

# Mechanisms of increased methane production through re-circulation of magnetic biomass carriers in an experimental continuously stirred tank reactor

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Magnetite particles were used in a semi-continuous process as magnetic biomass carriers to separate and re-introduce microorganisms in a CSTR reactor. In comparison to a control reactor the methane content during the semi-continuous process was elevated when magnetite particles were used. The difference was most apparent during the fermentative step directly after feeding and upon direct spiking with volatile fatty acids. Total DNA quantification of the separated magnetite particles revealed high association of microorganisms. Furthermore, quantitative real-time PCR analysis of the associated microbial consortia indicated that the hydrogenotrophic Methanobacteriales was overrepresented at the particle surface. Thus, the increased methane production could be coupled to both the crowding and shorter interspecies distances between the groups involved in anaerobic digestion, as well as a preferential adsorption of hydrogenotrophs. By bringing the hydrogenotrophs closer to the primary fermentative bacteria and increasing their relative number the produced hydrogen during acidogenesis is more effectively utilized and more carbon dioxide is converted to methane. Furthermore, by the same cause, the rate of acetogenesis increased as the hydrogenotrophs more effectively could consume the hydrogen produced and thereby keep the hydrogen partial pressure low.

**Keywords: anaerobic digestion; bio carrier; biofilm; magnetite; syntrophy**

## INTRODUCTION

One limiting factor in anaerobic digestion (AD) is the long generation time of anaerobic microorganisms and, in relation to this, the contradictory desire to keep a short hydraulic retention time (HRT) which can lead to a “wash-out” of microorganisms (Lalov *et al.*, 2001; Fernández *et al.*, 2008). Furthermore, the biogas production is limited by the amount of cells inside the reactor when substrate is in excess (Fernández *et al.*, 2008; Nicoletta *et al.*, 2000).

Microorganisms colonizing surfaces generate a biofilm *i.e.* a matrix-enclosed microbial layer of different species adhered. In biofilm reactors, *e.g.* *Packed-* or *Fixed* bed reactors the design take advantage of the microbial tendency to create biofilms by accumulating or retaining the active cells inside the reactor. *Fixed* bed reactors commonly have relatively small tanks with high throughput rates and short HRT, while the organic loading rate is high relative to reactor designs without carriers (Singh *et al.*, 2009). These *high rate biofilm* reactor configurations are designed to separate the HRT from the solids retention time (SRT) (Yang *et al.*, 2004; Muñoz *et al.*, 1997) and are mainly appropriate for liquids with dissolved organic content at low concentration as in many industrial wastewaters.

However, in continuously stirred tank reactors (CSTR), HRT and SRT are not separated and no carriers are used. Utilization of carriers are avoided since they would be washed out with the reactor outflow and likely increase the wash out of active cells that adhere to the carriers (Hulshoff Pol *et al.*, 2004). To overcome this problem a carrier surface material for microbial growth, which could be controlled and kept inside the reactor, may well prove to be a conceivable solution. This work evaluates such a material by using magnetic carriers.

### Biofilms

Bacterial adhesion to chemically inert materials depends on the properties of the carrier. The generation of biofilm on surfaces is dependent on the initial attachment and subsequent adhesion of microorganisms (Hall-Stoodley *et al.*, 2004) and is considered to comprise four phases. The first phase is the transportation to the surface (Loosdrecht *et al.*, 1990), the second phase is the physiochemical process of initial adhesion (Hermansson, 1999) whereas the third phase is accomplished by cell surface structures that mediate a firm anchorage. The fourth phase of adhesion is the colonization of the surface and starts with attachment of single cells followed by generation of micro colonies and biofilm (Hall-Stoodley *et al.*, 2004).

Biological factors influence adhesion and explain why the presence of specific microbial strains is more abundant and required for biofilm generation. For example, the methanogens *Methanosaeta spp.* is known to be responsible for initial adhesion to surfaces and *Methanosarcinales* are frequently found in aggregates and in inner layers of biofilm on carriers (Schmidt and Ahring, 1999; Sasaki *et al.*, 2007).

### Biofilm carriers

A promising carrier material should support the initial, reversible adherence, but also present an attractable surface for the irreversible, biological adherence. Several studies have showed the influence of material choice on adhesion patterns (Yang *et al.*, 2004; Picanco *et al.*, 2002).

Surface roughness is supposed to minimize the thermodynamically calculated free energy of initial adhesion (Li and Logan, 2004). Particle porosity is of importance, since porous particles contribute with larger surface area per particle. Microbial surface charge is principally negative and a material with positive surface charge contributes to a less repulsive force (Sheng *et al.*, 2008). Several

studies have reported elevated adhesion of methanogenic consortia to both porous and hydrophobic carriers (Chauhan *et al.*, 2005; Picanco *et al.*, 2001; Yang *et al.*, 2004).

