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Research paper

Effect of humic acids on the activity of pure and mixed methanogenic cultures

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ABSTRACT

The impact of humic acid (HA) on methanogenic activity was investigated. Methanogenic crushed granular sludge and pure cultures of mesophilic methanogens were incubated in batch cultures with HA. Initial methane production rates and substrate consumption rates were quantified. In the presence of 1 kg m^{-3} HA, the methane production rate of all hydrogenotrophic methanogens was inhibited by more than 75%, except *Methanospirillum hungatei* that was not inhibited up to 5 kg m^{-3} HA. The acetoclastic *Methanosarcina barkeri* was completely inhibited by HA ≥1 kg m^{-3}. However, *Methanosaeta concilii* was only slightly affected by HA up to 3 kg m^{-3}. When methanogenic granular sludge was incubated with HA, the specific methanogenic activity (SMA) tests showed less inhibition, when compared to the pure cultures of methanogens. The SMA test with H_2/CO_2, formate and acetate showed reduced initial methane production rate of 42%, 23% and 40%, respectively. Differences in HA susceptibility were explained by differences in cell wall structure.

1. Introduction

Methanogens are strictly anaerobic archaea that have diverse morphology and phylogeny. Their ecological niches are widely distributed. They can be found in aquatic sediments (marshes and swamps), stagnant soil (peat bogs and rice fields), marine geothermal vents, the digestive tract of animals (ruminants and termites) and in engineered anaerobic digesters [1]. Methanogens are sensitive to environmental factors. Wide range of organic compounds, such as long chain fatty acids, aromatic compounds, xenobiotics, and inorganic compounds such as ammonia and heavy metals have been described to affect the methanogenic activity [2].

Humic acids (HA) are charged polyelectrolyte complexes due to the presence of carboxylic, phenolic, ketonic, aromatic and aliphatic groups and interact with both living and non-living matter [3]. They can function as electron shuttles in anaerobic environments for fermentive, iron-reducing and sulphate-reducing bacteria, as well as for methanogenic archaea [4–8].

Although the role of HA in natural environments is known, their abundance, composition and effect in engineered systems (e.g. in anaerobic digesters) are not defined well in the literature. In an anaerobic digester environment, abundance and composition of HA mainly depend on the type of the feed [9]. HA concentrations can reach up to mass fraction of 1.5% of total solids in the treatment sludge and agricultural waste, such as manure and maize [9–11]. Abundance of HA in anaerobic digesters may negatively affect the overall conversion processes. Indeed, the negative effect of HA on hydrolysis step of anaerobic digestion was shown [9,12–14]. In addition, a decrease in methanogenic activity was observed in the presence of HA [12–15]. However, from these experiments it was not evident whether the methanogens were affected and if so, which physiological groups/phylotypes of methanogens were most vulnerable to HA inhibition. Thus, it is important to determine the physiological response of different methanogenic groups to get more information about the methane production in the anaerobic digesters, having higher HA concentrations.

In this study, important acetoclastic and hydrogenotrophic

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methanogenic groups, belonging to *Methanosaetaceae*, *Methanosarcinaceae*, *Methanospirillaceae*, and *Methanobacteriaceae*, were selected to test their methanogenic activity in the presence and absence of HA. These methanogenic groups were selected due to their high abundance in most of the anaerobic digesters [16]. The methanogenic activity of pure cultures was compared to anaerobic crushed methanogenic granular sludge from a full scale UASB (Upflow Anaerobic Sludge Blanket) reactor treating paper mill wastewater. In this scope, batch tests were set-up in identical conditions for both pure and mixed cultures. During the batch experiments, methanogenic activity of each experimental group was monitored with gas and organic acid measurements.

2. Materials and methods

2.1. Experimental set-up

The effect of humic acid (CAS Number 68131-04-4, Sigma-Aldrich, Zwijndrecht, The Netherlands) on mesophilic methanogens was investigated in batch tests. Crushed mesophilic anaerobic granular sludge and pure cultures of methanogens were tested. Batch incubations were performed in 120-cm^3 bottles with 50 cm^3 bicarbonate buffered mineral salts medium, supplemented with cysteine (0.96 kg m^{-3}), trace elements and a vitamin mixture. Additionally, 0.12 kg m^{-3} acetate was added to the hydrogenotrophic cultures (also when grown on formate) as additional carbon source [17,18]. The bottles were inoculated with a volume fraction of 10% of a culture pre-grown on the same substrate. Depending on the metabolic property of the strain, the growth substrates were H_2/CO_2 (a volume fraction of 80%/20%, respectively at 150 kPa), formate (final concentration: 1.68 kg m^{-3}) or acetate (final concentration: 1.2 kg m^{-3}), the latter two having a headspace of N_2/CO_2; a volume fraction of 80%/20%, respectively at150 kPa. In the assays 0, 1, 3 and 5 kg m^{-3} humic acid were tested, unless stated otherwise. The batch incubations were performed in duplicate and in the dark at 37 °C, pH 7. Methane (CH_4) production and hydrogen (H_2) consumption were monitored by gas chromatography. Liquid samples were collected to measure changes in acetate and formate concentrations.

