.52%	1792.19	Y	N
Chloroflexi sp. FUN14	*Chloroflexi*	70.87%	6.86	Y	N
Chloroflexi sp. FUN17	*Chloroflexi*	99.03%	2.64	Y	N
Chloroflexi sp. FUN22	*Chloroflexi*	93.20%	1.72	Y	Y
Chloroflexi sp. FUN25	*Chloroflexi*	81.55%	2.49	Y	N
Chloroflexi sp. FUN26	*Chloroflexi*	67.96%	1.17	Y	N
Chloroflexi sp. FUN28	*Chloroflexi*	66.02%	5.34	Y	N
Chloroflexi sp. FUN29	*Chloroflexi*	79.61%	4.21	Y	N
Chloroflexi sp. FUN30	*Chloroflexi*	60.19%	2.51	Y	N
Deltaproteobacteria sp. FUN37	*Proteobacteria*	100.00%	2.00	Y	N
***Elusimicrobia* sp. FUN39**	*Elusimicrobia*	96.12%	126.39	Y	N
***Elusimicrobia* sp. FUN40**	*Elusimicrobia*	70.87%	15.29	Y	N
Flexilinea flocculi FUN15	*Chloroflexi*	73.79%	1.46	Y	N
Ornatilinea sp. FUN23	*Chloroflexi*	94.17%	4.03	Y	N
***Peptococcaceae* sp. FUN46**	*Firmicutes*	100.00%	2974.01	Y	Y
***Propionibacterium* sp. FUN51**	*Actinobacteria*	99.05%	42.08	Y	N
Synergistaceae sp. FUN57	*Synergistetes*	90.48%	2.46	N	Y
***Syntrophobacterales* sp. FUN60**	*Proteobacteria*	95.24%	10.43	Y	Y
Syntrophobacterales sp. FUN61	*Proteobacteria*	86.67%	1.31	Y	Y
Syntrophobacterales sp. FUN62	*Proteobacteria*	96.19%	4.46	Y	Y
***Veillonellaceae* sp. FUN68**	*Firmicutes*	98.10%	1482.78	Y	N

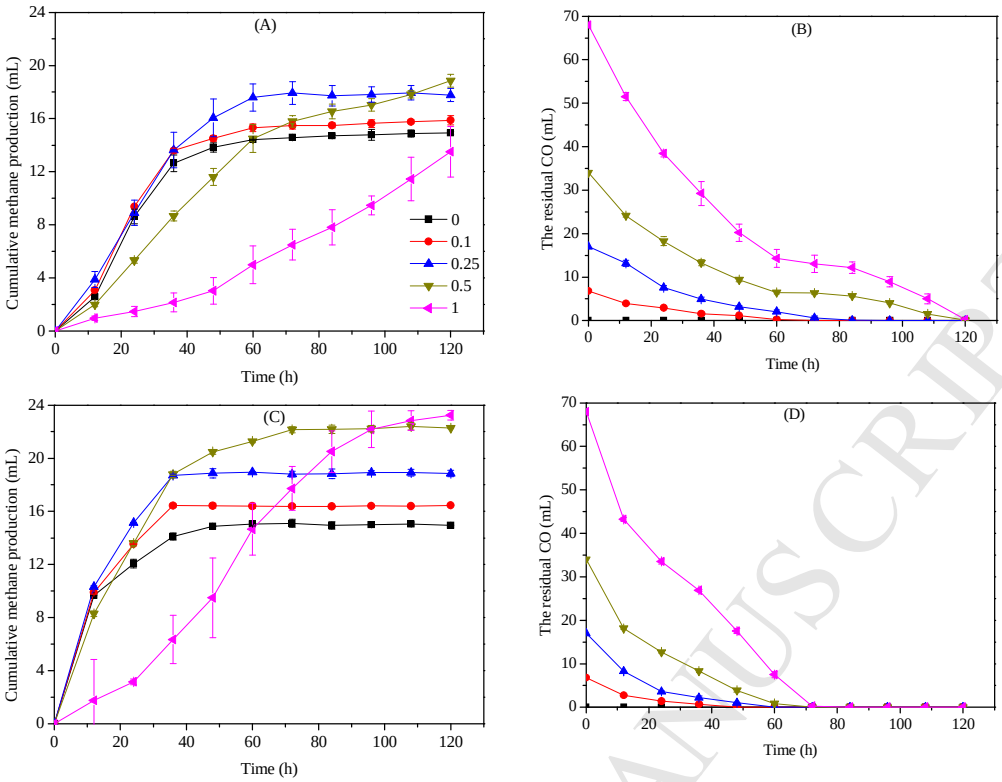

Fig 1. The cumulative CH_4 production and CO consumption of anaerobic granular sludge to degrade synthetic wastewater. (A) Cumulative CH_4 production in the first batch; (B) CO consumption in the first batch; (C) Cumulative CH_4 production in the sixth batch; (D) CO consumption in the sixth batch