The use of carriers in biofilm reactors may enhance the methane production in AD. Furthermore, a carrier material with preferential adhesion of methanogens would be an attainable technique to increase the biogas production even more. Several studies have evaluated carrier materials. Silva *et al.* showed that alumina-based ceramics presented the best adhesion of methanogenic consortia (Silva *et al.*, 2006), while Chauhan *et al.*, (2005) identified best adhesion on clay montmorillonite. Good adhesion of methanogens on clay and polymeric materials has also been noticed by Muñoz *et al.*, (1997) and Lalov *et al.*, (2001). Among metal carriers iron oxide and stainless steel have been shown to have good adhesion capacity (Zhao *et al.*, 2007; Sheng *et al.*, 2008; Li and Logan, 2004).

### Magnetic biofilm carriers

The use of magnetic carriers would circumvent problems with wash out in CSTRs since a magnetic separation will generate an accumulation of microorganisms when the carriers are retained. In other words, this application will separate HRT from SRT and, hence, allow for a shorter HRT with equal degradation or, identical SRT with the possibility to use a smaller reactor volume.

Iron (ferrite) particles have hydrophilic surfaces and are porous particles with great surface roughness. Magnetite is another possible carrier, which consists of magnetic iron oxide. Iron oxide has been shown to present, in a comparison of different metal oxides coatings, the highest adhesion values for several microbial strains. This favourable adhesion characteristic was attributed to the relatively hydrophobic and high surface roughness of iron oxide (Li and Logan, 2004). The surface of magnetite comprises of thick layers of different iron-oxides that provide large positive charge and thereby enables better electrostatic attraction of negatively charged microorganisms (Sheng *et al.*, 2008). Another available, but expensive, carrier could be the magnetic hydrophobic micro carriers used in the pharmaceutical industry, that consists of silica or polymeric materials *e.g.* polystyrene.

## METHODS

The particles used were magnetite particles (Electronic standard powder, Höganäs AB, Sweden), ferric iron particles (NC100.24, Höganäs AB, Sweden) and polystyrene particles with 20 % incorporated magnetite (Product id: 49664, Sigma-Aldrich, Germany).

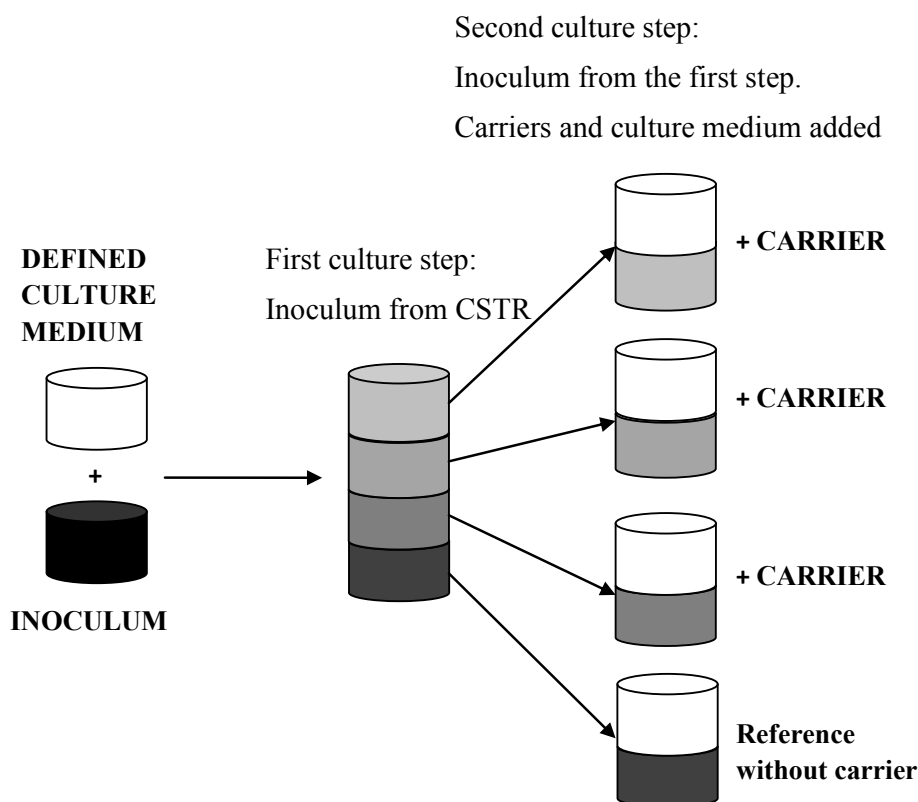
**Table 1:** Properties of selected magnetic carriers