2.2. Composition and the source of the humic acid

Humic acid (CAS Number 68131-04-4, Sigma-Aldrich, Zwijndrecht, The Netherlands) was used in the experiments. Only one batch of the humic acid was used throughout the experiments to avoid the composition changes during the production phase of the product. According to the manufacturer, the product may be produced from dead plants and brown coal. Alkaline extraction methods are applied to recover the humic acid. Molecular weight of the product is in the range of 2000–500000. The composition of the product includes polysaccharides, proteins, simple phenols, and chelated metal ions. Washing steps with deionized water are applied to remove the excess amount of the organic contaminants. Addition of HA to the experiments introduces a maximum amount of sodium (approximately 0.3 kg m^{-3}) and small amounts of calcium and iron in the anaerobic media [16]. Although an excess of sodium could potentially inhibit anaerobic digestion, sodium in HA, was still 10 folds lower than inhibitory sodium concentrations that were previously reported [19–21]. Moreover, the presence of the other cations such as potassium, magnesium and calcium, are likely to show antagonistic effects to sodium inhibition [19].

2.3. Growth conditions of methanogenic cultures and anaerobic sludge

In this study, *Methanosaeta concilii* (DSM 2139), *Methanosarcina barkeri* (DSM 800), *Methanobacterium formicicum* (DSM 1535), *Methanospirillum hungatei* (DSM 864) and *Methanobrevibacter arboriphilicus* (DSM 744) were used as pure cultures. All cultures were routinely grown at 37 °C in an anaerobic bicarbonate buffered medium [17,18]. Three subsequent transfers of each strain were made to ensure optimum growth conditions in the defined medium. After successful transfers, the microorganisms were used in the batch activity tests.

Granular methanogenic sludge was obtained from a UASB reactor treating pulp and paper industry effluents (Industriewater Eerbeek, The Netherlands). Sludge samples were collected on 10th of April 2014. Immediately after collection, granules were crushed under nitrogen gas flow in a 500-cm^3 serum bottle that contained 250 cm^3 phosphate saline buffer solution (0.1 kg m^{-3}, pH 7). The slurry obtained was transferred to a 500-cm^3 serum bottle and flushed with nitrogen gas. About 5 cm^3 of the prepared slurry (1 kg m^{-3} volatile solids) was used for the batch activity tests.

2.4. Analytical methods

2.4.1. Gas measurements

CH_4 and H_2 content of the gas phase was analysed with a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 2 m long, 3 mm internal diameter and 60–80 mesh packed column (Molsieve 13X) (Varian, Middelburg, The Netherlands). The column had a thermal conductivity detector that was operated at 70 mA, 150 °C. Argon was the carrier gas at a flow rate of 30 cm^3 min^{-1}. Gas samples (0.2 cm^3) were taken by syringe and the gas content was expanded to 1 cm^3 while the needles were in the rubber stopper, and injected to the column. All measurements were performed in duplicate and data was analysed using ChromQuest software (Thermo Scientific, Waltham, MA).

2.4.2. Organic acid measurements

Liquid samples were collected to determine acetate and formate concentrations. Liquid samples were centrifuged (11200 RCF, room temperature, 10 min) and filtered through a polypropylene filter (0.45 μm). The obtained supernatants were analysed by Thermo Scientific Spectrasystem HPLC system, equipped with a Varian Metacarb 67H 300 × 6.5 mm column kept at 45 °C, running with 0.5 kg m^{-3} sulphuric acid as eluent. The eluent had a flow rate of 0.8 cm^3 min^{-1}. The detector was a refractive index detector. Data was analysed using ChromQuest (Thermo Scientific, Waltham, MA).

3. Results and discussion

3.1. Effect of humic acid on methanogenic cultures

For all methanogenic pure cultures used in this study, the recovery of reducing equivalents in the form of CH_4, produced from H_2/CO_2, acetate and formate, was always higher than 85%.

3.1.1. Hydrogenotrophic methanogenesis

When *Methanobacterium formicicum* was grown on formate in the absence of HA, the maximum total amount of methane (0.2 mmol) was produced within one day (Fig. 2a). In the presence of HA, methane was also produced, but after a long lag phase of 20 days (Fig. 2b). The duration of the lag phase was similar for the cultures grown with different HA concentrations. During the lag phase, accumulation of trace amounts of H_2 was observed (to 0.027–0.035 mmol, Table 1). After day 20, the trace amounts of H_2