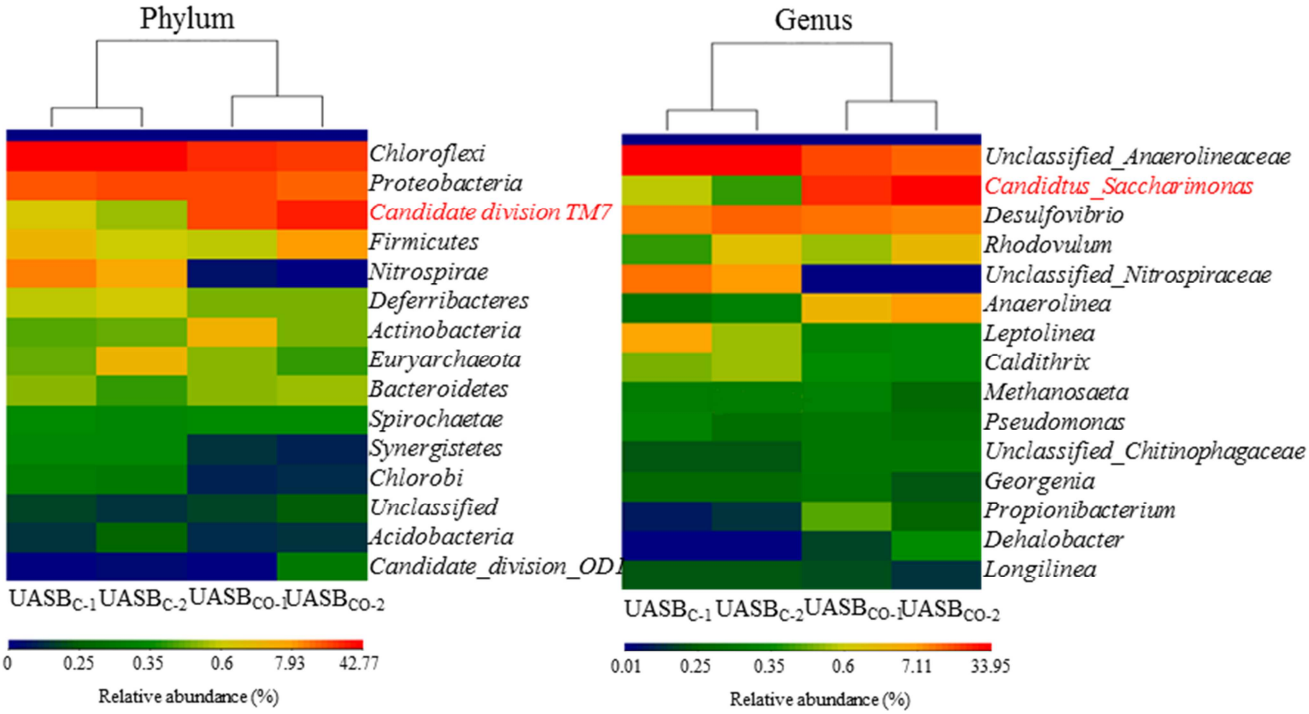

Fig 2. Phylum and genus level identification of the sequences obtained by high-throughput sequencing of 16S rRNA genes. $UASB_{C-1}$, $UASB_{C-2}$ are samples obtained from $UASB_{C}$ during steady-states, and $UASB_{CO-1}$, $UASB_{CO-2}$ are samples obtained from $UASB_{CO}$ during steady-states.

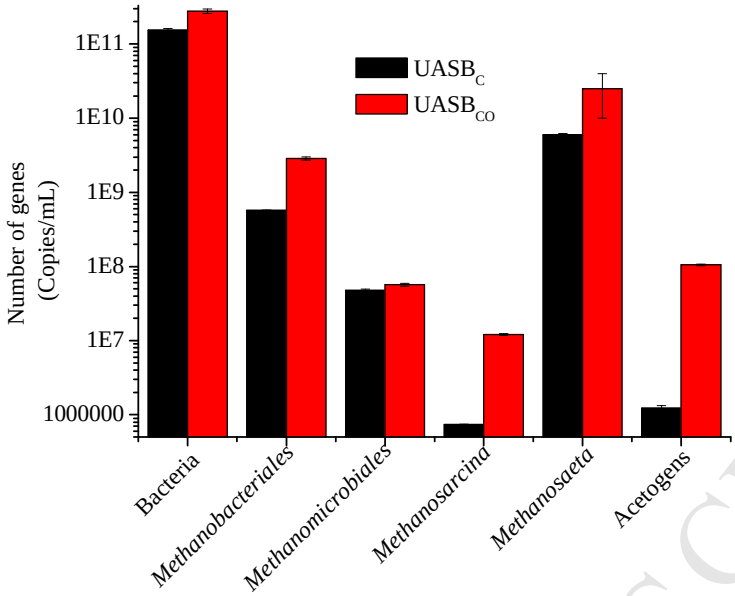

Fig 3. Abundances of bacteria, methanogens and acetogens in the samples obtained from $UASB_C$ and $UASB_{CO}$ during steady-states determined by qPCR