	Iron	Magnetite	Polystyrene
Approx. size	80 µm	50 µm	10 µm
Porosity	Medium	Low	Low
Hydrophobicity	Hydrophilic	More hydrophobic than Iron	Hydrophobic
Surface charge	-	Positive	-
Surface roughness	High	Medium	Low

### Batch experiments

Two sets of batch experiments were carried out under mesophilic conditions (38°C; Fig. 1). In the first set the concentrations of iron particles and DNA sampling schedule was examined. The second batch objective was to compare the amount of biomass and adhered microbial consortia on the three carriers. Each experiment was performed in triplicates, where each bottle (544 ml) contained 60 ml

inoculum and 240 ml defined culture medium prepared according to Karlsson *et al.*, (1999). Energy and carbon sources were provided by cellulose (filter paper, Munktell, Sweden) and peptone (Peptone C, Merck, Germany). 0.75 g of each was added per batch bottle yielding 2.5 g VS/L of each and a total of 5 g VS/L.



**Figure 1:** Schematic illustration of the culturing procedure. Both sets of batch experiments were performed in two steps where the microorganisms were cultured in defined anaerobic culture medium to obtain samples with low contamination from the inocula for accurate qPCR measurement. Inoculum for the batch experiment was extracted from the reactors of the CSTR experiment (see below) and the first culture step was used to purify the material prior to the second step, where inocula from the first (enriched) culture step was used. Both cultures were terminated at the end of the exponential phase of methane production to obtain vital inocula. The second step was terminated by separation of the biomass adhered to the magnetic particles from the residual digestate fluid by neodym magnets.

Gas production was measured by accounting for pressure (Testo 312-3, Germany) and gas samples were withdrawn for determination of methane content by FID gas chromatography, HP GC-5880A (Hewlett-Packard, USA), column Poraplot T, carrier gas N<sub>2</sub>.

### *1<sup>st</sup> set batch experiments*

Different concentrations (0.033, 0.5 and 3.33 g/L) of iron particles were used in the second step of the experiment (Fig. 1). When gas production had seized the magnetic material in the batch bottles was separated with neodym magnets. Adhered biomass were analyzed (weight and DNA) on the magnetically separated particles and be the basis on the 2<sup>nd</sup> batch experiment. The experiment was further performed to monitor the gas production kinetics to be able to terminate the following 2<sup>nd</sup> batch experiment at an appropriate time. At the time of termination the methane content should be high enough to ensure a complete and abundant methanogenic population whereas gas production should not reach a stagnant phase to avoid starvation and excessive cell death, and thereby false population composition in the RT-PCR analysis.

### *2<sup>nd</sup> set batch experiments*

The three different carriers were added in the second culture step (0.5 g/L; Fig 1) and the carriers with adhered biomass were separated from the batch material with magnetic field upon termination. Both the sedimented non-magnetic residual and the biomass adhered were quantified by qPCR and the ratio was measured. Selected sequences for amplification were the domains bacteria and archaea and the hydrogenotrophic and acetoclastic methanogens.

### **DNA extraction and real-time PCR**

FastDNA<sup>®</sup> SPIN Kit for Soil (MP Biomedicals, Solon, USA) was used for DNA extraction. Extracted DNA concentrations were determined by Quant-iT fluorescence technology (Invitrogen, Stockholm, Sweden). The extracted DNA was stored at -20°C. Quantitative real-time PCR (qPCR) was performed with LightCycler 480 System (Roche Diagnostics, USA) on hydrolysis probes assay as described in Yu *et al.*, (2005), but using a Black Hole Quencher1 on the probes. Primers and TaqMan<sup>™</sup> probes used for detection and quantification of the domains Bacteria and Archaea, for the orders Methanosarcinales, Methanobacteriales, Methanomicrobiales and Methanococcales (Yu *et al.*, 2005). All primers and probes were purchased from Sigma-Aldrich<sup>™</sup> (Stockholm, Sweden). Reaction mixtures of 20 µl contained 10 µl LightCycler<sup>®</sup>480 Probes Master (Roche Diagnostics GmbH, Mannheim, Germany), 500 nM of each forward and reverse primers, 200 nM of the corresponding TaqMan<sup>™</sup> probe, 3 µl PCR-grade water and 5 µl of template DNA (300 ng). Reaction conditions were 10 min incubation at 94°C, 45 cycles of denaturation at 94°C for 10 s, annealing and elongation at 60°C for 30 s (63°C for the MMB primer/probe set) followed by 40°C for 10 s.