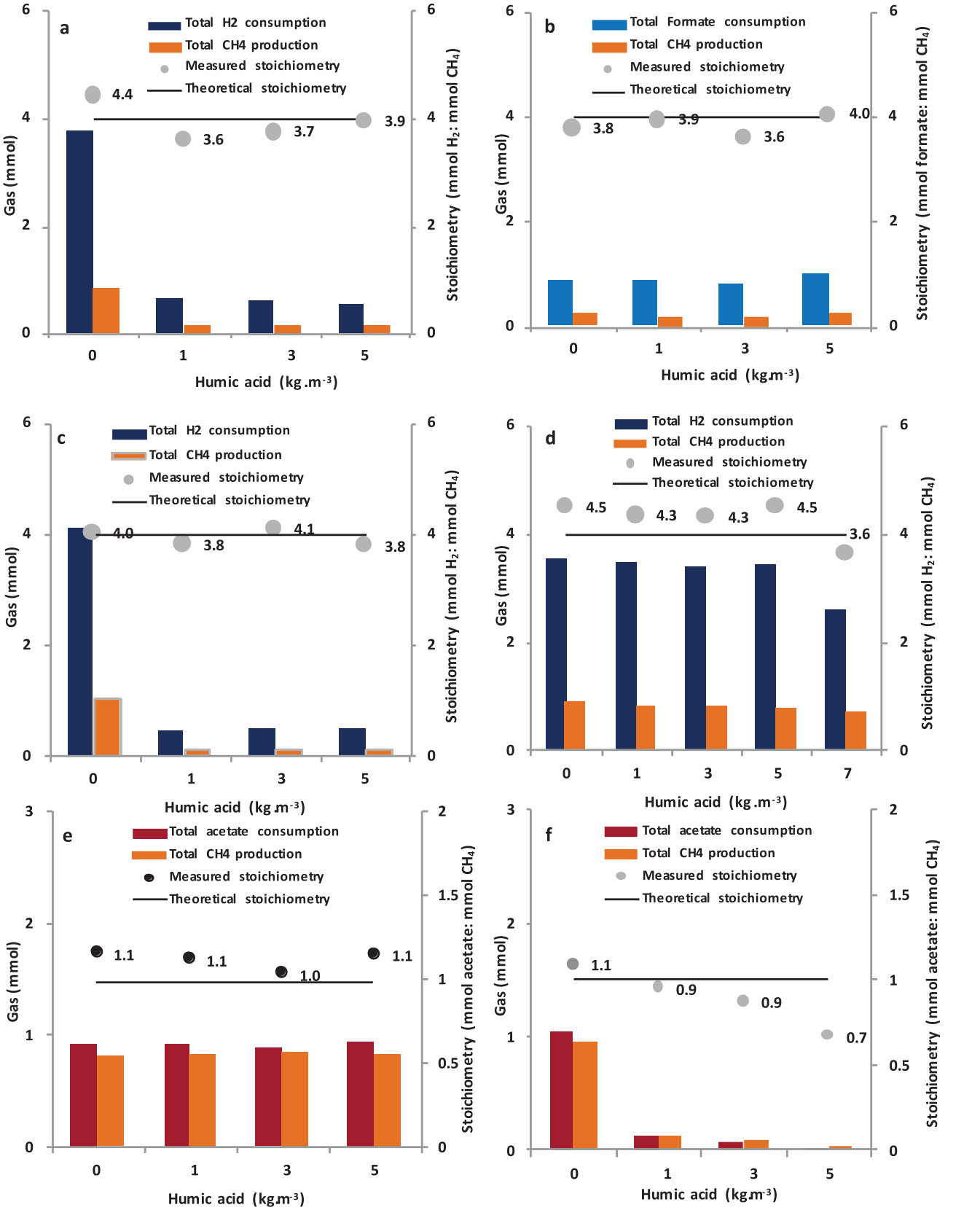

Fig. 1. The observed averaged stoichiometry of methanogenesis in the presence and absence of humic acid. The observed stoichiometry of *Methanobacterium formicicum* incubated with H_2/CO_2 a) and with formate b). The observed stoichiometry of *Methanobrevibacter arboriphilicus* c) and *Methanospirillum hungatei* d), both incubated with H_2/CO_2. The observed stoichiometry of *Methanosaeta concilii* e) and *Methanosarcina barkeri* f), both fed with acetate.