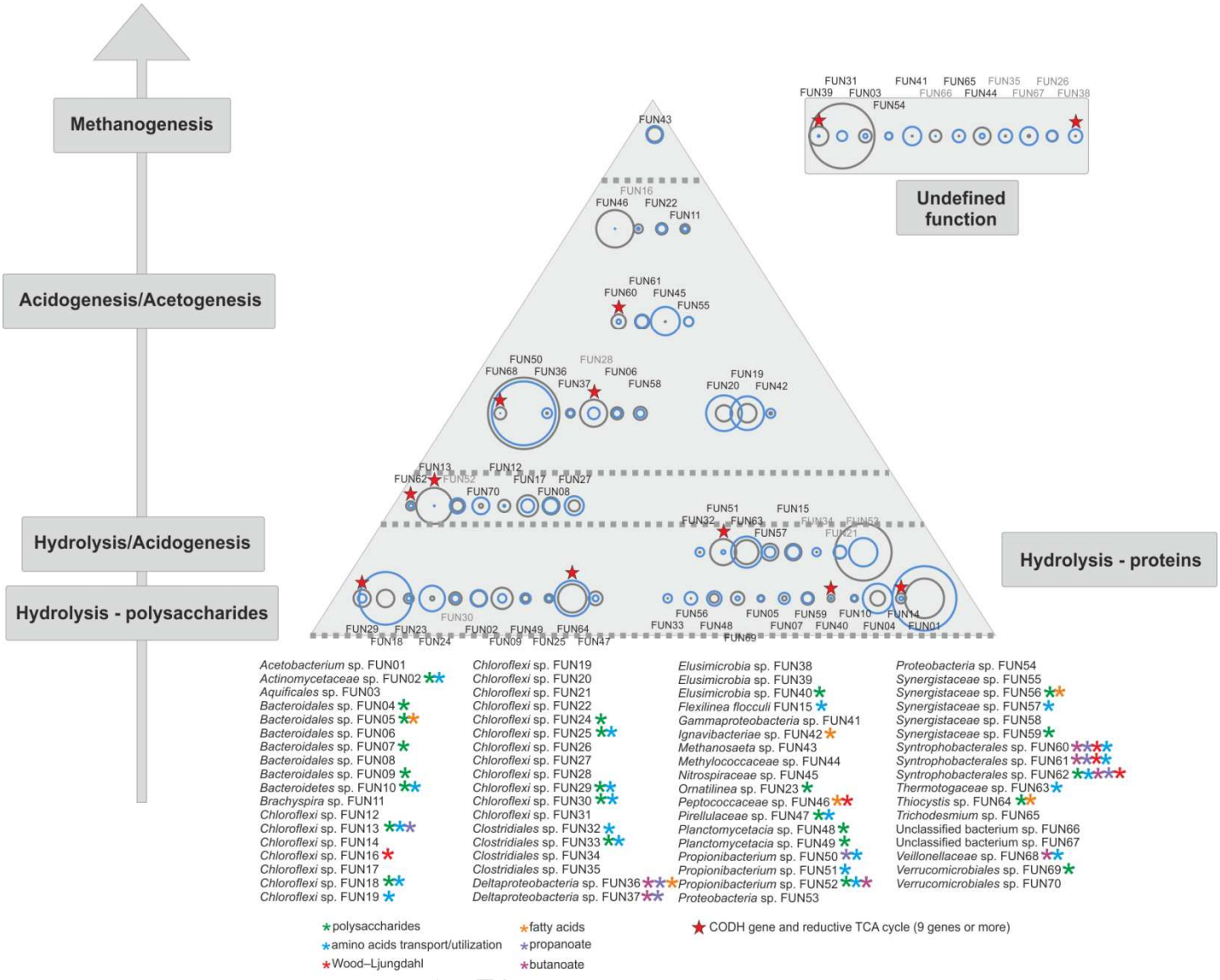

Fig 4. The genome bins (GBs) are represented in the context of the anaerobic digestion, starting from bottom (hydrolysis) to the top (methanogenesis). Functional roles were determined comparing the number of genes in each KEGG pathway/module among all the GBs of the microbiome. Those marked with asterisk(s) are in the top 10 % as number of genes in a specific KEGG pathway/module, while those marked with a red star encode at least one subunit of the CODH and 9 or more univocal genes in the reductive TCA cycle. Bubble size (area) is proportional to the abundance level calculated in the sample $UASB_C$ (blue) and in the $UASB_{CO}$ (grey). Only "FUN **" were shown in the triangle, which was unique and corresponded to the names of the GBs shown below the triangle. The GBs with names colored in light gray (in the triangle) are those with completeness lower than 70%.

Highlights:

- A new process for simultaneous biomethanation of organic wastewater and CO
- AGS could tolerate CO partial pressure as high as 0.5 atm after accumulation
- Gas-recirculation was crucial to increase the CO conversion
- Responses of AGS to the addition of CO were studied
- Metagenomic analysis revealed the potential CO-converting microorganisms