### **CSTR experiment**

An experiment with two semi continuous CSTR (9 L active volume) were operated at 38°C to study any effects of an accumulation of microorganisms on carriers amended to the slurry. Total gas production, methane content, syntrophic cooperation and process stability was used as means to evaluate possible influence in the processes. Inoculum from the AD of Nykvarn wastewater treatment plant in Linköping was used and a start-up period of three weeks was applied. Based on the batch result, magnetite (0.5 g/L) was added to one reactor and one reactor was a control. Gas production was measured online (MGC-10, Ritter, Germany) and methane content by Bluesense Gas sensor GmbH (Germany). Methane Tedlar balloons were used as gas buffers during feeding and volume adjustment.

A plastic container with four neodym magnets (Ø 23mm, Claes Olsson, Sweden) was designed to withhold the magnetic material in the outflow stream. This device was applied to the outlet for volume adjustment whenever a withdrawal of reactor sludge was made. When a withdrawal was completed, the retained magnetic material inside the container was reintroduced to the reactor.

### *Substrate and loading rate*

Dewatered mixed sludge (dry solids 6% and volatile solids 70%, Nykvarn wastewater treatment plant, Linköping, Sweden) was used as substrate. To avoid seasonal variation of the sludge, a batch was frozen and used during the study. 395 g of mixed sludge was fed daily, resulting in a retention time of 20 days and a loading rate of 1,84 kg VS/(m<sup>3</sup>\*d).

### *OLR-pulses*

The organic loading rate was transiently increased to observe differences in reactor stability and production performance. When these organic loading-pulses (Table 2) were used, VFA was measured daily and kinetics of gas production evaluated.

**Table 2:** Description of pulses of increased organic load applied.

Days after addition of particles	Organic loading rate (kg VS/m <sup>3</sup> *d)	Duration	Duration of VFA observation
20	3,69 mixed sludge (790 g)	1 day	5 days
41	5,44 mixed sludge (780 g) and glycerol (30 g)	1 day	5 days

*Spiking with VFA*

To provoke instability and study the reactors capacity to degrade VFA, propionic acid (45 mM; Baker, Holland) and butyric acid (5mM; Alfa Aesar GmbH & Co, Germany) were added to both reactors. No other substrate was added to the reactor during the sampling period (4, 24, 48, 72 and 96 h) after VFA addition.

*Additional monitored running parameters of CSTR experiment*

To control the process, pH, alkalinity, VFA, DS and VS were monitored regularly. pH was measured with a pH-meter (WTW Inolab, USA), at 25°C with a Hamilton electrode. Alkalinity was analyzed by titration to pH 5.4 with simultaneous removal of carbon dioxide by flushing with nitrogen gas on a Titrand 809 (Metrohm, Switzerland). VFA were analyzed twice a week with Perkin Elmer Clarus 550 Gas Chromatograph (Perkin Elmer, USA) with a packed Elite-FFAP column for acidic compounds. DS and VS was measured and calculated according to Swedish Standard method: SS 02 81 13: 1 1981-05-20.

**RESULTS AND DISCUSSION****Batch experiments***1<sup>st</sup> set batch experiments*

All amounts of carrier adhered enough biomass and DNA to perform qPCR analysis, but the lowest concentration was difficult to work with and was excluded (Table 1). The concentration of 0.5 g/L was used in all experiments, except the experiment with continuous reactors when 1 g magnetite/L was used. Thus, within this range of biomass carrier concentration, an increased carrier concentration was followed by an increasing adhered biomass.

**Table 3:** Amount of added iron carrier and the corresponding biomass separated with magnetic force and the attained DNA after purification.

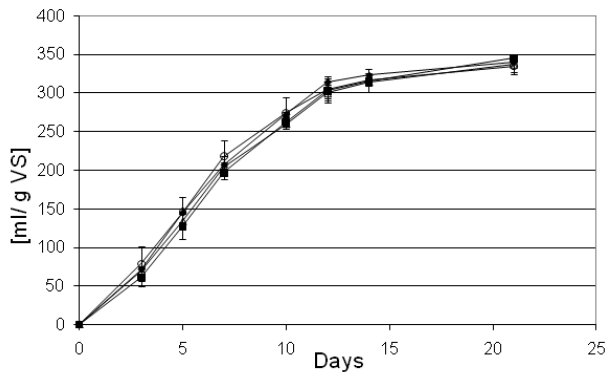
Carrier (g/L)	Carrier (g/flask)	Separated biomass (wet weight, ml)	Amount of DNA (µg/ml)
0.033	0.01	0.5	228
0.500	0.15	5	740
3.333	1	12	878

At day 14 the methane production was considered high enough to ensure a complete and abundant methanogenic population and this time point was later used to terminate the 2<sup>nd</sup> batch experiment for magnetic separation and DNA extraction.