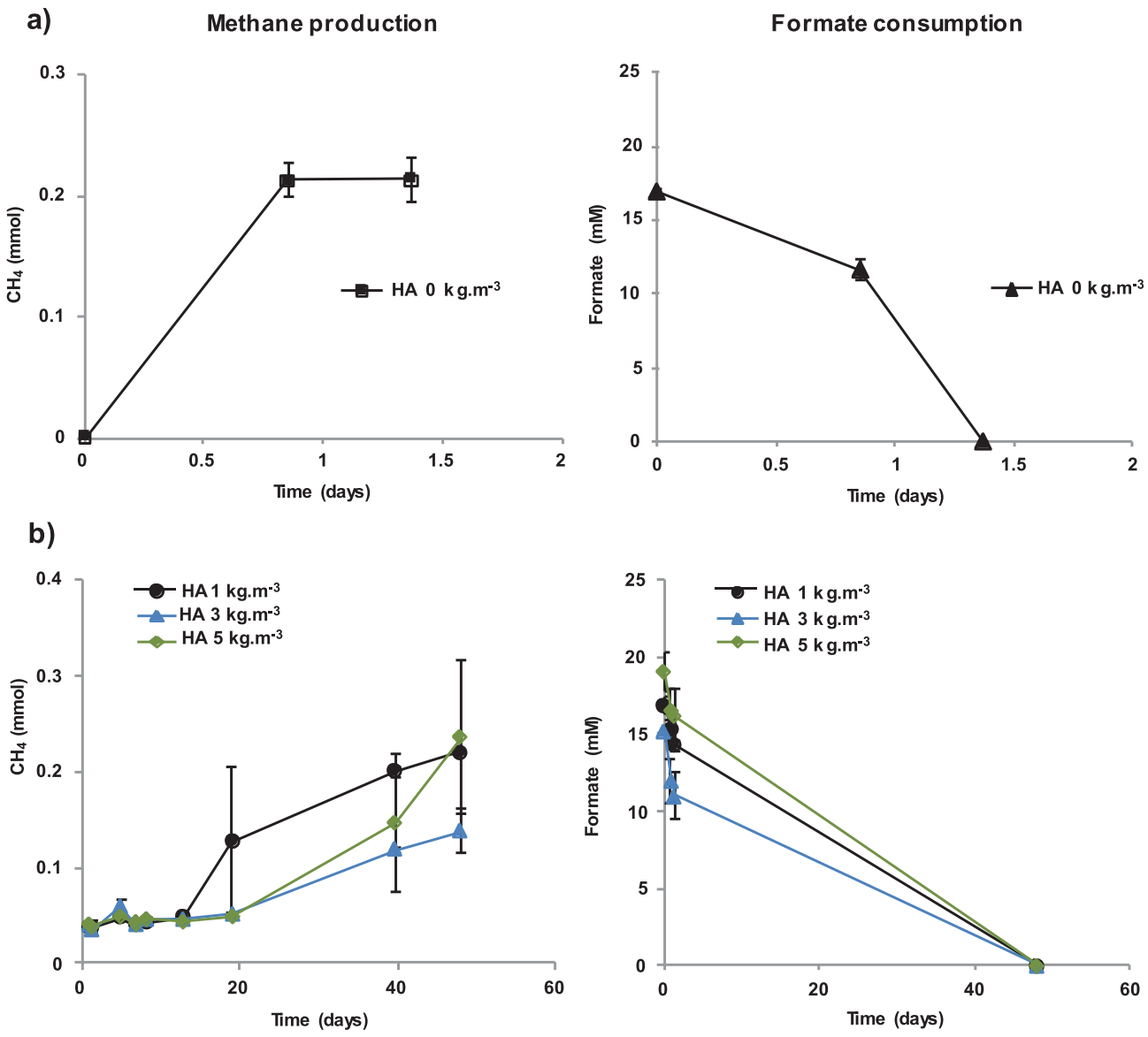

Fig. 2. Effect of humic acid (HA) on the methanogenic activity of *Methanobacterium formicicum*, fed with formate. Left panel: CH_4 production over time in the absence (a) and in the presence (b) of HA. Right panel: the corresponding formate consumption.

started to be consumed, which coincided with methane production, reaching the same level as the control (Fig. 2b). The observed initial CH_4 production rate was lower at higher HA concentrations (Table 1). The occurrence of H_2 production from formate may be a physiological response of *M. formicicum* to the presence of HA. It is known that H_2 formation by some methanogens is enhanced when the ambient H_2 concentration becomes low [22–24]. In this respect, the presence of HA can create a stress condition that inhibits the methanogenic process after formate cleavage. Methane production started after a relatively long lag phase when apparently sufficient excess reducing equivalent in the form of H_2 was obtained.

In batch incubations of *M. formicicum* with H_2/CO_2, methanogenic activity was inhibited at HA concentrations $\geq$1 kg m^{-3}. Addition of 1, 3 or 5 kg m^{-3} HA to the bottles resulted in a slow linear methane production (Table 1). In the absence of HA, this culture produced within 18 days 0.85 mmol methane (Fig. 3a). The total amount of produced methane at the end of the experiment was reduced by 79, 81 and 84% at 1, 3 and 5 kg m^{-3}, respectively (Table 1).

HA was also inhibitory to *Methanobrevibacter arboriphilicus* that was grown on H_2/CO_2. In the absence of HA, methane was produced at a linear rate and 1.02 mmol of methane was produced at the end of the experiment (Fig. 3b, Table 1). In the presence of HA, the total amount of methane produced was reduced by 89% and reduced methane production rates were observed for all tested HA concentrations (Table 1). As was the case for *M. formicicum*, HA was already inhibitory at 1 kg m^{-3} for *M. arboriphilicus*.

By contrast, *Methanospirillum hungatei* was not much affected by the presence of HA (Fig. 3c). In the absence of HA, 0.81 mmol methane was produced within 8 days. In the presence of 1 kg m^{-3} of HA, the total amount of produced methane was reduced by 9% and even at HA concentration of 7 kg m^{-3} the total amount of methane produced was only reduced by 13% (Table 1). The overall results showed that activity of *M. hungatei* was not much affected by the presence of HA.

An explanation for this lack of inhibition might be the complex, proteinaceous impermeable envelope layer (the sheath) of *M.*

Table 1
Initial methane production rates and total amounts of methane produced at the end of the experiment, by the different methanogenic pure cultures, in the absence and presence of humic acid. The presented values in the table are the average of representative duplicate measurements.

Microorganisms/Humic acid (kg.m^{-3})	Production rate (mmol.day^{-1})		Consumption rate (mmol.day^{-1})			Inhibition percentage of CH_4 production rate[a]	Inhibition percentage of total amount CH_4[b]
	H_2	CH_4	H_2	Acetate	Formate		
Methanobacterium formicicum							
fed with H_2/CO_2							
0	–	0.06	0.32	–	–	0	0
1	–	0.02	0.13	–	–	73	79
3	–	0.01	0.15	–	–	75	81
5	–	0.01	0.11	–	–	77	84
Methanobacterium formicicum							
fed with Formate							
0	–	0.25	–	–	6.26	0	0
1	0.01	0	–	–	1.92	99	5
3	0.01	0	–	–	3.78	99	6
5	0.01	0	–	–	2.87	99	0
Methanobrevibacter arboriphilicus							
fed with H_2/CO_2							
0	–	0.12	0.36	–	–	0	0
1	–	0.01	0.04	–	–	87	88
3	–	0.02	0.04	–	–	87	89
5	–	0.02	0.05	–	–	85	88
Methanospirillum hungatei							
fed with H_2/CO_2							
0	–	0.11	0.49	–	–	0	0
1	–	0.11	0.48	–	–	0	9
3	–	0.14	0.55	–	–	0	9
5	–	0.13	0.43	–	–	0	12
7	–	0.1	0.31	–	–	8	18
Methanosaeta concilii							
fed with Acetate							
0	–	0.06	–	0.07	–	0	0
1	–	0.06	–	0.07	–	0	0
3	–	0.05	–	0.06	–	6	0
5	–	0.04	–	0.07	–	21	0
Methanosarcina barkeri							
fed with Acetate							
0	–	0.03	–	0.03	–	0	0
1	–	0.02	–	0.01	–	45	86
3	–	0.01	–	0.01	–	69	92
5	–	0.01	–	0	–	74	97