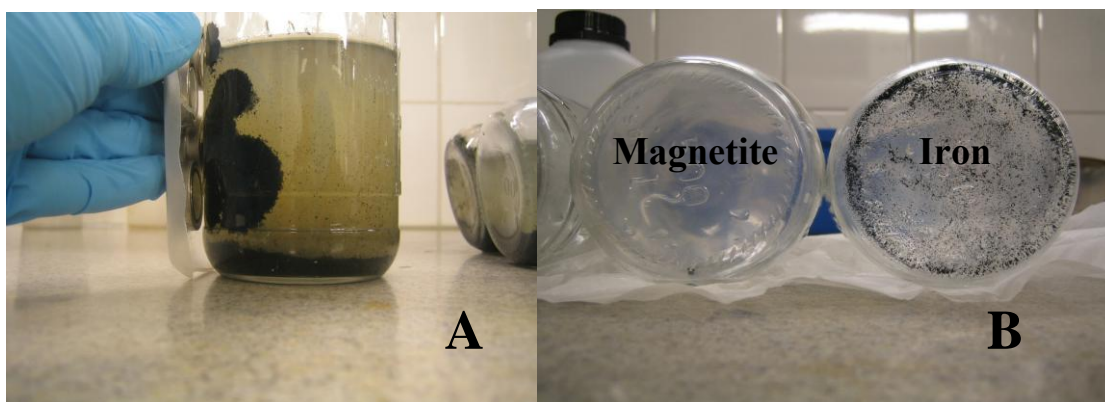
### 2<sup>nd</sup> set batch experiments

The methane production was essentially equal for all carriers and the reference (Fig. 2) and equal amounts of biogas had accumulated when the magnetite particles were separated from the digestate (day 14). In figure 3A the strength of magnetic separation of the biomass carriers is shown.

The particles were evaluated by the amount of biomass separated and their impact on the equipment (potential function in large scale biogas production). Magnetite particles were strongly attracted by the magnetic field and were harmless to the equipment. The iron particles, however, precipitated and attached to the bottle surface and also oxidized in brief contact with air, both which may prove harmful to the equipment (fig. 3B).



**Figure 2:** Accumulated methane gas for the alternative carriers and reference. The standard deviation is plotted in the graph. (■) iron, (●) polystyrene, (▼) magnetite, (○) reference.



**Figure 3:** (A) Photograph showing the strong magnetic separation of magnetite with adhered biomass in batch experiment. (B) Photograph showing two empty batch reactors after finished culturing. The left reactor had contained magnetite, while the right had contained iron particles and illustrates the precipitation of iron particles, which were firmly attached to the reactor glass bottom.

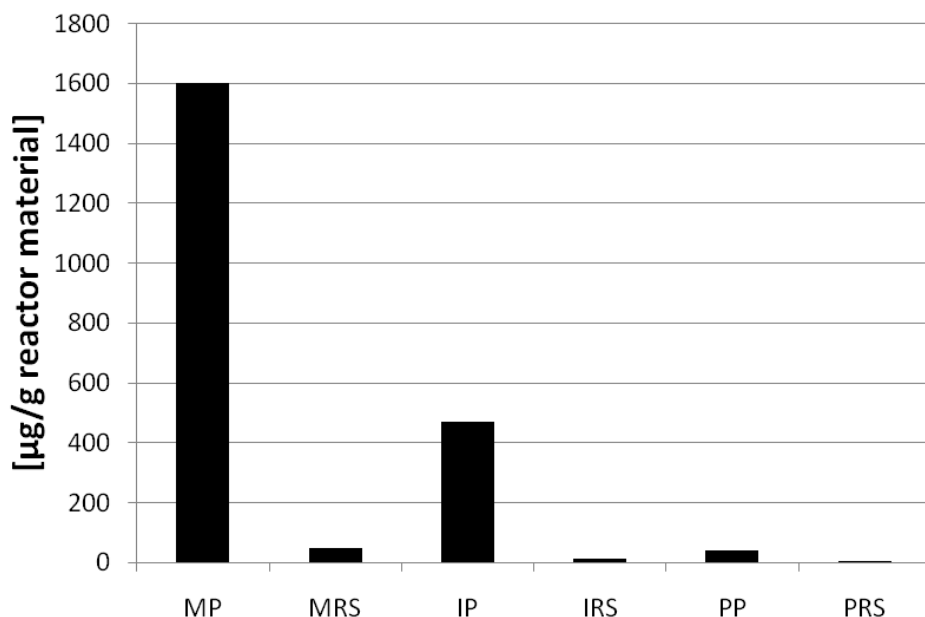
At day 14, one bottle of each triplicate was opened to perform magnetic separation and sampling for DNA extraction and qPCR. Amounts of magnetically extracted biomass are specified in Table 4 and showed the largest amount of associated biomass for magnetite.

**Table 4:** Wet weight of the magnetically separated biomass of alternative carriers.

Carrier	Separated biomass (g, wet weight)
Iron	2,03
Magnetite	3,86
Polystyrene	1,05

A complete separation of magnetite and iron was accomplished, while separation of paramagnetic polystyrene particles was incomplete since the magnetic force could not overcome the force of fluid flow. The DNA concentration was considerably larger in the magnetically separated material than in the non-magnetic residual fluid (Fig. 4). The high DNA concentration on the magnetic material indicates that the biomass consists of microorganisms.

A thirty-four time larger DNA concentration was observed for separated magnetite in respect to the non-magnetic internal standard (Fig. 4) and reintroduction of this magnetic separate during a continuous process could be expected to result in an accumulation of biomass inside the reactor.



**Figure 4:** Approximate amount of DNA extracted from different fractions, when normalized for weight of reactor material used for DNA isolation. (MP) magnetite particles, (MRS) magnetite residual slurry, (IP) iron particles, (IRS) iron residual slurry, (PP) polystyrene particles, (PRS) polystyrene residual slurry.

Based on the above results, magnetite was selected as the most promising particle for a CSTR application, since it displayed no harmful effects and further, attained the largest amount of biomass when separated from the digestate.

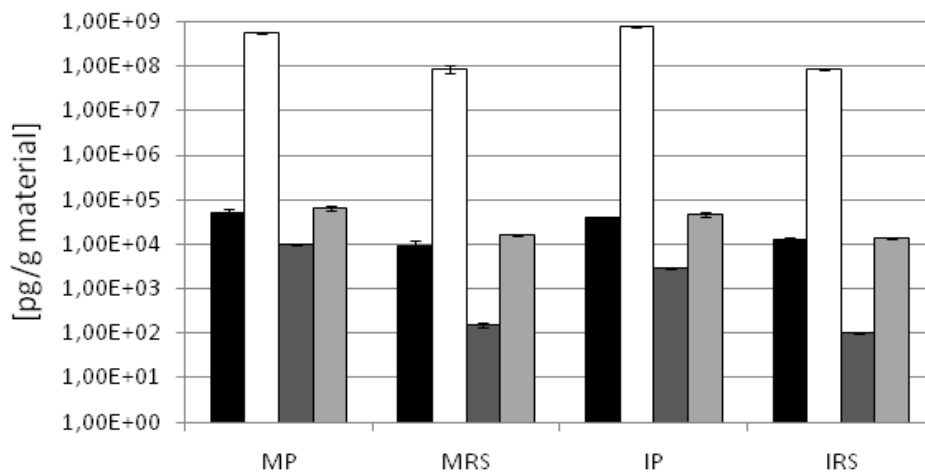
### Quantitative Real-Time PCR analysis

To determine if any preferential microbial adhesion could be observed on the magnetic particles, compared to the community in the residual, the ratio of microorganisms were examined. Only magnetite and iron particles yielded high enough DNA concentration for further qPCR analyses. Figure 5 shows that in comparison to iron, magnetite accumulated a slightly higher concentration of Archaea and the acetoclastic Methanosarcinales (MSL) and, in particular, the hydrogenotrophic Methanobacteriales (MBT).

More importantly, the ratio of microorganisms associated with the particles is not the same as the ratio of organisms in the residual slurry, but have changed in a way that both particle types showed increased proportion of associated MBT. In the case of magnetite, MBT increased almost two orders of magnitude, which is considerably higher than the increase of the other microorganisms



associated with the magnetite particles. Thus, in the case of magnetite particles, the result point to a preferential adsorption of hydrogenotrophic Methanobacteriales.



**Figure 5:** The amounts of target sequence DNA per gram isolated material. Reference slurry was concentrated by a factor of 15, while the particles were used as obtained after magnetic separation. Annotation is: (MP) magnetite particles, (MRS) magnetite residual slurry, (IP) iron particles, (IRS) iron residual slurry. Color coding is; (black) archaea, (white) bacteria, (dark grey) hydrogenotrophic *Methanobacteriales*, (light grey) acetoclastic *Methanosarcinales*.