[a] The total amount of methane refers to the average amount of methane produced from duplicate experiments at the end of the experiment. To calculate the percentage of the methane production rates and the total amount of methane in the presence of HA, the methanogenic activity in the control bottles (without HA) was considered 100%.
[b] Methane production rates were calculated as initial production rates relative to the initial methane production rate in the control bottles without humic acid.

hungatei, which might prevent HA to penetrate inside the cells [25]. In contrast, *M. formicicum* has a much thinner pseudomurein surface envelope. Experiments by Prokhotskaya and Steinberg [26] on the effect of HA on cyanobacteria and eukaryotic algae support this hypothesis. Cyanobacteria were more susceptible to HA inhibition than eukaryotic algae, because of their difference in cell wall structure. The observed inhibitory effects of HA on *M. formicicum* and *M. arboriphilicus* may be explained by the accumulation of HA inside the cells. Once HA is concentrated inside the cells, electron transport system of the methanogens might be altered due to the negative charge and the electron shuttling properties of the HA. Alternatively, reducing equivalents inside the cells might be transported through the cell membrane to the exterior of the cells, where HA acts as an electron acceptor. Such potential losses of reducing equivalents will suppress microbial growth. However, at present it is not clear which reactions or enzymes in the cell are affected by HA.

3.1.2. Acetoclastic methanogenesis

Methanosaeta concilii grown on acetate was not much affected by HA. With all tested conditions, methane production reached 0.8 mmol after 20 days and acetate was completely converted (Fig. 4a). Furthermore, the CH_4 production rate was not strongly affected by HA (Table 1). In contrast, the acetoclastic activity of *Methanosarcina barkeri* was strongly affected by the presence of HA (Fig. 4b and Table 1). At HA concentrations of 1, 3 and 5 kg m^{-3}, the total amount of methane decreased with 86, 92 and 96%, respectively (Table 1). Inhibition of methane production from acetate by *M. barkeri* in the presence of the anthraquinone-2, 6-disulfonate (AQDS) was also observed by Bond and Lovley [27].

However, in that study Fe (III)-containing growth media were used and it was not clear whether AQDS was indeed the inhibitory compound.

The differences in HA sensitivity between the two acetoclastic methanogens can also be due to the proteinaceous cell wall of *M. concilii*, which may prevent HA to enter the cells as described above for *M. hungatei* [25]. *M. barkeri* lacks such a thick cell wall [25].

3.1.3. Methanogenic activity of crushed granular sludge

Batch tests were performed using crushed granular sludge in the presence and absence of HA and with either H_2, formate or acetate as growth substrates. When H_2 was used as an electron donor, HA had only a small inhibitory effect compared to the results with the pure cultures of hydrogenotrophic methanogens (Fig. 5a and Fig. 3). HA concentrations of 1, 3 and 5 kg m^{-3} had similar inhibitory effects on the methane production rates, but total methane produced at the end of the experiment was only slightly affected by the HA concentration (Table 2).