## CSTR experiment

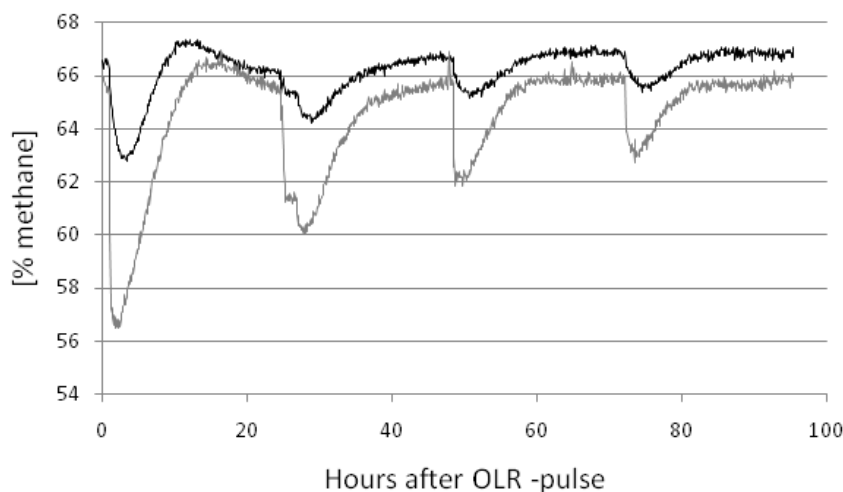
### *Magnetic separation*

The CSTR experiment was performed with magnetite as magnetic carrier to evaluate the technique of magnetic retention in a continuous process. Average amount of separated magnetic particles with associated biomass were 68 g from an average total amount of 481 g withdrawn material each day. Thus, about 14% of the outtake was reintroduced at each volume adjustment consisting of between 15 and 23% DS, with VS of DS of between 24 and 49%. DS in the CSTR increased from approx. 2.7 % to 3.4 % as a consequence of accumulated biomass, but also the added magnetite. However, although the biomass in the reactor likely increased this could not be registered in differences in gas production compared to the control reactor.

### *OLR-pulse 1 & 2*

Small differences in accumulated methane were observed during OLR-pulse 1. The methane and biogas production was 5.4 and 3.6% higher in the reactor without carriers and no accumulation of VFA was observed. The OLR-pulse 2 was high enough to provoke an accumulation of VFA, which peaked at 16 mM of total VFA for the reactor without magnetic carriers, while the reactor with carriers peaked at 14 mM. After about 70 hours, both processes resumed stable conditions with low VFA concentrations.

However, one notable difference was the methane production upon addition of new substrate, where the reactor with magnetic carrier displayed a smaller drop in methane concentration at each loading (Figure 6).



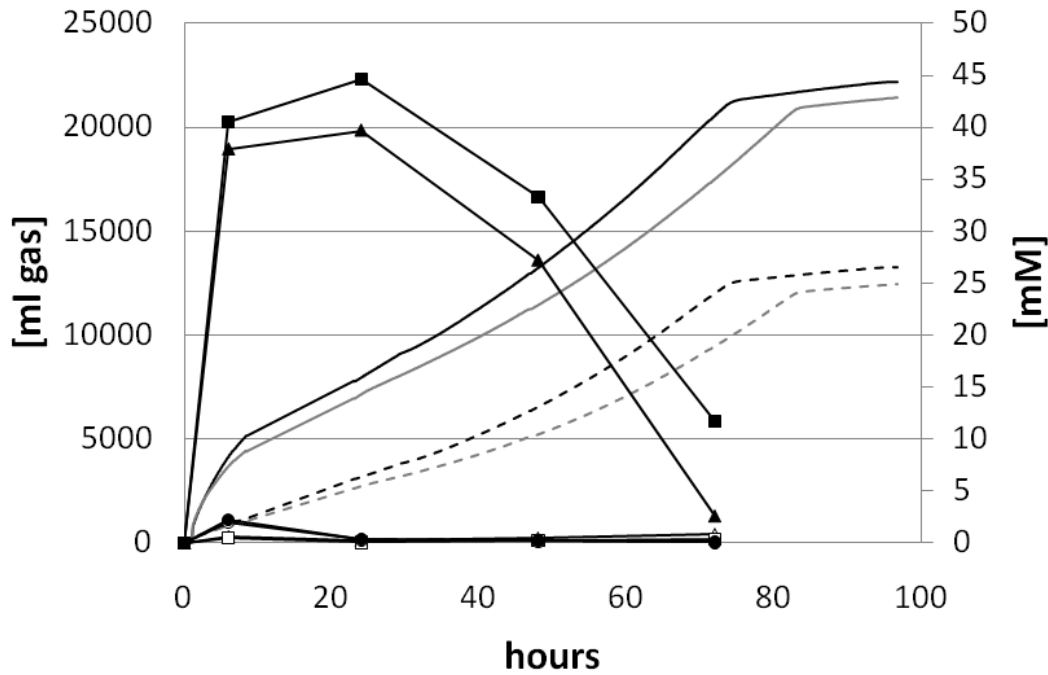
**Figure 6:** Methane concentration during a week after OLR-pulse 1 of double organic loading rate with mixed sludge ( $2 \times 1.8 \text{ kg VS/m}^3 \cdot \text{d}$ ). (—) reactor with magnetite particles, (---) control.