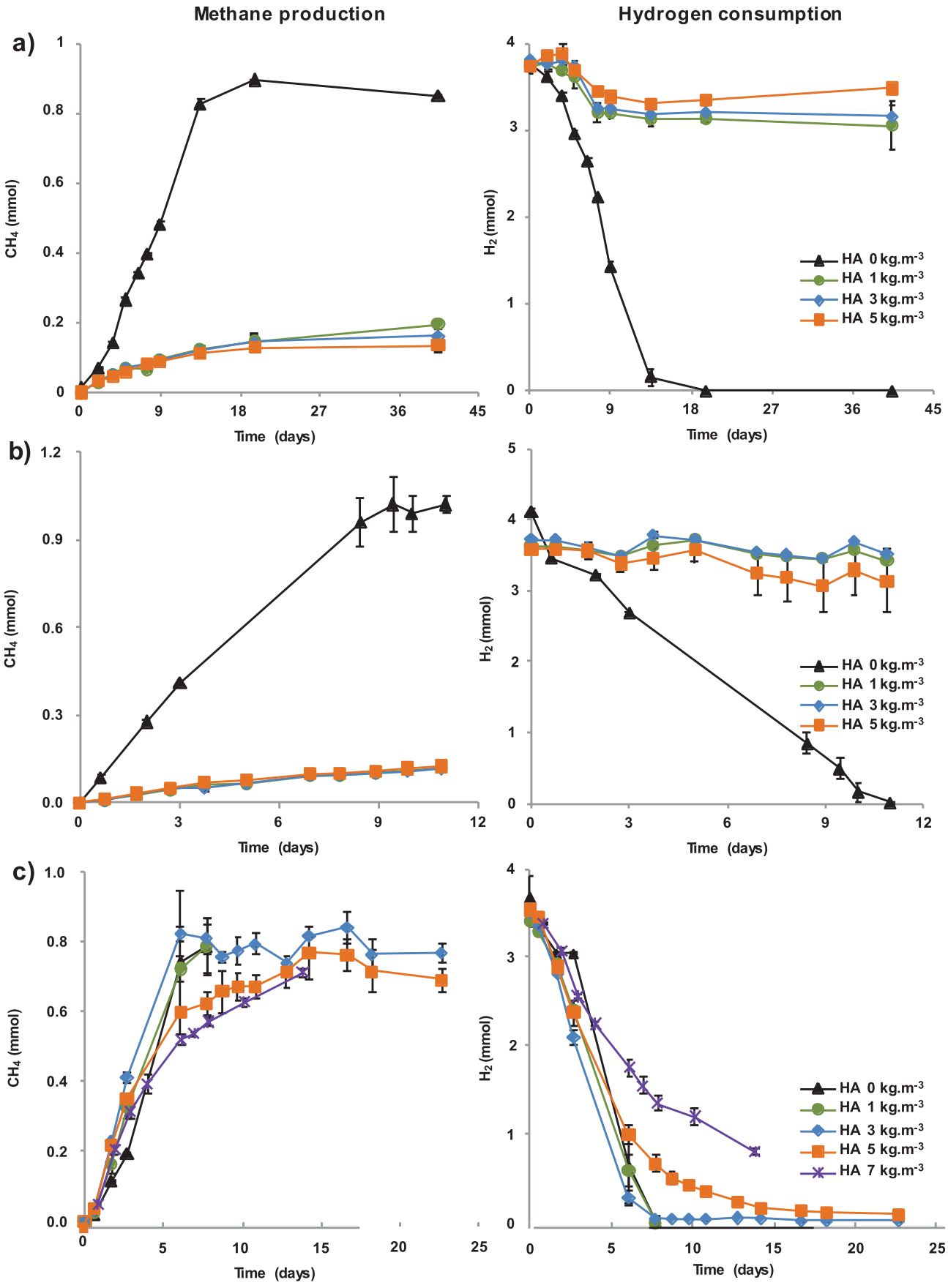

Fig. 3. Effect of humic acid on methane production of a) *Methanobacterium formicicum*, b) *Methanobrevibacter arboriphilicus* and c) *Methanospirillum hungatei*. Left panel: CH_4 production over time. Right panel: the corresponding H_2 consumption.

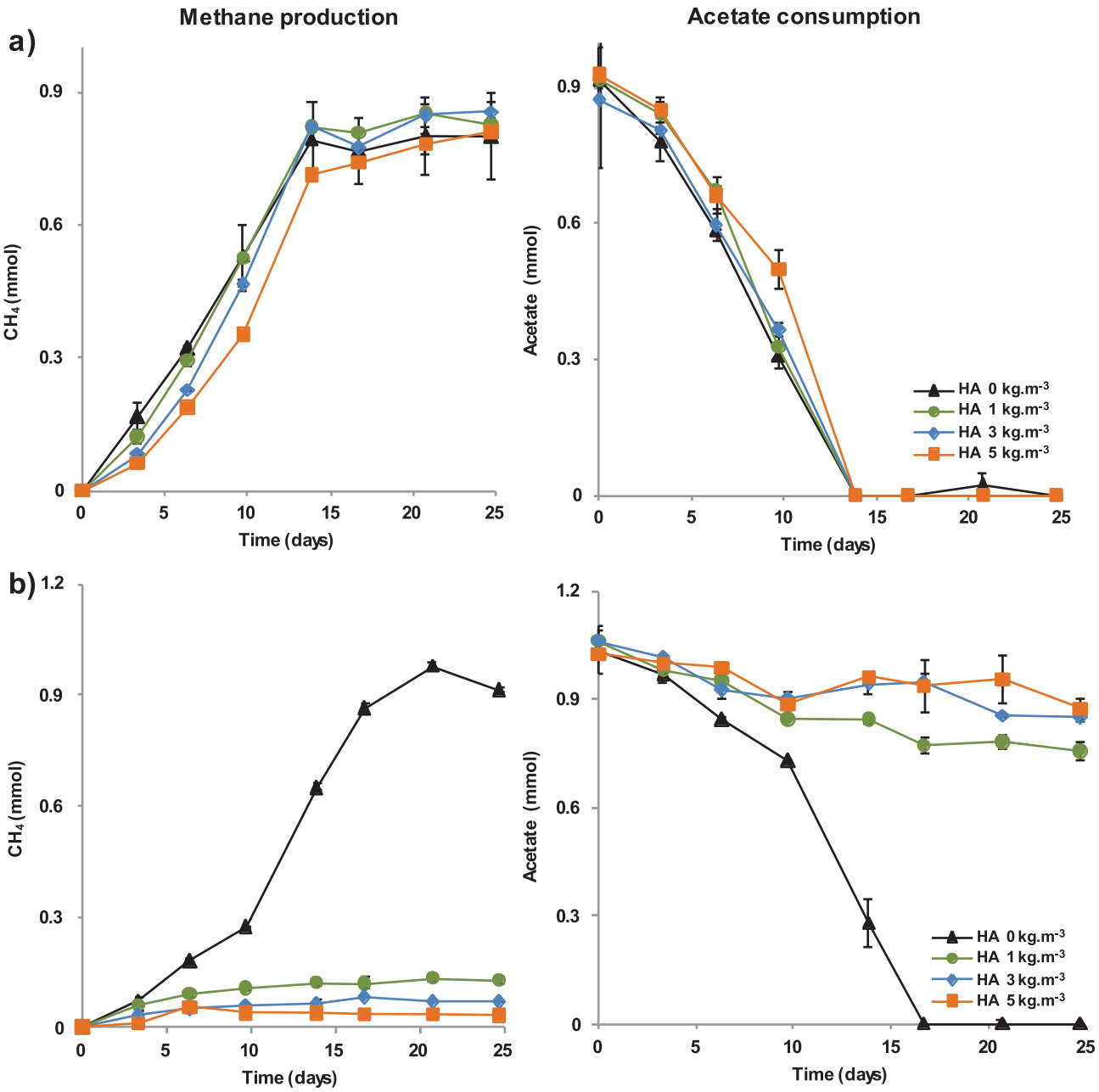

Fig. 4. Effect of humic acid on methanogenic activity of a) *Methanosaeta concilii*, b) *Methanosarcina barkeri*. Left panel: CH_4 production over time. Right panel: the corresponding acetate consumption.

Results of the formate fed batch tests showed a very rapid conversion of formate to methane (Table 2 and Fig. 5b). In one day, all added formate was converted to methane. Overall, the total methane produced at the end of the experiment was hardly affected by the presence of HA. A maximum reduction of 23% in total methane production was observed at 5 kg m^{-3} of HA (Table 2). As observed in the *M. formicicum* incubations, trace amounts of H_2 were formed in all the incubations, but the H_2 was consumed at the end of the experiments (Table 2).

In the acetate fed batch incubations, the total amount of methane produced in the control bottle reached 0.97 mmol within 5 days (Fig. 5c). Addition of 1 kg m^{-3} HA, did not affect the total methane produced at the end of the experiment nor the rate of production (Fig. 5c and Table 2). However, addition of 3 kg m^{-3} of HA reduced the CH_4 production rate, whereas the total methane produced was not strongly affected (Table 2). Addition of 5 kg m^{-3} HA resulted in 24% reduction in total amount of methane produced and the methane production rate was reduced by 40% (Table 2).

The results from the batch activity tests with H_2, formate and acetate showed that methanogenesis with anaerobic crushed granular sludge was not strongly affected by the presence of HA. When the crushed granular sludge was fed with formate or acetate, maximum observed reduction in the total amount of CH_4 produced was 24% at 5 kg m^{-3} of HA (Table 2). Apparently, the mixed methanogenic population present in the crushed granular sludge is