The methane content naturally decrease after addition of organic substrate as the fermentation of organic material produce a sudden increase of hydrogen and carbon dioxide immediately after “feeding”. Hence all the hydrogen and carbon dioxide cannot be utilized by hydrogenotrophic methanogens for methane production, and carbon dioxide and hydrogen are lost. A possible interpretation of the behavior is that the reactor with magnetic carrier, with increased biomass on the particle surfaces and a preferential adsorption of hydrogenotrophic methanogens, can take better advantage of this window of excess carbon dioxide and hydrogen, leading to enhanced methane production. From the above results it could be inferred that possible positive effects of retaining the biomass on magnetic particles could not be observed because of the nature of the substrate. It is well established that the rate limiting step in AD of wastewater sludge is the hydrolysis of polymeric substances (Appels *et al.*, 2008). If this was the case also in the experimental reactors, effects in down-stream process would remain un-noticed despite increased biomass.

#### *Spiking with VFA*

In order to further elucidate at what metabolic stage the addition of magnetic particles has its biggest influence VFA, in the form of propionic and butyric acid, was added to the continuous reactors. In theory, fermentation products including VFA other than acetic acid are degraded by syntrophic bacteria during acetogenesis and their activity is dependent on hydrogenotrophic activity to keep the  $p_{\text{H}_2}$  low. Thus, a more efficient cooperation would be demonstrated by a faster degradation of VFA and higher rate of methane production. In turn this would also lead to a greater resistance to process instability inflicted by VFA addition.

Figure 7 illustrates the higher gas production kinetics and increased methane concentration of the reactor with magnetite particles that was the result after spiking with VFA. The production rate of gas decreased sharply when all VFA was degraded, which occurred about 10 hours earlier in the magnetite reactor than in the control. Consistent with the gas production it was also found that the VFA were degraded faster in the magnetite reactor.



**Figure 7:** Gas production and VFA degradation after spiking with 50 mM VFA. (—■) gas production magnetite reactor, (—●) gas production in control, (- - - ▲) methane production magnetite reactor, (- - - ■) methane production control. Gas production and methane content was not monitored between hour 8 and 23 due to electrical failure, production during this period was estimated by linear regression. (▲) total VFA in magnetite reactor (-■-) total VFA in control.

Since the addition of VFA separates the acetogenesis and methanogenesis from the other steps of AD, the differences in methane content and VFA degradation observed during the spiking experiment can be concluded to be a direct effect of an enhanced syntrophic cooperation in the presence of carriers.

Hydrogen functions as an interspecies electron carrier between syntrophs and methanogens and a rapid removal of the hydrogen produced by acetogens is necessary to keep the partial pressure of hydrogen low. Thus, the increased degradation rate of VFA is likely an effect of low  $P_{H_2}$  near syntrophs, and the low  $P_{H_2}$  is in turn a consequence of the accumulated biomass inside the reactor. The biomass adhering to the magnetite particles contain more microorganisms in close proximity and furthermore display a preferential adsorption of hydrogenotrophic methanogens that more effectively can utilize the hydrogen produced during acidogenesis and acetogenesis. This will in turn make the methanogenesis more effective, since the methane production will not be rate limited by the diffusion rate of hydrogen and, thus, support the syntrophic cooperation.

## CONCLUSION

The technique of reintroducing magnetic biomass carriers does indeed function with satisfactory results in respect to accumulated biomass of microorganisms inside the reactor. Also, for several reasons, magnetite proved to be a promising carrier for magnetic separation and re-introduction of biomass in CSTR. An extensive colonization of magnetite is present, with preferential adhesion of hydrogenotrophic Methanobacteriales. The AD with magnetite biomass carriers presented elevated degrading rates of VFA and produced gas of higher methane content when subjected to increased organic loading. These characteristics can be ascribed to an enhanced syntrophic cooperation between hydrogenotrophic methanogens and syntrophs and the increased population of vital microorganisms in the reactor.

However, these properties was not translated to higher degradation kinetics or increased methane content during the normal reactor process, since the increased syntrophic cooperation is not the rate limiting step of AD of sludge from waste water treatment plants. Thus, it is necessary to further study the effects of retaining biomass in the reactor by magnetic separation of biomass adhered to magnetic particles as the technique might prove more beneficial in reactors treating more easily hydrolyzed substrates leading to higher VFA loading.

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