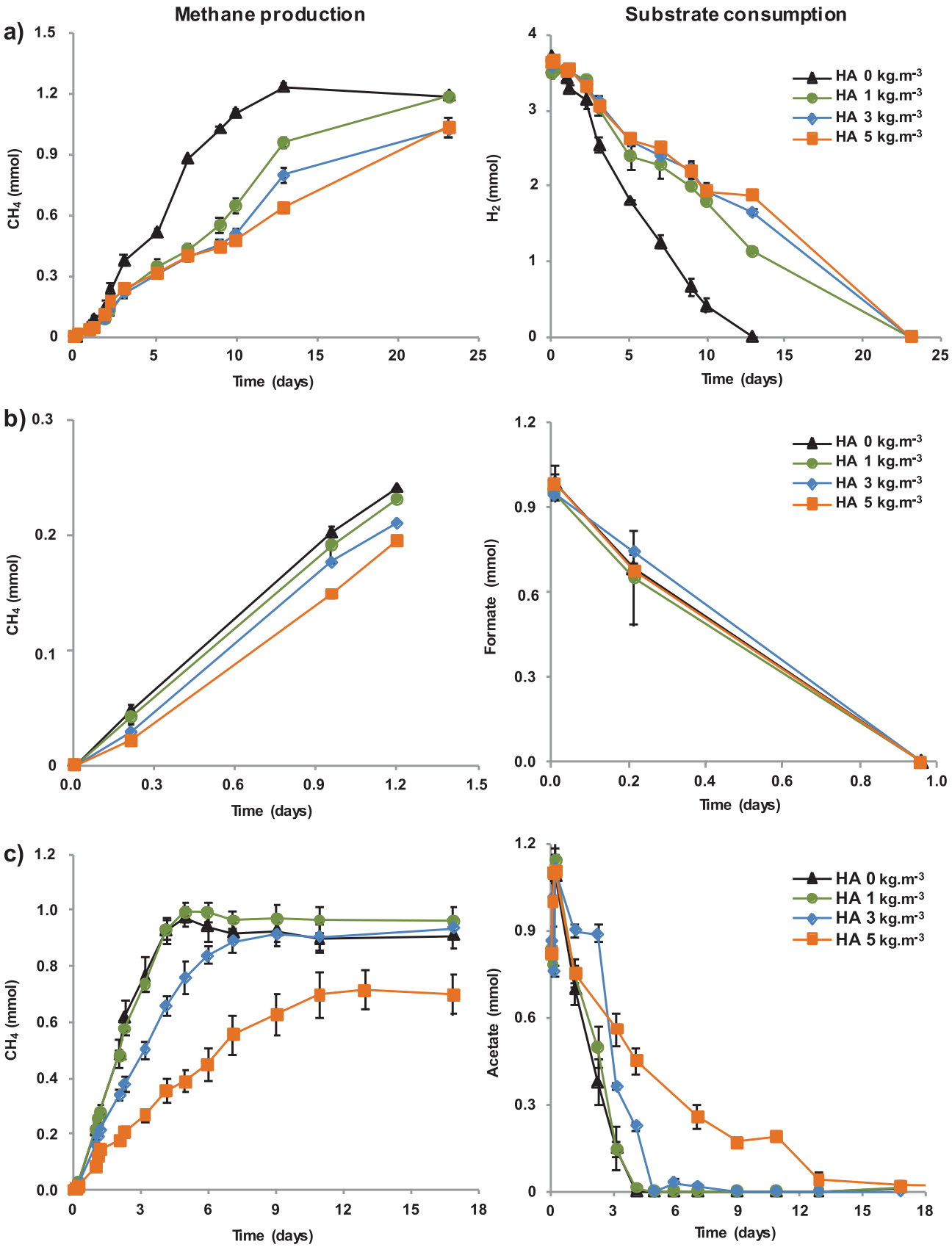

Fig. 5. Effect of humic acids on methanogenic activity of crushed granular sludge, incubated with H_2/CO_2 (a), with formate (b) and with acetate (c). Left panel:CH_4 production over time. Right panel: the corresponding substrate consumption.

Table 2
Initial methane production rates and total amounts of methane produced at the end of the experiment, by sludge from the Eerbeek paper mill digester, in absence and presence of humic acid. The presented values in the table are the average of representative duplicate measurements.

Humic acid (kg.m^{-3})	Production rate (mmol.day^{-1})		Consumption rate (mmol.day^{-1})			Inhibition percentage of CH_4 production rate[a]	Inhibition percentage of total amount CH4[b]
	H_2	CH_4	H_2	Acetate	Formate		
Eeerbeek sludge Fed with H_2/CO_2							
0	–	0.07	0.35	–	–	0	0
1	–	0.04	0.14	–	–	42	0
3	–	0.04	0.15	–	–	43	13
5	–	0.04	0.19	–	–	42	12
Eeerbeek sludge fed with Formate							
0	0	0.21	–	–	1.01	0	0
1	0	0.2	–	–	0.97	5	2
3	0	0.18	–	–	1	13	12
5	0	0.16	–	–	1.01	23	24
Eeerbeek sludge fed with Acetate							
0	–	0.24	–	0.32	–	0	0
1	–	0.23	–	0.32	–	2	0
3	–	0.16	–	0.24	–	33	1
5	–	0.14	–	0.15	–	40	24

[a] The total amount of methane refers to the average amount of methane produced from duplicate experiments at the end of the experiment. To calculate the percentage inhibition of the methane production rates and the total amount of methane in the presence of HA, the methanogenic activity in the control bottles (without HA) was considered 100%.
[b] Methane production rates were calculated as initial production rates relative to the initial methane production rate in the control bottles without humic acid.

sufficiently diverse to sustain methane production regardless of HA presence. However, results show a clearly increasing gap in the stoichiometry of substrate conversion to methane production with increasing amounts of added HA (Fig. 1f). At 3 kg m^{-3} of HA, the gap in reducing equivalents balance was about 30%, when acetate was added as the substrate. Likely, in the presence of HA, reducing equivalents are diffusing or transported out of the archaeal cell leading to reduced methane formation and reduction of oxidised HA moieties. The latter would mean a drop in the biomethane production potential of a substrate when HA concentrations in the medium are high. Methanogenic populations in the used inoculum sludge were previously characterized by Roest et al. [28] and Worm et al. [29]. These authors found that *M. concilii* was the main acetoclastic methanogen, whereas, *M. formicicum* and *M. hungatei* were both found as the main hydrogenotrophic methanogens. Therefore, obtained results were consistent with the results for each of the pure methanogenic cultures. The methanogenic activity of crushed granular sludge, fed with H_2, was inhibited less strongly by HA than the inhibition observed when *M. formicicum* was grown in pure culture. When crushed granular sludge and the pure culture of *M. concilii* were incubated separately with acetate, HA was inhibiting at concentrations higher than 3 kg m^{-3}. In addition, these results support the results of recent study of Azman et al. [15]. In that study, lab-scale biogas reactors, fed with cellulose and xylan were operated in the presence/absence of humic acids for 220 days. HA concentrations of the inhibition reactor was increased up to 8 kg m^{-3}. Archaeal community dynamics were monitored with next generation sequencing of the 16 S rRNA genes. Long term monitoring study showed that the relative abundance of the hydrogenotrophic methanogens, *Methanobacteriaceae,* reduced whereas the relative abundance of *Methanosaetaceae* increased with the elevating HA concentrations. The results from this study and the study of Azman et al. [15] indicated that *M. concilii* and *M. hungatei* can maintain methanogenesis in anaerobic digesters, containing high levels of HA. Due to their insensitivity to HA inhibition, *M. hungatei* and *M. concilii* can be candidates of interest to bio-augmentation studies in anaerobic reactors that are suffering from HA dependent losses in methane yields. However, this hypothesis should be tested to prove the stability of the added methanogens within the reactors.

4. Conclusions

The effect of HA on methanogenic activity was demonstrated using pure cultures and mixed cultures. With the exception of *Methanospirillum hungatei*, all pure cultures of hydrogenotrophic methanogens tested were severely affected by addition of HA. Of the acetoclastic methanogens tested, *Methanosaeta concilii* was not affected by HA, whereas *Methanosarcina barkeri* was severely affected by HA. Anaerobic sludge was less affected by the addition of HA. However, a clear gap in the reducing equivalent balance was observed, probably due to HA acting as an alternative electron acceptor and resulting in reduced methane production in the presence of HA.

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